THE CONTENTS OF THIS THESIS

INTRODUCTION

Despite several thousand years of interest in the question, the nature and properties of the mind remain obscure, and so do the properties of the interaction between the brain and the mind, that is, how thoughts, memories and subjective feelings can emanate from physical entities such as proteins at specific positions in the brain. Human beings can learn, feel, reason, be creative, be self-conscious *et cetera,* and sometimes we attribute some aspects of these abilities only to ourselves, and not to other animals. All these abilities involve the brain. Historically, most theories of mind and behaviour have been formulated in the fields of philosophy and psychology. Before Cajal in the beginning of the 20th century proposed the brain to be built up of neurons that communicated with each other through spaces between cells, the brain was believed to be one big cell.[1] The brain is much more complex than other organs and it is also more inaccessible for exploration. Still, we have been able to investigate several aspects of the mind in action.

By studying subjects with brain injuries or specific cognitive impairments, rather than healthy subjects, brain regions involved in specific cognitive processes have been identified. The famous case of Phineas Gage (1848), who changed after he got a pole stuck through frontal parts of his brain, illustrates how certain frontal brain regions are involved in motivation, personality and in understanding the consequences of actions.[2] The frontal cortex is more than four times larger in humans than in non-human primates, and is involved in controlling most aspects of human behaviour. There is evidence that the development and growth of the frontal cortex is abnormal in autism.[3] The influence of factors that may affect brain development is investigated in relation to autism in paper VII.

The amygdala is a brain region that is crucial for emotional behaviours and that is activated by emotional stimuli, especially by fear and threat.[4,5] The activity of the amygdala affects or depends on mood and anxiety. Amygdala activity during emotional experience in subjects with social phobia is investigated in papers I & II.

Several findings regarding which neurotransmitters are involved in which behaviours have been come across by chance. The plant Rauwolfia serpentina has been ingested for centuries and can reduce psychosis and induce suicidal behaviour, effects that are due to the active substance Reserpine, which prevents storage and thus release of monoamines including serotonin and dopamine. Narcotic drugs also illustrate the involvement of specific neurotransmitters in *e.g.* happiness[A] or psychosis; when the mechanism of action of these substances becomes clear, a specific neurotransmitter can be linked to the emotion or behaviour. An aspect of the mind that is perceived as being impaired or abnormal in subjects diagnosed with a psychiatric disorder can be studied in those subjects; if that property improves with pharmacological treatment or is modified by genetic variation in genes with known gene products (proteins), that aspect of the mind may be linked to a neurotransmitter system.

The neurotransmitter serotonin is involved in controlling mood and anxiety, as demonstrated *e.g.* by the effectiveness of serotonergic drugs in reducing depressive and anxious symptoms, and by the induction of depressed mood when serotonin synthesis is inhibited in subjects with family members with depression.[6,7] The relationship between genetic variation in serotonin-related genes, on the one hand, and mood and anxiety-related traits, on the other, is investigated in papers I-V. Sex steroids affect the prenatal development of the brain, and the possible influence of sex steroid-related genetic variation on personality traits, autism and transsexualism is analysed in papers VI-VIII.

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Comparisons of the frequency of genetic variations between subjects with psychiatric conditions (cases) and subjects without (controls) are used to find genetic variations that may influence the psychiatric trait. The identification of a genetic variant that affects a phenotype (the disease or trait) implies the elucidation of the original code underlying the increased susceptibility for that phenotype, *i.e.* one aspect of the aetiology (aitia=cause, logos=discourse) of the phenotype that is innate. By the identification of a relationship between a genetic variant and a phenotype, that gene, its product, and the pathways this product is involved in, can be connected to the phenotype.

Almost all psychiatric conditions are partly heritable. The heritability for autism is approximately 80% and that for depression and social phobia approximately 50%.[8-10] The genes that underlie this heritability are still largely unknown. Possible reasons for this are (i) that genes interact with each other and with the environment – a genetic variation may give rise to increased susceptibility for a disease in one person that carries it but not in another, possibly due to different variants on other locations (loci) in the genome or different environmental exposure, (ii) that different combinations of genetic variants may give rise to the same phenotype, and (iii) that rare variants are common in the genome – one person may have an increased vulnerability due to one such rare variant, whereas another person with increased vulnerability carries a different rare susceptibility variant. Paper IX introduces a new method that detects effects of combinations of genetic variations with increased probability.

When searching for susceptibility variants for psychiatric traits it is important to take environmental risk factors into consideration – by doing so, genetic variants that interact with the environmental exposure can be detected. Similarly, when searching for environmental risk factors, it is important to know which genes are involved in the heritable part of the aetiology. Risk factors for depression, including stressful life events and possible susceptibility genes, as well as the inter-relationship between these, are investigated in paper V.

The aims of this thesis are threefold. First, the influence of variation in serotonin-related candidate genes on mood disorders, and brain processes that appear to be involved in mood and anxiety disorders, as well as the influence of genetic variation in a neurotrophic factor on the serotonin transporter, which is important for the function of the serotonergic system, were explored. Second, variation in sex steroid-related genes was related to personality traits, autism and transsexualism. Third, a method that restricts the search for effects of combinations of genetic variants to certain patterns was introduced and shown to be better at detecting these two-locus effects.

BACKGROUND TO GENETICS

NATURE & NURTURE

The location of the soul or mind and the influence of nature on our mind were debated long before the concept of the genetic code was introduced. Hippocrates (460 BC – 370 BC) and Plato (430 BC - 350 BC) were the first to localize the mind in our heads. After them, Aristotle (380 BC - 320 BC) placed the rational soul in the heart, and his theories were leading for centuries. Descartes (1596-1650) influenced many scientific areas, philosophy of the mind being one of them; he placed the link between the body and the mind in the pineal gland. One of his successors was Locke (1632-1704), whose ideas were influenced by those of Descartes in many ways. Locke is known for the conceptualization of the mind as a "tabula rasa", a blank slate, and is frequently pointed out as the extreme-nurturist that ascribed all influence on the mind to nurture or environment, and none to nature or genes. Except for his use of the tabula rasa expression, his controversial opinion that Christian moral principles were *not* innate may have contributed to this interpretation. Locke believed that ideas, the components of the mind, came from experience (experience of the external through perception and experience of the internal through introspection), in contrast to Descartes, who stated that the ideas were innate and activated by experience. But, more importantly, Locke believed the mental *abilities* to be innate, *i.e.* that we are born with the ability to think, memorize and to use our senses. He also proposed personality traits and talents to be innate, a notion that is in line with current findings of considerable heritability estimates for such traits.[11-14]

HERITABILITY

Heritability is the proportion of phenotypic *variation* in a population that is attributable to genetic variation. The proportion not explained by genetic variation is believed to be attributable to variation in environmental exposure.

Estimation of heritability

Twin studies have been the major source of information regarding the respective contributions of genes and environment to a trait. One way of estimating heritability, is by comparing resemblances between monozygotic (MZ) twins, who share all their genes (however see[15]), and dizygotic (DZ) twins, who share on average half of their genes. MZ twins are hence twice as genetically similar than the average DZ twin pair, and the heritability is estimated as two times the difference in correlation for the trait: $2 \cdot (r_{MZ} - r_{DZ})$.

Even better at elucidating genetic and environmental components, albeit naturally more rare, are adoption studies, which compare the similarity between twins or siblings who are brought up together with the similarity of those brought up apart. The similarity between offspring and biological parents can also be compared to that between offspring and adoption parents.

Shared and non-shared environment

The proportion of variation in the phenotypic trait attributable to the environment is divided into shared and non-shared environmental effects. Shared environmental factors reflect environmental exposure that makes the two siblings more alike. Shared environment, c^2, is

estimated by the DZ correlation minus half the heritability (the degree to which DZ twins share the same genes), *i.e.* $c^2 = r_{DZ} - (h^2/2)$. Unique or non-shared environmental variance, e^2, reflects the degree to which identical twins raised together are dissimilar and is estimated as $e^2 = 1 - r_{MZ}$.

Historically, clustering of a trait in a family, such as two siblings affected by the same disease, was largely believed to be due to the environment they shared, *e.g.* their common upbringing. The contribution of shared environment to complex traits (see below) has however often turned out to be very low, whereas the contribution of genes and non-shared environment both generally are large.

MODES OF INHERITANCE

Mendelian traits

Mendel (1822-1884) studied the inheritance of traits in pea plants and found that it follows particular laws, which were later named Mendelian laws. The principles of Mendelian inheritance are the following: Consider a *locus* (which is a position in the genome) with two possible variants or *alleles*, *A* and *a,* and the trait or *phenotype* colour, which can take two forms: red and green. Assume that the presence of the *A* allele results in green colour and that the genotype *a/a* is the only genotype that results in red colour. Each parent transmits one of their alleles to their offspring. Two red parents will then always have red offspring. However, if one of the parents is green, the genotype of this parent can be either *A/a* or *A/A*. If this parent carries the *A/A* genotype and gets offspring with a red parent, then all offspring will be green, since all of them will carry an *A* allele. On the other hand, if the green parent carries the *A/a* genotype, offspring will be green and red in equal proportions, half of them will carry the *a/a* genotype and the other half will carry the *A/a* genotype. The inheritance of a Mendelian trait follows this pattern; the proportion of affected individuals can hence be predicted from the traits of the parents and grand-parents. For a dominant trait, inherited with a dominant mode of inheritance, only one susceptibility allele is required for the trait to appear, whereas for a recessive trait, two alleles are required for the trait to be expressed.

Complex traits

A complex trait does not follow a Mendelian mode of inheritance, and its aetiology depends both on genes and environment, including the involvement of different susceptibility genes in different subjects and also of combinations of genetic variants (see locus heterogeneity and gene-gene interactions below in the GENES section). Most psychiatric disorders are complex, *e.g.* autism, mood disorders and anxiety disorders. Despite extensive research aimed at finding genes for complex traits, no strategies have been successful in finding genes that explain the high heritabilities.

Gene-environment interaction

Neither genes nor the environment acts in isolation. Instead, genes and environment *interact* in influencing traits. A gene and an environment are said to interact when a gene has different effects on *e.g.* disease risk in different environments. Gene-environment correlation is the influence of genes on environmental exposure. For example, exposure to stressful life events has been shown to be heritable.[16-18]

THE INHERITED CODE

DNA

A deoxyribonucleic acid (DNA) molecule looks like a spiral staircase. The nucleotides or bases, *i.e.* adenine (A), cytosine (C), guanine (G) and thymine (T) bind to each other in a specific manner (A-T and C-G), thus forming the base pairs that constitute the steps of the stairway. A and G are purines, whereas C and T are pyrimidines. The edges of the staircase are made up of sugars called deoxyriboses and of phosphate groups. Humans have 23 chromosome pairs in the nucleus of each cell. Each of these chromosomes is a DNA molecule. One member of a chromosome pair originates from the mother and one from the father. Although Delbruck suggested the chemical structure of the chromosomes to mediate heritable properties in the 1930s, the structure of DNA was discovered first in 1953[19]. In Figure 1 the chromosome has just replicated (duplicated) in the meta-phase of the cell-division cycle (mitosis) – the process when one mother cell divides into two daughter cells – and the chromosome is attached to the new chromosome copy at the centromer. When the cell is not dividing, DNA is packaged by proteins into chromatin to fit in the cell nucleus. Any location, or locus, in the genome, is made up of two variants or alleles, one situated on the maternal and one on the paternal chromosome of the chromosome pair. A combination of such alleles on the same parental chromosome is called a haplotype.
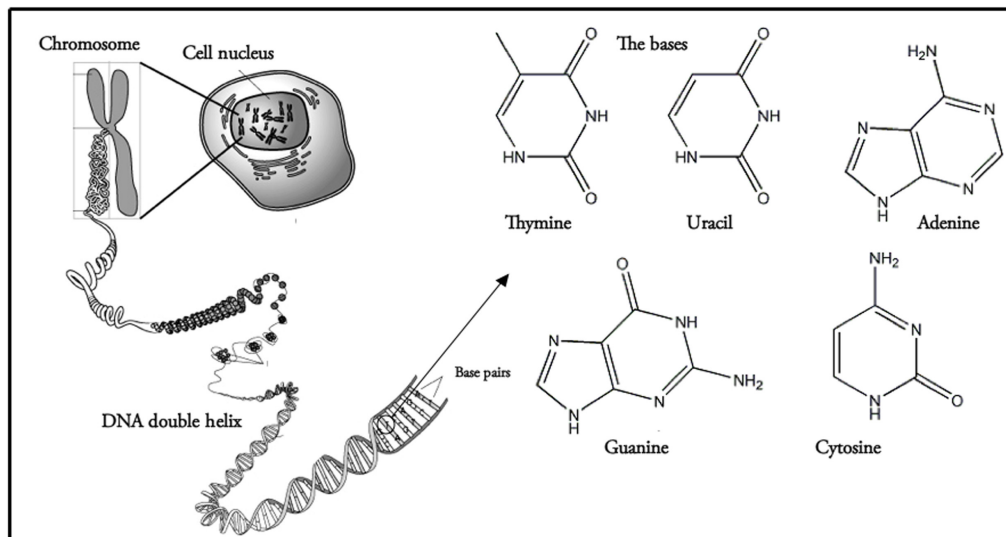
Figure 1. One cell nucleus contains 23 chromosome pairs. The chromosome in the figure has just replicated and is attached to the new chromosome copy. It consists of DNA, which is built up of the chemicals depicted in the picture, *i.e.* adenine, cytosine, guanine and thymine.

Gene composition

A gene is composed of exons, which are elements encoding amino acids, the building-stones of proteins, and by introns, which are non-coding elements. The regulatory region upstream of the gene is called the promoter and contains motifs where transcription factors bind. Transcription factors are proteins required for the expression, or *transcription*, of genes.

Transcription is the transformation of DNA to RNA, in which T is substituted for uracil (U) and the introns are spliced off. The region downstream the gene, the 3' untranslated region (UTR), holds several elements that regulate RNA stability and translation. In the *translation* process, which takes place outside of the nucleus, the ribosome reads codons, *i.e.* every three nucleotides of the messenger RNA (mRNA), and builds the protein from the amino acids that these codons encode.

GENETIC VARIATION

Evolution occurs when heritable differences become more common or rare in a population, usually because the properties promote or reduce survival and reproduction, leading to a natural selection of those best suited for their environment. Genetic variation is thought to be under constant evolutionary pressure.[20]

Crossovers and recombination

Meiosis is the division of a cell into four gametes. A gamete contains one chromosome of each type and fuses with another gamete during conception in all organisms that reproduce sexually. In the prophase of meiosis, the two chromosomes of a pair, one of maternal and one of paternal origin, replicate and exchange genetic material at crossover points called chiasmata. One crossover creates new combinations of alleles (haplotypes) in half of the gametes (two of the four gametes produced by one cell, see Figure 2). Crossovers result in increased genetic variation in nature and thus enable acceleration of evolution by natural selection and formation of new genetically unique individuals.

Figure 2. One crossover event creates recombined chromosomes in two out of four gametes.

Females display approximately 50% higher rates of crossovers than men (some species do not display crossovers in males). Some regions of the genome experience a larger rate of crossovers. Due to this and also that selection causes some crossovers not to survive, observed regions of increased crossover rates, so-called hot spots of recombination, are believed to be located in regions where presumably either variation is important, or conservation is not important.

If crossovers occur in uneven numbers between two loci, so-called recombination events can be observed. A recombination between two loci denotes the event that the two different grandparents contribute with one allele each at the two loci of that haplotype. When no recombination between two loci has occurred, it means that the haplotype contains two alleles which originate from the same grandparent. For loci in close proximity, at most one crossover occurs per generation, meaning that the recombination fraction directly measures genetic

distance as determined by crossover probability. Recombination events are the basis for gene finding strategies such as linkage and association analysis[21].

Polymorphisms

In the year of 2003, the human genome organization (HUGO) succeeded in sequencing the whole human genome of one person[22]. Our genomes are identical to over 99.9% but still differ on many locations. For example, one individual could have an ACGTTTT<u>A</u>-sequence in an important region of a gene encoding a protein necessary for the function of a neurotransmitter system, whereas another individual carries an ACGTTTT<u>T</u>-sequence at the same location, and this single nucleotide polymorphism (SNP), polymorph meaning it takes many (=poly) forms (=morphus), may implicate an increased or reduced vulnerability for disease. Since chromosomes come in pairs, such a polymorphic locus can give rise to three genotypes. An individual can thus carry one of the three genotypes *AA*, *AT* or *TT* on that specific locus.

SNPs are the most common sort of genetic variation. Other sorts of polymorphisms are *e.g.* insertion/deletion polymorphisms and repeat polymorphisms, so-called variable number of tandem repeats (VNTRs). Copy number variations are deletions or duplications of sequences that are longer than 1000 base pairs. A population that displays random mating is in Hardy Weinberg equilibrium (HWE), *i.e.* the state in which the proportions of genotypes in the population depends only on the allele frequencies.

The functional consequences of polymorphisms can be several. An SNP can be situated in an exon where it may lead to an exchange of which amino acid is coded for, which in turn can affect protein folding and/or function. This sort of SNP is called non-synonymous. In contrast, synonymous SNPs are situated in coding regions but do not change the amino acid sequence. Repeat polymorphisms in exons can encode stretches of amino acids; a CAG repeat may thus encode a repeat of the amino acid glutamine. The length of such a stretch of amino acids may affect protein function. Repeat polymorphisms of other sizes, such as the di-nucleotide repeats may affect the reading frame, leading to altered amino acid sequence or a truncated protein due to a premature stop codon. Polymorphisms can be situated in the promoter region where they may affect expression efficiency and protein amount. Polymorphisms in the UTR regions may affect RNA stability or they may be located in motifs for microRNAs, which inhibit translation. Intronic polymorphisms may influence splicing or other regulatory mechanisms.[23]

Linkage disequilibrium

Loci A and B, locus A with alleles *A* and *a* and locus B with alleles *B* and *b*, are in linkage equilibrium (LE) when the occurrence of *e.g.* allele *A* and allele *B* in a haplotype are independent events, and the haplotype frequency consequently can be determined as the product of the two allele frequencies, P(haplotype *A-B*)=$P(A)\cdot P(B)$. Linkage disequilibrium (LD) is measured by comparing the observed haplotype frequency with that expected if the loci had been in LE. When two loci are located closer to each other and are in linkage (see below), it is more likely that the occurrence of two of their alleles in a haplotype is non-random. Measures of LD do however not only depend on genetic distance, but also on allele frequencies and the time passed since the polymorphism first appeared. LD between two loci can also be the result of population stratification (see below).

D' is a measure of LD and is determined as the ratio between D and D_{max}. Absolute LD, r^2, is determined as D^2 divided by the product of all allele frequencies. Only when r^2 is equal to its max value (=1) do two specific alleles *always* occur together on a haplotype, leading to the existence of two haplotypes only; the allele of locus B on a haplotype can then be absolutely

determined by the allele of locus A. The locus B allele can not be determined from the allele at locus A when D' equals to its max value (=1) and r^2 is smaller than 1.

Haplotypes and haplotype structure

A haplotype is a combination of alleles on a chromosome. The haplotypes that two-locus genotypes consist of can be determined when at least one of the two loci is homozygous. Thus, for the two-locus genotype composed of the two genotypes *A/a* and *B/B*, the two haplotypes are *A-B* and *a-B*. However, for a two-locus genotype of two heterozygous loci with genotypes *A/a* and *B/b*, the haplotypic *phase* can not be determined: allele *A* can be on the same haplotype as allele *b*, or on the same haplotype as allele *B*, the two possibilities being the possible phases. When calculating LD measures between such loci, the haplotype frequencies need to be estimated; this is usually done by means of the expectation-maximization algorithm.

Haplotypes consisting of alleles that are in high LD in a population are called haplotype blocks. Two haplotype blocks may be separated by hot spots of recombination. Haplotype blocks are meaningful entities for association analyses since an allele that is located on a certain haplotype, even though it has not been measured, can indirectly give evidence of association. The HapMap project has defined so-called haplotype tag SNPs, which are SNPs that are supposed to cover the majority of variation in a gene.[24]

GENE FINDING STRATEGIES

Linkage analysis

Linkage analysis uses genetic information from families with many affected subjects to determine which genomic regions that are inherited together with the disease. In this manner, the chromosomal regions harbouring the relevant disease-causing genetic variations can be identified.

The basis of parametric linkage analysis is the recombination fraction, *i.e.* the fraction of offspring for which recombination has occurred between two loci on a chromosome. Two loci are completely unlinked when recombinants and non-recombinants are expected in equal proportions (recombination fraction 0.5), as when two loci are situated on different chromosomes. Linkage analysis measures how much the observed recombination fraction between two loci deviates from 0.5 and localizes the disease locus to a map interval bounded by crossovers. Parametric linkage analysis has been successful for Mendelian traits.

The basis of nonparametric linkage analysis is the number of alleles shared by affected sibpairs that are identical by descent (IBD). For a locus that is inherited with the disease locus (or is the disease locus), a sibpair that shares more alleles IBD is expected to have more similar phenotypes, *e.g.* both sibs are expected to be affected by a disease if one of them are. Nonparametric linkage analysis measures the deviation of the observed number of shared alleles IBD from the distribution that is expected when no disease locus is linked to the investigated locus. Non-parametric linkage analysis is used for complex traits but has not been successful.[21]

Association analysis

Association analysis is a comparison of genotype or allele frequencies between cases and controls (so-called case-control study) or a comparison of trait means between genotypes, or a comparison of the number of times an allele is transmitted or non-transmitted from a healthy parent to an affected offspring, the latter often analysed using transmission disequilibrium tests. If the investigated locus is close to the disease locus, the disease-related variant is more likely to

be transmitted on the same haplotype as the measured locus since it is less likely that any crossovers have occurred between the two loci. Association studies with dense markers have been used to follow up the results of linkage analysis, to further delimit the region that carry the disease-related gene or polymorphism.

Association studies can also be performed using candidate genes, *i.e.* genes whose products are linked to the trait. The investigated polymorphisms in these genes may be candidate polymorphisms, *i.e.* polymorphisms that affect protein amount or function, a strategy that not is dependent on recombination, or, they may be polymorphisms that are be in LD with functional polymorphisms. Association analysis may be a more powerful method than linkage analysis for identifying polymorphisms with small to moderate effect sizes on complex traits.[25]

> **The studies of papers I-VIII are all association studies. Papers I-III and VI investigate continuous outcome variables, whereas papers IV-V and VII-VIII are focused on dichotomous traits. Paper VII also includes a family-based association study.**

Genome-wide association studies are becoming more feasible because of new technologies that can genotype many SNPs simultaneously. When many polymorphisms are investigated, the effect sizes need to be rather large (the *p*-values[B] need to be small) for an effect to be considered significant, since the multiple testing needs to be controlled for. One test in 20 becomes significant simply due to chance. A recent genome-wide association study investigated seven diseases. Although the sample sizes were relatively large, 2000 cases and 3000 controls, the *p*-value[B] needed to be under $5 \cdot 10^{-7}$ to be considered significant, and the power was only around 40% for finding variations with relative risks of 1.3, and 80% for finding those with relative risks of 1.5 (relative risk = probability of disease when carrying one genotype / probability of disease when carrying another genotype).[26] The large size of association studies may thus affect the chance of finding genes with small effect sizes and increases the need for powerful gene analysis tests.

Spurious associations can arise due to population stratification. Allele frequencies differ between populations, even between regions within Sweden. Population stratification refers to the combination of two subpopulations that display different allele frequencies and different trait means, leading to spurious association between an allele and a trait. Even if there is no factual association between a locus and a trait in either of the subpopulations the trait mean can become very different for the three genotypes of the locus when the two populations are pooled.[27]

COMPLEX GENETICS

Association analysis strategies

Research in psychiatric genetics of the last decade has mostly been devoted to studies of the relationship between one polymorphism and one trait, resulting in findings of polymorphisms with small effects, explaining approximately 1-5% of the variation in the studied trait. Although many associations have been reported, only a few have been replicated so many times that they now are considered to be established. Possible explanations for the inconsistencies in one-polymorphism-one-disease studies may be interactions between genes and locus heterogeneity, but incomplete penetrances, uncertainties in the age of onset for the conditions, and the notion that many of the polymorphisms that associations are reported for are probably neither necessary, nor sufficient for disease onset, are also of importance. More recently,

investigations of complex traits have tried to take the combined influence of several loci into account,[e.g.28] thus considering the possibilities that different variants are susceptibility loci in different subjects, and that genes may interact with each other.

A rare disease was previously believed to be related to rare variants, whereas common polymorphisms were believed to increase the risk for common diseases (the rare disease – rare variant and common disease - common variant hypothesis). This view, together with the view that a rare variant more often *causes* a disease, than merely increases the risk for it, has now largely been abandoned. Rare variants seem to increase the risk also for common diseases, although different rare variants are present in different subjects with that disease (locus heterogeneity), leading to the necessity for huge sample sizes for these variants to be found. Similarly, quite common variants can be risk factors for rare diseases, possibly because they interact with other susceptibility polymorphisms (gene-gene interaction).

Three different approaches used when searching for susceptibility loci for complex diseases are: (i) to look at the diagnosis as a whole, ignoring clinical heterogeneity or even pooling diagnoses that have overlapping heritability, (ii) to reduce phenotypic heterogeneity by investigating phenotypes that are less clinically heterogeneous than are diagnoses, such as specific symptoms, and (iii) to investigate phenotypes considered to be more closely related to the genetic effect than are symptoms or diagnoses.

The first strategy is preferable when the different heterogeneous symptoms of a complex disease are believed to arise from the same genetic aberrations. The second strategy is applied when different aspects of disease are believed to be influenced by independent genes.

In favour of a view where one genetic variation can influence several aspects of disease, the same rare variants can sometimes give rise to very different autism-related phenotypes[29-31] and also to different diagnoses.[32] Supporting the second strategy, the evidence for the involvement of some genes in autism aetiology has been strengthened by reducing phenotypic heterogeneity, either by focusing on subjects with language impairment or on subjects with savant skills.[C 33,34]

The third approach has also been fruitful. Based on the assumption that the effect of a gene on a protein concentration or on a brain process is larger than that on a specific disease, it has become more common to investigate the relationship between one gene and one so-called intermediate phenotype, meaning a phenotype that possibly mediates the effect of the gene on the disorder. If the intermediate phenotype, *e.g.* a brain process alteration, is specific for a condition as well as heritable and is showing intermediate values for first-degree relatives, it is called an endophenotype. This strategy has led to findings of polymorphisms that explain a larger proportion of variance in the intermediate trait, compared to the effect sizes of studies that focus on diagnoses. If an intermediate phenotype is more common in, but not specific for, a certain condition, this does however not imply that a larger proportion of the variance in the *condition* is explained by that polymorphism. For example, hyper-responsiveness of the amygdala during observation of emotional stimuli[D] is an intermediate phenotype that is observed with higher frequency in depressive and anxious subjects than in controls, but which is not specific for subjects with these diagnoses.[35,36] The association of genetic variation in the serotonin transporter promoter region with activity within the amygdala has been much more consistent than that with clinical diagnoses of mood and anxiety disorders or with related temperamental measures.[37,38]

Locus heterogeneity

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A phenotype is genetically heterogeneous when it has a genetically different aetiology in different individuals, *i.e.* when different polymorphisms can increase the risk for *e.g.* a disease *independently* of each other. One example is Alzheimer's disease. Mutations in one gene (encoding the amyloid precursor protein) lead to a Mendelian dominant inheritance of the disease, but are present only in very few Alzheimer families in the world.[39] Mutations in another gene (presenilin 1) also show high penetrance and give rise to a substantially increased disease risk. However, neither of these polymorphisms explains a large proportion of the affected individuals (the so-called population-attributable risk). Instead, another more common allele (the apolipoprotein E4 allele) increases the risk for sporadic (in contrast to familial) Alzheimer's disease,[40] a risk that is further increased by environmental risk factors. Depression is yet another condition for which there are rare variants with high penetrance, although most cases of depression are not explained by these. Amino acid substitutions in a serotonin synthesis enzyme thus have been shown to be much more common in depressive subjects than in controls, but they have only been found in very few subjects.[41,42]Notably, when several steps in a disease-related pathway are susceptible to interruption, it is reasonable to expect that locus heterogeneity is an important aspect of the genetic part of the aetiology of that disease.

One mathematical definition of locus heterogeneity has been described by Risch.[43] He defines a new sort of penetrances as well as so-called penetrance summands, which are obtained by applying the law of total probability to the penetrances-like entities. The penetrance-like entities could be interpreted as the probability of being A-affected or B-affected given genotype on locus A and locus B, respectively, *i.e.* P(A-affected|A-locus genotype) and P(B-affected|B-locus genotype). The penetrance summands could be interpreted as the probability of being A-affected and the probability of being B-affected, meaning that one disease is subdivided into two subdiseases, A and B, with exactly the same symptoms, only that the risk for subdisease A is influenced by locus A only, and that the risk for subdisease B is influenced by locus B only. This locus heterogeneity model is described like this:

$$P(\text{affected}|AA,BB)=P(\text{A-affected}|AA)+P(\text{B-affected}|BB)-P(\text{A-affected}|AA)\cdot P(\text{B-affected}|BB)$$

$$P(\text{affected})=P(\text{A-affected})+P(\text{B-affected})-P(\text{A-affected})\cdot P(\text{B-affected}).$$

The subdiseases A and B are only theoretical, and neither their prevalence, nor the penetrance-like entities can be determined. However, by applying common statistical rules to the above formulas, a relationship between two-locus penetrances, the two marginal penetrances and the disease prevalence can be found. A conceptualization of gene-gene interaction as departure from locus heterogeneity may be considered a reasonable theoretical definition; a test for assessing gene-gene interactions may then be designed to search for effects that deviate from the relationship expected by locus heterogeneity.

Gene-gene interaction

Gene-gene interactions are possibly one of the reasons why one variant, allele *A*, causes an increased risk for disease in one person, but not in another. Different individuals have different genetic backgrounds; the variants that allele *A* can interact with are therefore different in two subjects. Gene-gene interactions are probably major contributors to variation in complex disease.[44,45] However, although gene-gene interaction analyses are performed more frequently and several interactions have been reported, there are, as yet, no *established* gene-gene interactions for psychiatric traits.

The definitions and interpretations of the terms epistasis and gene-gene interaction are many. The original definition of epistasis, expressed by Bateson in 1909,[46] was that the effect of one locus on the phenotype is masked by the presence of a certain allele of a second locus acting on the phenotype. By masked was meant that carriers of the masking allele *B* did not have different phenotypes for the three genotypes of the masked locus A. If the phenotype is colour, then carriers of the *A* allele are black, whereas *a/a* homozygotes are white. But this is the case only when the genotype of the interacting locus is *b/b*; whenever the masking *B* allele is present at locus B, there is no difference in colour between genotypes at locus A (Figure 3).

	bb	Bb	BB
aa	white	gray	gray
Aa	black	gray	gray
AA	black	gray	gray

Figure 3. The original definition of epistasis.

The Bateson definition does not always overlap with that of epistatic interaction or epistacy, described by Fisher in 1918 as departure from additivity between the effects of two loci.[47] As pointed out by Phillips and Cordell,[48,49] the definition of epistasis has been widened, causing confusion in terminology and interpretation. The expressions gene-gene interaction and epistasis are usually used interchangeably and, although the definition usually includes that one locus alters the effect of another locus, the precise definition depends on which model is used for interaction analysis. The Fisher definition of epistatic interaction was further developed in the fifties[50] into the present conceptualization of interaction as the interaction term in a regression framework.

When interactions are synergistic, the effect of one polymorphism is potentiated by a genotype of the other locus. In contrast, antagonistic interaction means that the combined effect is smaller than the individual effects. Interaction is absent when the effects of two loci are independent, *i.e.* when the effects of the two loci are additive. These different sorts of interaction are depicted in Figure 4.
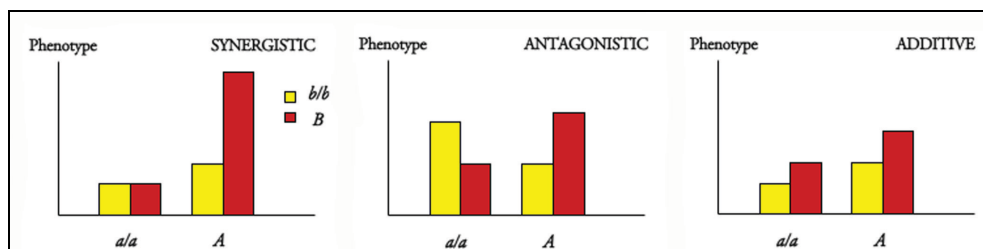
Figure 4. Different sorts of interaction.

Reasons for interaction analysis

The benefits of analysing interactions are: (i) that a larger proportion of the variance in a trait may be explained, (ii) that genes that are not found when ignoring interactions, due to small individual effects, may be identified, and (iii) that relevant biological mechanisms may be elucidated (although statistical interactions do not imply interactions on a physical or mechanistic level).

Several authors have pointed out the necessity for separation of synergistic and antagonistic interactions.[51] For synergistic interactions, detection of the loci involved, *i.e.* point (ii), is not a

principal problem. By including interactions in the models, however, a larger proportion of the variance in the complex trait can be explained. It is worth noting that for the original definition of epistasis as described by Bateson, the single loci would be *detectable* without the need for interaction analysis.

The second point above – stating that interactions may need to be analysed for loci to be detected – is particularly relevant for antagonistic interactions. If locus A only has an effect when allele *B* is present at locus B or if locus A has effects in different directions depending on B-locus-genotype, then locus A may not be found when the loci are investigated separately. Antagonistic interactions are believed to be responsible for inconsistencies across studies, including failures in attempts to replicate strong findings.[44] For large (*i.e.* that include many polymorphisms) association studies with the aim of detecting all loci implicated in disease, it may hence be important to include analysis of such interactions. Since the antagonistic interactions are the most statistically challenging, especially when marginal effects are absent (so-called disordinal interactions or pure epistatic interactions[52,53]), statisticians have been fascinated by them, and therefore made a point in investigating models with no marginal effects, most of them largely non-monotone.[52,54-57]

Monotone models

A monotone single-locus model assumes that the alleles within a locus display monotone effect patterns, meaning that the penetrance – if the trait is dichotomous – or the mean value – if the trait is continuous – of the heterozygote is not outside the interval defined by the penetrances or mean values for the two homozygotes. Treating genotype as a covariate – 0, 1, and 2 representing the number of risk alleles – in a regression analysis restricts the test to this monotone pattern of effect. A two-locus monotone model for a dichotomous trait similarly assumes that the two-locus penetrance matrix is monotone, *i.e.* that $f_{ij} \leq f_{kl}$ for $i \leq k$ and $j \leq l$, where f_{ij} are the two-locus penetrances and i and j designates the number of *A*-alleles and *B*-alleles in those genotypes.[58] A test restricted to monotone models hence does not detect effect patterns that are non-monotone, *e.g.* when the double heterozygote displays the largest penetrance or trait mean.

> As shown in paper IX, a monotone penetrance matrix always has marginal effects, provided that either of the two loci is related to disease risk.

Regression analyses

Regression analysis is a method for obtaining a regression equation, in which the dependent response variable is a function of the independent or explanatory variables. The parameters of the function are estimated in a manner as to best fit the different values of the dependent and independent variables, usually using the least squares method.[59,60] In linear regression, the regression function is a line, representing the predicted value for each genotype. In logistic regression the dependent variable is dichotomous, representing *e.g.* presence and absence of the trait investigated. The logistic regression equation predicts the logarithm of the odds of being affected, *i.e.* the logarithm of the probability of being affected divided by the probability of not being affected. The logistic regression output can be expressed in terms of odds ratios (ORs). When the two loci are reduced to two meaningful genotypes each: *A* and ¬*A*, and similarly for locus B, in a full model including the individual loci and the interaction term, the OR for locus A is determined under the reference genotype of locus B (¬*B*) in this manner:

$$OR_{A\neg B} = \frac{f_{A\neg B} / h_{A\neg B}}{f_{\neg A\neg B} / h_{\neg A\neg B}},$$

where $f_{A\neg B}$ is the two-locus penetrance for the two-locus genotype $A,\neg B$, and $h_{A\neg B}$ is the probability of being unaffected given that genotype. The interaction term is the ratio, R, between the OR for the interaction and the product of the ORs for the individual loci, or equivalently, the ratio between the OR for locus A under B as reference and the OR for locus A under $\neg B$ as reference:

$$R = \frac{OR_{A_B}}{OR_{A\neg B}} = \frac{(f_{AB} / h_{AB}) / (f_{\neg AB} / h_{\neg AB})}{(f_{A\neg B} / h_{A\neg B}) / (f_{\neg A\neg B} / h_{\neg A\neg B})}.$$

Logistic regression was used in papers II, IV, V, VII and VIII, in paper II to control for the possible influence of other factors on the relationship between polymorphisms and the dependent variable, and in the other papers to explore the possible presence of interactions between polymorphisms. Stepwise backward elimination was used to eliminate non-significant variables from the logistic regression equation in papers IV and VIII.

When regression analysis is used, the interaction between two polymorphisms needs to explain an additional proportion of the variance, compared to that explained by the polymorphisms alone, in order to be significant. Several methods that claim to analyse gene-gene interactions do not compare the likelihood of a two-locus combination with that of the single-locus model and hence do not have this requirement.[54-57] These methods detect joint effects, which may or may not be interactive, but they can not distinguish between interactive and non-interactive joint effects. When the main goal is to find as many susceptibility loci as possible amongst many loci, the definition of interaction is however not a central issue. In contrast, the definitions of interaction and non-interaction are important when trying to elucidate the nature of the combined effect of two known susceptibility loci.

One strategy for finding interactions is a two-step approach, meaning that single-locus effects first are ascertained at a less strict significance level than that usually used, and that subsequent two-locus effects are analysed only between the loci that have passed step one.[44,45] Antagonistic interactions without any marginal effects will however not be found with this method.

Power considerations
When considering if interactions should be included in the analyses or not, there are also other aspects to take into consideration. The most important of these factors is statistical power[B], *i.e.* the probability of finding existing effects. When only considering single-locus effects, the number of tests increases linearly with the number of loci included in the study – one test for every locus. Since an increased number of tests implies that more of them could become significant by chance, the significance level required becomes stricter in a linear manner for every locus added to the study, due to control for multiple testing. When considering all possible pairs of loci, the number of tests increases quadratically with the number of loci

included. For n loci, the number of tests thus is $n\cdot(n-1)/2$. The number of tests that need to be controlled for is hence much larger, resulting in a reduced power.

The difference in power between the two courses of action, *i.e.* either investigating single-locus effects only, or investigating two-locus effects, depends on the difference in size between the epistatic effect and the corresponding marginal effects; the power of finding the loci involved in an interaction may be higher when searching for marginal effects if the marginal effects of the two loci acting in epistasis are relatively high compared to the epistatic effect. However, if the size of the two-locus effect is considerably higher than the single-locus effect(s), then the two-locus approach will be more powerful. Marchini et al.[45] have found the two-locus approach to "win" over the single-locus approach for several monotone two-locus models, in spite of the large difference in the number of tests performed.

> The need for tests with increased power was met in paper IX, where a test that is restricted to search for monotone two-locus effects is introduced. The power of finding monotone effect patterns was shown to increase by approximately 10 percent units, compared to an unrestricted test, by using this method.

TRAITS

MOOD & ANXIETY

Symptoms of major depressive disorder

Major depressive disorder (MDD) is, according to DSM-IV (Diagnostic and Statistical Manual of Mental Disorders, fourth edition), characterized by at least five of the following symptoms: (i) depressed mood, (ii) diminished interest or pleasure in almost all activities, (iii) weight or appetite gain or loss, (iv) insomnia or hypersomnia, (v) psychomotor agitation or retardation, (vi) fatigue or loss of energy, (vii) feelings of worthlessness, (viii) diminished ability to concentrate and (ix) recurrent thoughts of death (not just fear of) or suicide attempt or plan. Either of item (i) or (ii) needs to be present. The symptoms need to be present for at least two weeks and most symptoms must be present most of the day, nearly every day.

Symptoms of anxiety disorders

According to DSM-IV the anxiety disorders include generalized anxiety disorder (GAD), social phobia, panic disorder, specific phobias, obsessive-compulsive disorder (OCD) and post-traumatic stress disorder (PTSD). To what extent OCD should be regarded as an anxiety disorder has however been questioned, and it is also often pointed out that GAD may be more closely related to depression than to the other anxiety disorders.

Social phobia is characterized by excessive and unreasonable fear provoked by exposure to, or anticipation of, unfamiliar people in social or performance situations, and includes feelings of embarrassment and/or humiliation. GAD is characterized by excessive anxiety and worry during most days, lasting for at least six months. At least three of the symptoms restlessness, fatigue, concentration problems, irritability, muscular tension and sleep disturbance need to be present, and the symptoms must affect social or occupational function. Panic disorder implicates recurrent panic attacks, which are characterized by discrete periods of fear in the absence of real danger, accompanied by at least four symptoms such as shortness of breath, feelings of choking, palpitation, sweating, trembling or shaking, chest pain, a feeling of losing control or going crazy and fear of dying. The main features of specific phobia are excessive and irrational fear or anxiety provoked by exposure to or anticipation of a specific feared object or situation, such as spiders (arachnophobia) or heights (acrophobia). Subjects with OCD suffer from obsessions, *i.e.* anxiogenic recurrent and persistent thoughts and impulses, and compulsions, *i.e.* repetitive behaviours that the subject feels driven to perform in response to the obsession, and which lead to anxiety relief. PTSD is characterized *e.g.* by the re-experiencing of a traumatic event, that, when it occurred caused substantial fear or horror.

Symptoms of premenstrual dysphoric disorder

Premenstrual dysphoric disorder (PMDD) is characterized by irritable and depressed mood. Common complaints during the luteal phase of the menstrual cycle are mood symptoms, including irritability, sadness and affective lability, the latter expressed as sentimentality or tearfulness, and somatic symptoms including *e.g.* breast tenderness, bloating and headaches.

The DSM-IV diagnosis PMDD requires presence of at least one of the core mood symptoms, *i.e.* irritability, depressed mood, anxiety or affective lability, and also requires for the symptoms to be present during the luteal phase and absent during the follicular phase of the menstrual cycle and to affect professional, social or family function. The most prominent symptom is usually irritability.[61] During the luteal phase, the risk for suicidal attempts is increased in women with PMDD, and the severity of the mood-induced impairments is of the same magnitude as that for patients with MDD during depressive episodes.[62]

Prevalence of mood & anxiety disorders

Depression and anxiety disorders are more common in women than in men; the lifetime prevalence of depression is approximately 20-40% in women and 10-20% in men.[63-66] The lifetime prevalence for any anxiety disorder is approximately 17%.[67] The 7-12% prevalence for social phobia is the largest among the anxiety disorders.[63,68-70] The prevalence of PMDD, a disorder that only affects fertile women, is approximately 5% in this group.[61]

Comorbidity between depression & anxiety

Comorbid anxiety disorders are common in depression, including GAD, panic disorder and phobias; as an example, over 70% of subjects with GAD have a history of depression[71] and up to 90% of patients with anxiety disorders experience a depressive episode some time in life.[72] High levels of anxiety-related traits, such as neuroticism, have been shown to predict, or at least to be strongly related, to depression.[73,74]

Heritability of mood & anxiety disorders

The heritability of unipolar depression is under 50%.[8,75,76] This is a low heritability compared to that of *e.g.* bipolar disorder, displaying a heritability of almost 90%.[77] Non-heritable factors may thus be assumed to play a considerable role for the aetiology. Childhood trauma and stressful life events are associated with an increased risk for depression[78-80]; the risk for depression is doubled by exposure to childhood trauma and approximately 50% of those who suffer childhood trauma display a depressive episode in their lifetime.[81-85] The variation in vulnerability to trauma between individuals is probably due to genes, a notion that is supported by findings showing interactive effects between genetic variation on the one hand, and stressful life events just prior to depression onset, or childhood maltreatment, on the other, in predicting depression.[86] Also the exposure to stressful life events is however heritable,[18] and the genes of the parents may increase the risk for exposure to stressful life events of their offspring. Given that genetic variation can influence environmental exposure, it is possible that some alleged gene-environment interactions are instead due to interactions between genes, one of the interacting genes being the one that influences the environmental exposure. The predictive value of stressful life events for depression is also rendered complicated by (i) the increased reporting of life events as stressful when depressed,[87] especially when reports of stressful life events and depression are given simultaneously,[88-90] (ii) the possibility that the same genetic variants may increase the risk for exposure to stressful life events and the risk for depression,[89] and (iii) the possibility that personality traits associated with depression, and/or depression *per se,* increase the risk of being exposed to stressful life events.[91] Most of the anxiety disorders, including social phobia, display heritabilities of approximately 50%[9,92] as do anxiety-related personality traits [93] and PMDD.[94] The heritability for depression has been reported to be partly shared with that for anxiety disorders and with that for anxiety-related traits, thus indicating that the same genes may affect these different traits.[74,95-97]

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Pharmacological treatments of mood & anxiety disorders

The most commonly prescribed antidepressant drugs are serotonin reuptake inhibitors (SRIs, see below in the SEROTONIN section), which are effective also for anxiety disorders and for PMDD.[98-103] These drugs display an approximately 4-week-long delayed onset of antidepressant effect and an approximately 8-week-long delay of anxiety-reducing action; moreover, during the initial period of administration they may enhance anxiety, particularly in subjects with panic disorder.[104-106] For PMDD, however, the response is instant and SRIs can hence be administered intermittently (*i.e.* only during the luteal phase of the menstrual cycle).[101,107] Anxiety-related traits, as measured with the scales harm avoidance and self-directedness (see PERSONALITY), may also display reductions after chronic SRI treatment, the latter also in healthy individuals.[108,109] Also anxiety-reducing are benzodiazepines, which are agonists on the gamma-aminobutyric acid (GABA) receptor, subtype A, and thus reduce excitability of the central nervous system. Benzodiazepines can be used to counteract anxiety-related symptoms upon initial use of SRIs, and in addition to being anxiolytic, benzodiazepines also have sedative, muscle-relaxing and anticonvulsant effects. Longterm use of benzodiazepines leads to addiction.

Brain regions implicated in mood & anxiety

The brain cortex is divided into Brodmann areas (BA), defined and numbered by Brodmann[110] and based on the cytoarchitectural organization of neurons he observed in the cortex (Figure 5).[111] Several regions have been proposed as those responsible for mood and anxiety symptoms, including *e.g.* regions related to fear, emotional memory, stress and reward. Two of these are the hippocampus and amygdala, which are depicted in Figure 5 and discussed below.

Hippocampus

The involvement of the hippocampus in depression has been suggested by: (i) the discovery of reduced hippocampal volume in a subgroup of depressed patients,[112-117] (ii) the dysregulation of the hypothalamus-pituitary-adrenal (HPA) axis, which is partly regulated by the hippocampus, in this disorder,[118] and (iii) animal studies showing chronic stress and chronic glucocorticoid treatment to induce depressive-like symptoms and reduced hippocampal plasticity, including *e.g.* reduced dendritic sprouting and reduced neoneurogenesis,[119-122] all of which are reversed by different antidepressant treatments.[115,123-127] However, several antidepressant treatments are effective in animal models[E] also when neoneurogenesis is inhibited[115,128]; moreover, the importance of neoneurogenesis in the adult organism is probably different in rodents and humans. The work of Pasko Rakic thus suggests that if new neurons are produced in adult human brains, they would probably not be incorporated into established networks[129] and hence not be of functional importance, possibly because such incorporation would impair longterm memory or increase irrelevant connections between neurons.[130] Although extensively investigated in relation to depression, lesion of the hippocampus causes impairments of explicit memory[F] but does not induce depressive symptoms,[131] thus arguing against the notion that hippocampal dysfunction plays an important role in depression aetiology.

Since MZ twins discordant for depression are discordant also for hippocampal volume, reduced hippocampal volume in depression does not seem to be related to the genetic part of the aetiology for depression.[132] It may, however, be associated with environmental risk factors

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for depression, and thus possibly to the environmental part of depression aetiology. Reduced hippocampal volume has been reported for first-episode cases,[112] suggesting that it is not a consequence of depression. The hippocampus reduction has been shown to correlate with stressful life events prior to depression onset and the volume has been reported to be more reduced in depressed subjects who have experienced childhood trauma than in those who have not.[133-137] A small hippocampal volume may also be a risk factor for PTSD, since both subjects with PTSD and their unaffected relatives display reduced volumes.[138]
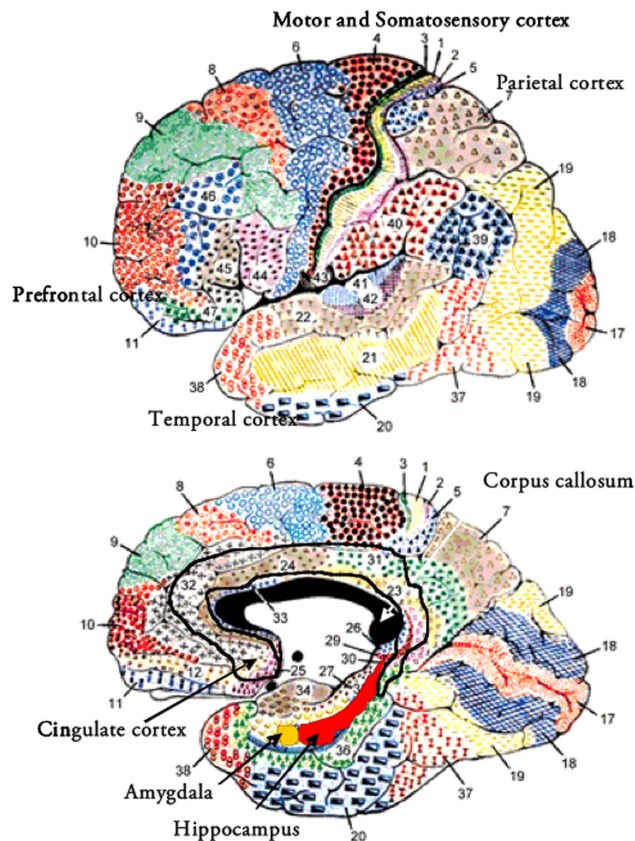
Figure 5. Brain regions.

The amygdala

The amygdala reacts to emotional stimuli, with largest activation seen for threat and fears.[139-144] It is involved in the acquisition and expression of fear and fear conditioning,[E 5,145-148] and in the enhanced memory displayed for emotional stimuli, as compared to neutral. The magnitude of the amygdala engagement during encoding of emotional stimuli correlates with retrieval performance.[149-154] A reduced volume of the amygdala may be associated with an increased acquisition of fear and conditioned fear response, as well as with an elevated rise of cortisol levels to stress.[155] Lesions of the amygdala results in social disinhibition, emotional blunting, reduced fear conditioning and in the absence of reactions associated with emotional states[5,156,157], whereas electric stimulation of the amygdala may increase glucocorticoid levels and fear-induced attention, vigilance and freezing.[158]

The volume of the amygdala may be reduced in depression.[159,160] The reactivity of the amygdala to emotional faces, *i.e.* the difference in amygdala activity when a person is presented with an emotional face as compared to when he or she is presented with a neutral face (reactivity = activity$_{emotional}$–activity$_{neutral}$), has been shown to be increased in depressive subjects[36,161-163](however see also[164]). The amygdala reactivity may be increased during the premenstrual phase in women with PMDD.[165] Several anxiety disorders including social phobia,[166-175] specific phobias,[167] PTSD[167,176-180] and GAD[181] also display exaggerated amygdala reactivation to emotional stimuli and anxiety provocation, with larger effects seen for aversive than for positive stimuli[177]. In addition, increased levels of anxiety-related traits and elevated anxiety sensitivity have been associated with increased amygdala reactivity to emotional stimuli.[172,182] Interestingly, the emotionally valenced stimuli need not reach conscious awareness to engage the amygdala.[36,144,183]

The exaggerated amygdala reactivity in depression and anxiety disorders has been shown to be reduced by antidepressant and anxiety-reducing treatments, including pharmacological treatment, cognitive-behavioural therapy and sleep deprivation,[36,69,184-187] an attenuation that correlates with response and also is present in healthy individuals,[188,189] in whom chronic SRI treatment may reduce hostility and anxiety-related traits.[109,190,191]

Depression also appears to be related to increased amygdala metabolism or activity at rest,[192-196] an intermediate phenotype that may correlate with depression severity.[197] Also amygdala metabolism appears to be reduced by antidepressant treatment.[185]

Amygdala reactivity to angry faces is investigated in paper I. Subjects with social phobia and controls both displayed enhanced activation when presented with the angry faces; however, there was no significant difference in reactivity between these groups. Variation in serotonin-related genes was however linked to amygdala reactivity.

The interaction of frontal regions with the amygdala
Stimulation of regions in the prefrontal cortex (PFC) can impair amygdala-dependent processes such as fear conditioning.[5,156,157] Reduced inhibitory prefrontal control over amygdala activation may be related to the increased response of the amygdala in depression and anxiety; moreover, this inhibitory control may be affected by chronic stress exposure.[163,180,181,198-200]

Both the amygdala and the anterior cingulate cortex (ACC) display lower volumes in depression,[159,201] and a compromised connectivity between these two regions has been suggested to be related to depression and anxiety[202]. The ACC is activated during induction of happy and sad mood[203] and during distal threat.[204] A circuitry involving connections between the amygdala and different parts of the ACC may be important in emotional processing, and this circuitry shows lower connectivity in carriers of certain variants of a polymorphism in the promoter of the gene encoding the protein responsible for serotonin reuptake, explaining 30% of the variation in anxiety-related personality traits.[202,205] In depressed patients, the rest activity and metabolism of the ACC has been reported to be reduced.[185,201] The ACC is also implicated in antidepressant response; antidepressant treatment may downregulate the resting activity of the subgenual region of the ACC in responders[185,206] and stimulation of the subgenual ACC has been reported to relieve symptoms of depression.[207]

Neural correlates of the placebo response in depression and anxiety
Placebo refers to the beneficial outcome of a treatment known to have no specific effect for the condition being treated, but in the efficacy of which the patient believes. Approximately 20 -

50% respond to placebo in clinical placebo-controlled studies of antidepressant and anxiety-reducing effect.[208-212] The response to placebo is believed to be due to expectancies.[213] It should however be emphasized that some of the symptom reduction often attributed the influence of placebo in antidepressant drug trials may be due to other factors, such as spontaneous recovery.

Placebo administration not only influences behaviour and symptomathology but also disorder-related brain activity.[214,215] Several of the changes observed after both pharmacological and psychological treatment of depression and anxiety,[187] are observed also after placebo treatment,[213,216] *e.g.* the reduction of activity in limbic regions.[215] In addition, subjects responding to placebo usually display an increased activity of the rostral ACC, possibly reflecting a conflict resolution between expectations and experience.[213] Subjective reports of unpleasant feelings and amygdala response to unpleasant emotional stimuli can be reduced by falsely telling subjects that they are receiving anxiolytics, when they have previously experienced the effect of the drug.[217]

> **In paper II, amygdala activity during public speaking was measured before and after chronic placebo treatment. Responders displayed reduced public speaking-induced amygdala activity after placebo treatment. The reduced amygdala activity was only observed in carriers of certain serotonin-related genotypes.**

PERSONALITY TRAITS

Personality traits can be assessed by different questionnaires. Two of these are the Karolinska Scales of Personality (KSP) and the Temperament and Character Inventory (TCI). Personality as assessed with both these scales has been shown to be partly heritable.[11,14,93]

Karolinska Scales of Personality

KSP consists of 135 items forming 15 subscales. These subscales are often classified into four factors covering different dimensions of temperament: neuroticism, psychoticism, non-conformity and extraversion.[93,218-220]

The neuroticism factor of KSP measures anxiety and depression-related traits and includes the subscales psychic and somatic anxiety, muscular tension, psychastenia, socialization, guilt and inhibition of aggression. The psychic anxiety subscale includes items about feeling worried, anticipatory anxiety for minor things, bad self-confidence, not speaking up for oneself, being easily offended, shyness and insecurity around strangers and acquaintances. Somatic anxiety includes restlessness without reason and not feeling at ease, as well as several somatic symptoms such as heart pounding loud or sweating and occasional feelings of panic. Subjects scoring high on muscular tension are tense and un-relaxed, those scoring high on psychastenia are easily fatigued, those with high socialization are satisfied with their life situation and those with high guilt often feel remorseful and ashamed about thoughts and actions. Inhibition of aggression means that the subject acknowledges when being badly treated and gets upset without saying or showing it.

The non-conformity factor in KSP is related to anger, and is characterized by high verbal and indirect aggression, *i.e.* easily getting into quarrels, telling or shouting at people when not agreeing with them and expressing anger by slamming doors or throwing things. The non-conformity factor also includes being easily irritated and lacking patience with people, and

having low scores on the social desirability subscale, *i.e.* that it is unimportant for the subject to be liked and to be seen as a good person.

KSP also includes the personality factors extraversion and psychoticism, the former consisting of the subscales impulsiveness and monotony avoidance and the latter of the subscales suspicion and detachment. Impulsiveness measures non-planning and acting on the spur of the moment, and monotony avoidance measures how much the subject avoids routine and desires action. Detachment means avoiding involvement in others, and a highly suspicious individual believes people to laugh at or offend him or her and also wonders for what reasons people are nice to him or her (distrusting motives).

Temperament and character inventory

TCI is based on a self-administered true/false questionnaire and is designed to assess personality along four temperament dimensions – novelty seeking, harm avoidance, reward dependence and persistence – and along three character dimensions – self-directedness, cooperativeness, and self-transcendence.[221,222] The original TCI scales have been translated into Swedish.[223]

The harm avoidance (HA) dimension of TCI measures anxiety- and depression-related traits and includes questions regarding anticipatory anxiety, fear of uncertainty, shyness, behavioural inhibition, fatigability and asthenia; the majority of items deals with anticipatory worries and anxiety, especially fear of the unfamiliar (9 items out of 20), shyness and fear of strangers and social interaction with strangers (5 items), and lack of strength (3 items). The dimensions self-directedness and possibly also reward dependence may also be related to anxiety. High scores on self-directedness indicates high will-power and choosing behaviours that optimizes the chance of reaching goals, whereas low scores indicate that the subject does not believe that he or she is able to control his or her choices, that the subject wants others to solve his or her problems and the wish for superpowers. Reward dependence measures the degree to which the subjects depends on approval from others and lacks independence. High scores are also indicative of a person that is sentimental, talks a lot about his or her feelings and is empathic and helpful.

The temperament dimension novelty seeking includes questions on impulsivity and flexibility, on being outgoing versus reserved, and on the wish to try new things. Several items also address wasting money, not liking laws and lying. The temperament dimension persistence is a measure of how hard the individual tries to achieve well and works hard even when exhausted. High scores on the character dimension cooperativeness reflect a person who is accepting, empathic, non-egoistic and who likes to help others. The self-transcendence scale consists of questions regarding how much the subject is devoted to a religion or philosophy and to what degree he or she believes in supernatural things such as clairvoyance, how much he or she has sacrificed for world peace and justice, and how much he or she has experienced feelings of spiritual connection with nature when relaxed.

THE SEROTONERGIC SYSTEM

The development of the serotonergic system

Serotonergic neurons reside in the raphe nuclei, caudal to the isthmus, and project to most parts of the brain. Rostral parts of the raphe, including the nucleus raphe pontis, the median raphe (nucleus centralis superior) and the dorsal raphe, project to the forebrain, whereas caudal parts, including nucleus raphe obscurus, magnus and pallidus, project to the spinal cord. The division of the raphe into caudal and rostral parts displays an approximate overlap with gene expression profiles.[224]

An intricate network of essential factors initiates the formation of serotonergic neurons. Sonic hedgehog and fibroblast growth factor 8 (FGF8) are essential for the rostral and the caudal raphe neurons, respectively, and FGF4 and NKX2-2 for serotonergic specification.[225-227] The interaction between NKX2-2 and NKX6-1 is important for expression of *GATA3* and *GATA2*, which encode GATA-binding protein 2 and 3 and which induce the expression of *LMX1B* and *FEV* (Pet1 in animals). The latter two are required for the expression of enzymes of importance for serotonin synthesis – the tryptophan hydroxylases (TPH1 & TPH2) – and for the expression of the serotonin transporter.[225]

Serotonin synthesis and turnover

The serotonin precursor tryptophan is an essential amino acid that is actively transported into the brain. In the rate-limiting step of serotonin synthesis, it is converted by TPH to 5-hydroxytryptophan (5-HTP), which subsequently is converted into serotonin (5-hydroxytryptamine; 5-HT) by aromatic amino acid decarboxylase (AADC).

In adults,[228,229] TPH1 is expressed primarily in the periphery and in the pineal gland,[230,231] whereas TPH2 is brain-specific[232,233]; its expression is at least fourfold to that of TPH1 in all brain regions except for the pineal gland.[232,234] TPH can be inhibited by *para*-chlorophenylalanine (*p*CPA), with serotonin depletion as a consequence. Serotonin is degraded by monoamine oxidase, preferably the A subtype (MAOA), to 5-hydroxyindole-acetic acid (5-HIAA).

Serotonin receptors

There are at least 17 serotonin receptor subtypes, all of which are G-protein coupled, except for the 5-HT3 receptor, which is a ligand-gated ion channel composed of five subunits. The serotonergic autoreceptors of type 1A and 1B – 5-HT1A situated on soma and dendrites and 5-HT1B on nerve terminals – exert negative feedback on serotonergic neurotransmission. Whereas stimulation of 5-HT1A autoreceptors primarily inhibits the firing of serotonergic neurons [235], 5-HT1B receptors decrease synthesis rate and release.[236,237] Both these receptor subtypes are also present postsynaptically where they decrease excitability of the postsynaptic neuron.

The serotonin transporter

The serotonin transporter (also referred to as 5-HTT or SERT) performs sodium-dependent transport of serotonin from the synaptic cleft back into the nerve terminal, so-called serotonin reuptake, thus determining the duration and intensity of the influence of the transmitter on

serotonergic receptors. The serotonin transporter is the target of antidepressant SRIs, which inhibit serotonin reuptake from the synaptic cleft.

By inhibiting serotonin reuptake, the SRIs cause an acute, approximately fivefold, increase in extracellular serotonin levels,[238,239] with the largest elevation observed in the raphe nuclei where the density of the transporter is highest.[240-244] As extracellular serotonin levels increase, the negative feedback system (5-HT1A and 5-HT1B receptors)[245] is activated, leading to a reduced firing of serotonergic neurons and a reduced synthesis and release of serotonin.[235,246-249] The reduction in serotonergic nerve cell firing induced by SRIs is blocked by 5-HT1A antagonists.[250] The increased extracellular serotonin levels seen after acute SRI treatment represents the net effect of serotonin transporter inhibition and 5-HT1A-mediated negative feedback; 5-HT1A thus restrains the SRI-induced elevation in extracellular serotonin levels as illustrated by an increased elevation of serotonin when a 5-HT1A antagonist is simultaneously administered.[251]

Administration of SRIs leads to reduced serotonin brain tissue concentration, as measured in animals,[252] and 5-HIAA levels are reduced after acute or chronic SRI treatment, both in lumbar cerebrospinal fluid (CSF) and in jugular venous blood.[253,254]

Mice failing to express the *5-HTT,* due to genetic manipulation (*5-HTT* knock-outs), display increased baseline extracellular serotonin levels, 50% reductions in the number of serotonergic neurons, substantially reduced firing rates in the dorsal raphe, altered serotonin receptor function and approximately 50% reduction in serotonin tissue concentrations.[255-261] Similar changes are observed also in heterozygous knock-outs and also for animals with transient inhibition of the serotonin transporter perinatally.[262]

SEROTONIN IN MOOD & ANXIETY

The influence of serotonin on mood & anxiety

The majority of antidepressants act on the serotonergic system. MAO inhibitors thus block the degradation of serotonin and tricyclic antidepressants (TCAs), serotonin and noradrenaline reuptake inhibitors (SNRIs) and selective serotonin reuptake inhibitors (SSRIs) all inhibit the serotonin transporter (*i.e.* they are SRIs). Chronic treatment with SRIs is effective both for depression and anxiety disorders.

Administration of 5-HTP may give rise to an amelioration of depressive symptoms,[263] whereas depletion of the serotonin precursor tryptophan[G] may induce depressive mood.[264] In particular, it leads to relapse of depressive subjects in remission[265-267] and reduces mood in subjects with a family history of depression.[6,7,265,268,269] Serotonin depletion by *p*CPA also appears to induce relapse in depressed patients.[270] In animals, however, depletion of serotonin using *p*CPA results in reduced anxiety-like behaviour and increased aggression, feeding and sexual behaviours[271-273] and acute depletion of tryptophan does not appear to affect anxiety-related behaviour,[274,275] whereas repeated depletion may increase anxiety-related behaviours.[276]

Serotonin-related biological markers in mood & anxiety disorders

As yet, no studies aiming to measure extracellular serotonin concentrations in different regions of the living human brain have been published. Depression have by some been suggested to be associated with reduced plasma tryptophan availability[277] and with reduced increase in 5-HTP after tryptophan administration.[278] In women with PMDD, 5-HTP levels may be lower during phases of PMD symptoms.[279]

The serotonin-releasing agents fenfluramine and mCPP (1-(3-chlorophenyl)-piperazine) normally induce a serotonin-dependent increase in prolactin, an increase that has been reported to be blunted in depressive subjects,[280] in suicide attempters,[281] and in subjects with PMDD, PTSD and OCD, in subjects with high levels of anxiety-related traits, and in monkeys displaying impaired social function.[282-287] Serotonin uptake into platelets, the density of serotonin transporters in platelets and the MAO activity in platelets have also been reported to be abnormal in depression,[288,289] and women with PMDD have been reported to have reduced density of platelet serotonin transporters.[290]

Levels of the serotonin metabolite 5-HIAA are lower in the lumbar CSF of suicide attempters with major depression[291-295] and other psychiatric diagnoses.[296] This relationship may be specific for a violent, aggressive or impulsive subgroup of depressed suicidal patients. 5-HIAA levels in CSF have been reported to be increased in subjects with depression and in subjects with comorbid depression and anxiety.[297-299] 5-HIAA levels in lumbar CSF may however not be a good measure of serotonin turnover, since it only measures a small portion of the 5-HIAA produced in the brain,[300] and since several studies have failed to find a correlation between ventricular and lumbar 5-HIAA.[301] 5-HIAA levels as measured in jugular venous blood have also been shown to be substantially increased[H] in subjects with depression or anxiety disorders.[302,303]

> Some papers in this thesis find variation in the genes encoding the TPH2, the serotonin transporter, the 5-HT3 receptor and GATA2 to be related to mood and/or anxiety-related phenotypes (papers I-II, IV, V). In paper III, the density of serotonin transporters and 5-HT1A receptors in the human brain is investigated in relation to genetic variation in a neurotrophic factor.

The serotonin transporter in mood & anxiety disorders
Serotonin transporter availability has been shown to be decreased post-mortem in the PFC and brainstem of suicide victims[304-307] and *in vivo* in the raphe of depressed subjects and remitted depressed subjects,[308,309] as well as in the raphe and amygdala of drug-naïve depressed subjects[310,311] (see also[312]). The reduction has in some studies been shown either to correlate with anxiety severity or to be more closely related to levels of anxiety than to depression.[308,313,314]

The observed low availability of the transporter can be due to a number of factors. It could be compensatory to other deficits in the serotonergic system. It could also be due to reduced serotonergic innervation and/or to a reduced number of serotonergic neurons. Notably, a reduction of the number of neurons in the dorsal raphe has been reported for patients with mood disorders[315]; however, an increased density of the same magnitude has been reported for depressed suicides.[316] Although one of the above-mentioned studies was conducted on drug-naïve subjects, it is worth noting that longterm SRI treatment may reduce the expression of the serotonin transporter.[317-320]

The reduced serotonin transporter availability could be related to serotonin-related heritable factors that increase the risk for depression (see 5-HTTLPR in SEROTONIN-RELATED GENES), or to environmental factors that increase the risk for depression; early life stress has been shown to reduce transporter availability in most parts of the brain including raphe, amygdala and ACC in rhesus monkeys.[321] A reduced availability of serotonin transporters may also be related to depression-induced adaptive changes.

Both single photon emission computed tomography (SPECT) and positron emission tomography (PET) with numerous different radioligands are used for *in vivo* measurement of the availability of receptors and transporters in the brain. Some discrepancies between studies are expected due to the use of different tracers. The SPECT tracer ^{123}I-β-CIT can measure density of both the dopamine and the serotonin transporters but at different time intervals after administration.[322] The most commonly used PET radioligands for assessment of serotonin transporter availability are [11C]DASB (3-amino-4-(2-dimethylaminomethyl-phenyl-sulfanyl)-benzonitrile) and [11C]MADAM ([11C]N,N-dimethyl-2-(2-amino-4-methylphenyl thio)benzylamine), both of which are selective for the serotonin transporter and display low non-specific binding.[323-326] Competition with endogenous serotonin has recently been shown for [11C]DASB,[327] but may be true also for other tracers,[323] thus complicating the interpretation of the results by implicating a lower binding when endogenous serotonin levels are high.

Since amygdala rest activity and reactivity may be increased in depression and anxiety disorders, the finding of low serotonin transporter availability in depression and anxiety is in line with the notion that low transporter availability is associated with high amygdala reactivity to fearful faces as measured by PET and fMRI in the same subjects.[328]

The 5-HT1A receptor in mood & anxiety disorders
The density of 5-HT1A receptors can be measured *in vivo* using the high-affinity 5-HT1A antagonist and PET radioligand [11C]WAY100635.[329] The density of 5-HT1A receptors appears to be reduced in social phobia[330] and panic disorder.[331] The results of studies investigating the 5-HT1A in depression and suicide diverge: Some report decreased *in vivo* and post-mortem expression of the 5-HT1A receptor in depression and in depressed suicides,[332-337] whereas others report that 5-HT1A density is increased in depression and suicide victims and that the increase may correlate with depression severity.[304,338-341] In women with PMDD, the density of 5-HT1A receptors does not appear to display normal fluctuation during the cycle.[342] SRIs have been shown to reduce 5-HT1A density in several brain regions, but not in raphe.[343]

TPH2 levels in relation to depression
Increased TPH2 levels as well as increased TPH2 protein per neuron have been found in the raphe nuclei of depressed suicides,[344-348] an increase that has been proposed to reflect a response to serotonin deficiency.[346] Findings in depressed suicide victims may, however, not be representative for the whole group of depressed subjects, as illustrated by the increased 5-HIAA levels in depressed subjects and decreased levels in depressed suicides. In animal models, chronic antidepressant treatment has been shown to increase levels of TPH2, especially in raphe.[228,349]

Behavioural effects of manipulation of the serotonergic system in mice
Serotonin transporter gene (*5-HTT*) knock-out mice exhibit increased depression- and anxiety-like behaviours and reduced aggressive behaviours,[257,350-353] changes that are observed also in heterozygous knock-outs and also by perinatal inhibition of either the transporter or MAOA.[262,354-358] The phenotype invoked by early transporter inhibition can not be reversed by adult rescue of *5-HTT* expression.[262]

Knock-out mice for the 5-HT1A receptor gene, *HTR1A*, show anxiety-like behaviours.[359-361] The *HTR1A* knock-out phenotype can be reversed by partial rescue of 5-HT1A expression in the frontal cortex and hippocampus during a critical period in early postnatal development,[362] thus suggesting that serotonergic transmission via postsynaptic 5-HT1A receptors is important

during this time and that the effect of the knock-out is due to the lack of postsynaptic 5-HT1A receptors during this time. However, by blocking the 5-HT1A early in life, the depression-like phenotype in *5-HTT* knock-out mice can be rescued.[363] Agonists of the 5-HT1A receptor display anxiety-reducing effects in animal models,[364-366] a mechanism that probably is mediated by somatodendritic autoreceptors, thus mimicking the anxiety-reducing effect of serotonin depletion.

5-HT3 antagonists display anxiety-reducing and anti-aggressive effects in rodents, primates and humans. In line with this finding, deletion of the gene that encodes the 5-HT3A subunit, *HTR3A,* produces a phenotype characterized by reduced anxiety.[367,368]

Serotonin and antidepressants effect

The effects of SRIs in depression and different anxiety disorders are apparent first after approximately 4 weeks of treatment.[103-105,369] Subjects with PMDD, however, show instant reduction of irritability after acute SRI treatment.[99,101]

The initial period is sometimes characterized by increased anxiety especially for subjects with panic disorder.[106] In animal models, acute SRI treatment increases fear conditioning, whereas chronic treatment reduces it.[370] The increased initial anxiety observed in animals has been shown to be inhibited by serotonin receptor antagonists, thus indicating serotonergic mediation.[371,372]

There are several theories regarding the delayed effect of SRIs. One suggests the reason for the delayed antidepressant response to be that serotonin levels display a gradual increase due to desensitization of 5-HT1A autoreceptors, as suggested by studies indicating that 5-HT1A desensitization is necessary for antidepressant effect[373] and that administration of 5-HT1A receptor partial agonists improves or accelerates the antidepressant response of SRIs.[374,375]

Serotonin levels are however substantially increased after acute SRI treatment, as supported (i) by studies of extracellular serotonin levels in animals,[238,239] (ii) by studies of serotonin in cisternal CSF in monkeys,[376,377] (iii) by the acute onset of therapeutic effect in PMDD, which appears to be mediated by increased serotonin levels,[378] and (iv) by the acute onset of serotonin-dependent side effects, including sexual side effects and increased nausea.[379,380] Moreover, a marked acute increase in extracellular levels of serotonin, as obtained by means of the serotonin-releasing agents mCPP and fenfluramine, does not lead to an instant antidepressant or anti-anxiety response. Also, although the 5-HT1A receptor appears to be desensitized by chronic SRI treatment,[238,381] deletion of 5-HT1A receptors does not seem to affect the timing of the antidepressant response.[382] In addition, 5-HT1A receptors have been shown to retain the capacity to restrain SRI-induced extracellular serotonin levels also after chronic treatment with SRIs, and 5-HT1A antagonists hence still add to the increase in extracellular serotonin levels after chronic SRI treatment.[251]

Another theory of the delayed therapeutic effect is that the increased hippocampal neoneurogenesis, observed after approximately four weeks treatment in rodents,[383] reverses hippocampal pathology, thus leading to antidepressant-like response. The timing of antidepressant response in animal models is however not affected by deletion of new progenitor cells[382]; moreover, to what extent neoneurogenesis takes place, and/or is warranted, in the human hippocampus, remains a matter of controversy. Other theories of the delayed antidepressant response are based on the notion that serotonin induces an upregulation of the expression of so-called neurotrophic factors that facilitate plasticity and restore network function[384-387] (see the SEROTONIN & BDNF section).

In line with these network-related hypotheses, the findings of Zhou et al.[388] suggest that the effect of antidepressants is due to structural, as opposed to biochemical, changes in the serotonergic system. In this study, increased serotonin fiber density and branching in layers four and five of the fronto-parietal cortex and in the limbic regions was found after chronic administration of antidepressants. These observations were made in the absence of any effects on *5-HTT* or *TPH2* expression.

Also in favour of the notion that antidepressants enhance plasticity, several authors have reported increased sprouting of axons and dendrites of hippocampal neurons after treatment with several antidepressant treatment methods in animal models,[123-125] an increase that is accompanied by decreased stress sensitivity.

SEROTONIN-RELATED GENES

Polymorphisms in the *5-HTT*

The most thoroughly studied serotonin-related gene is the one encoding the serotonin transporter, *5-HTT*, which is also called *SLC6A4*, it being the 4th member of the 6th solute carrier family. It is situated on chromosome 17q11-12 and consists of 14 exons.[389]

The 5-HTTLPR is an insertion/deletion polymorphism in the promoter region of the *5-HTT*, resulting in one short allele (S allele) with 14 similar repeat units and one long allele (L allele) with 16 similar repeat units. An SNP, rs25531, situated in repeat 6 of the 5-HTTLPR, was first believed to be located on the L allele of the 5-HTTLPR only, due to different reports of breakpoints for the insertion/deletion. However, since it is repeats 7 and 8 that are present only on the L allele, also the S allele carries this polymorphism.[390-393] Out of those carrying the long 5-HTTLPR allele, 10% carry a G allele on the rs25531 locus, resulting in altered affinity for the AP2 transcription factor, and thus lower promoter activity, rendering the serotonin transporter availability similar to that of carriers of the S allele.[394] In addition to this polymorphism, another SNP, rs25532 (C/T), situated in repeat 14 of the 5-HTTLPR, has been found; a haplotype with the L_{AC} allele of the 5-HTTLPR$_{rs25531\ and\ 25532}$ and the C allele of rs16965628 located in intron 1 has been shown to constitute a high-expressing haplotype.[395] Association studies of the 5-HTTLPR are described in the next section.

A rare gain-of-function mutation, Ile425Val, encoding the transmembrane region 8 of the serotonin transporter, has been found in exon 9. The rare Val allele, which is in absolute LD with the L allele of the 5-HTTLPR, shows increased serotonin transporter function and has been associated with OCD and comorbid disorders including Asperger syndrome and social phobia.[396,397] The Gly56Ala (rs6355) is a rare gain-of-function mutation in exon 2, for which the Ala allele increases transporter function by approximately 75%, and that may confer susceptibility to autism.[398]

Intron 2 of the *5-HTT* holds a VNTR polymorphism called STin2.[399] It has three common alleles of length nine, ten and twelve repeats. The common 12 allele of STin2 may display increased transcriptional activity[400,401] but measures of the availability of the serotonin transporter *in vivo* in the human brain suggest the 10/10 genotype to be associated with increased transporter availability.[402] The 12-allele has been associated with anxiety disorders, including OCD and GAD,[403] and possibly also with increased anxiety-related traits.[218]

Association studies of the 5-HTTLPR

5-HTTLPR and serotonergic transmission
The S allele of the 5-HTTLPR gives rise to lower *in vitro* transcriptional activity of the *5-HTT*.[404] Three PET and SPECT studies measuring *in vivo* transporter availability show lower availability for carriers of the S allele,[402,405,406] although one of them observed the lowest levels for heterozygotes. Another study showed lowest availability in SL carriers and highest in SS carriers.[309] Moreover, some studies have been negative.[407] In combination with the rs25531, carriers of the S or L_G alleles have been reported to display lower availability.[394,408]

The S allele has also been associated with low serotonergic function as measured by a reduced prolactin response to serotonin-releasing agents,[409,410] as well as with reduced platelet serotonin uptake[411] The same allele has also been associated with increased 5-HIAA levels in both humans and monkeys.[298,302,412]

5-HTTLPR and depression & anxiety
Since chronic inhibition of serotonin reuptake by antidepressant drugs reduces depression and anxiety, the 5-HTTLPR has been intensively investigated in the context of its possible relation to the aetiology of these disorders. Paradoxically, given that SRIs inhibit the serotonin transporter and are effective in reducing depression and anxiety, the association for the 5-HTTLPR is in the direction that the S allele, associated with low serotonin transporter function, appears to *increase* the risk for depression and anxiety. The S allele has thus been reported to be associated with anxiety and anxiety-related traits as measured with certain scales,[218,404,413-415] and the same allele has also been associated with attention bias towards anxiety-related stimuli.[416] Although several studies have found suggestive evidence of an association between the S allele and depression, a meta-analysis found that such an increased risk exists for bipolar disorder only,[37] and one study showed that approximately 40% of the association with depression was mediated by the influence of the polymorphism on anxiety-related personality traits.[417] Notably, several studies support the notion that high anxiety-related traits increase the risk for depression[73] and that they also display shared heritability with depression.[74,97]

How the S allele exerts its effect remains uncertain. Since transient inhibition of the transporter perinatally increases adult anxiety- and depression-related traits, and substantially reduces the density of serotonergic neurons in animals, it has been suggested that the anxiety- and depression-related phenotypes observed in carriers of the S allele may be due to low levels of the serotonin transporter (and thus possibly increased extracellular serotonin levels) during some critical moment in development.[262]

5-HTTLPR and stressful life events & depression
Carriers of the 5-HTTLPR S allele react stronger to stress as measured by an increased startle response[418] and increased fear conditioning.[419] The first specific gene-environment interaction reported for a psychiatric trait was a synergistic interaction between the 5-HTTLPR S allele and stressful life events (SLEs) with respect to risk for depression; subjects who had experienced a SLEs between the ages 21 and 25 thus had more depressive symptoms, depression and suicide attempts at age 26 only if they were also carriers of the S allele. The enhanced risk was increased by increased number of SLEs. The possibility that the gene-environment interaction was due to a gene-gene interaction, where the second gene were to increase the exposure to SLE, was considered unlikely since no interaction was observed between the 5-HTTLPR and

SLEs when the life event occurred after the onset of depression. Also subjects who had been maltreated as children had increased prevalence of depression at age 26.[86] The 5-HTTLPR-SLE interaction was later replicated for the SS genotype[420]; carriers of this genotype were thus more sensitive to the depressogenic effects of SLEs and had a sevenfold risk of developing depression after SLEs, whereas the increased risk after exposure to SLEs for carriers of the LL genotype was only twofold. This interaction has also been replicated for depression in children after removal from their families [421] and for the genotypic combination of the 5-HTTLPR and rs25531.[422] Carriers of the S or L_G alleles also report that they experience more anxious mood on stressful days.[423]

Studies in monkeys show evidence of increased neuroendocrine responses, including increased ACTH response to stress, in S carriers (of the orthologous 5-HTTLPR). In females, this increased response was present only in monkeys who had been reared in stressful environments.[424] Interaction between the S allele and stressful environments has also been reported for related phenotypes in monkeys[425] In addition, monkeys reared in stressful environments may display increased 5-HIAA levels only if they also carry the S allele.[412]

The majority of the studies reporting an interaction between the 5-HTTLPR and SLEs do not observe a main effect of the 5-HTTLPR; to explain this, it has been proposed that carriers of the S allele may be influenced by both positive and negative life events,[37] a proposition that is supported by the notion that depressed children carrying the S allele show increased response to social support compared to LL carriers[421] and also by findings of increased emotional processing in S carriers, irrespective of whether the valence of the emotion is positive or negative.[426]

In paper V, the 5-HTTLPR S allele, in combination with another polymorphism, is associated with an enhanced exposure to controllable SLEs. The S allele is however associated with avoidance of controllable SLEs in subjects with high anxiety-related personality traits, whereas the correlation between anxiety-related personality traits, on the one hand, and the number of SLEs experienced during the last year, on the other, is positive in carriers of the LL genotype.

5-HTTLPR and reactivity of the amygdala
The 5-HTTLPR has been investigated in the context of the increased amygdala reactivity seen in depression. In healthy subjects, the 5-HTTLPR S allele has thus been associated with enhanced activity to emotional faces and words compared to the activity elicited by neutral faces or neutral geometrical objects.[35,38,199,427,428] Since the S allele may reduce the expression of the serotonin transporter, this finding is in line with the negative correlation observed between low serotonin transporter binding and enhanced amygdala reactivity.[328] The increased amygdala reactivity to emotional faces for the S allele, and for the L_G allele of the 5-HTTLPR$_{rs25531}$, has been shown to be valid for both healthy and depressed subjects, and also when the faces are shown for such a short period of time that the perception does not reach consciousness.[426] The effects of the 5-HTTLPR S allele on emotional perception have been shown to be additive with other functional polymorphisms that appear to affect amygdala reactivity.[429,430]

5-HTTLPR and connectivity between the amygdala and the anterior cingulate cortex (ACC)
Although the influence of the S allele on amygdala reactivity does not appear to explain variation in anxiety- and depression-related traits,[35] reduced functional connectivity between

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the amygdala and parts of the ACC during exposure to fearful faces, observed in S carriers, may explain 30% of the variance in harm avoidance.[202] The S allele has also been associated with increased resting metabolism of the ACC[431] and low volume of the perigenual ACC.[201,202] Another study showed stronger coupling between an adjacent region, BA10 in the ventromedial PFC, and amygdala in S carriers, an increased activity that has been proposed to be compensatory to the increased activity of the amygdala.[202,428]

5-HTTLPR and baseline amygdala activity

An alternative interpretation to the elevated activation of the amygdala in response to emotional conditions compared to neutral ones in carriers of the S allele, proposed by Canli et al.[432], is that S carriers may display lower activation to the neutral conditions. Indeed, the activation to neutral conditions was shown to be lower than that while resting in S carriers. This contrast was subsequently shown to be driven by higher activity during the rest condition[433,434] (rather than lower response to the neutral stimulus). This finding was proposed to be due to an increased anticipatory anxiety in carriers of the S allele, which then would be reduced when the task begins,[435] but was subsequently shown to be independent of state anxiety.[434] Rest activity (as compared to response to neutral faces) correlated with the number of SLEs experienced in S carriers only.[433] In LL carriers, this correlation was negative, *i.e.* the more exposure to SLEs, the smaller the rest activity of the amygdala.

In spite of this genetic influence on amygdala reactivity, both in healthy and depressed subjects,[199,429] and in spite of the fact that increased amygdala activity has been observed also in familial depression,[193] genetic influence on amygdala reactivity may be unrelated to genetic risk for depression and anxiety. When comparing the amygdala reactivity of MZ twins concordant and discordant for high and low depression and anxiety risk, it was thus found that a twin with high risk for depression or anxiety due to exposure to SLEs, displayed higher amygdala reactivity to negative emotional faces compared to its discordant low-risk twin. However, for twins concordant for high depression- and anxiety-related traits, amygdala reactivity was low, and significantly lower than that for twins concordant for low such traits,[436] suggesting that amygdala reactivity is increased only in those high-risk subjects that exhibit increased risk due to environmental and not genetic factors. The results of this twin study did not seem to be influenced by the genotype for 5-HTTLPR.

5-HTTLPR and tryptophan depletion

For subjects who are in remission from depression, tryptophan depletion has been reported to lead to relapse and increased amygdala, hippocampal and subgenual ACC metabolism only in LL or L_AL_A carriers[437,438]; in contrast, non-depressed SS-carriers show more effect of tryptophan depletion on anxiety and depression scores than carriers of the LL genotype,[438-440] as well as impaired recognition of fearful faces.[441] An increased effect of tryptophan depletion in carriers of the S allele is observed also in healthy subjects who have a family history of depression, indicating that the differential effect of the 5-HTTLPR on the effects of tryptophan depletion is not related to its possible effect on the aetiology of depression but that it is a consequence either of disease or of antidepressant medication; although unmedicated for at least three months before tryptophan depletion, these depressed subjects had previously been treated with antidepressants. The differential relationship between genotype and tryptophan depletion in subjects with and without depression was proposed to be due to different regulatory tone in the serotonergic system in cases and controls.[438] A differential response to tryptophan depletion in healthy S and LL carriers has also been found for motivation; in S carriers, tryptophan

depletion thus leads to a reduced motivation and memory performance, whereas carriers of the LL genotype exhibit increased motivation after tryptophan depletion.[439,442]

Polymorphisms in the tryptophan hydroxylase genes *TPH1* and *TPH2*

TPH1 and TPH2 share approximately 70% amino acid homology and are encoded by genes situated on chromosomes 11p15 and 12q21, respectively. Whereas TPH1 is expressed in raphe during development,[228,229] TPH2 is much more abundant in the raphe and in other brain regions, except for the pineal gland, in adult organisms.[229-232,234,443]

TPH1

Polymorphisms in *TPH1* have been associated with mood-related phenotypes; the A allele of the A218C, situated in intron 7 of the gene, has been related to anger, depression, anxiety, compromised antidepressant response and suicide.[444-451] This influence may be due to the activity of TPH1 during development.[229]

Structure of TPH2

TPH2 has 11 exons. Strong LD has been found over *TPH2*, in one study between the promoter region (~400 upstream) and intron 5[452] and in another from the promoter region (~800 upstream) to intron 8[453], the latter revealing a structure where every other allele of the haplotype was the rare variant, suggesting selection to maintain the ancient chromosomes. The 212121 (1 for the common allele and 2 for the rare) haplotype was associated with suicide, depression and anxiety, and also with lower serotonin turnover as measured by 5-HIAA levels, which have been shown to be partly heritable in monkeys and possibly also in humans.[454,455] Haghighi et al. recently found the existence of a truncated form of *TPH2*, lacking the catalytic site encoded by exon 11. This truncated variant was found to be composed of 7 exons only, and parts of intron 5 of the long more abundant *TPH2* variant belong to exon 6 of the short, truncated *TPH2* variant. No associations between polymorphisms and the relative amount of the two forms were reported.[456]

TPH2 exon polymorphisms

In the exon (11) that encodes the catalytic site of the TPH2 enzyme, the Arg441His SNP (G1463A) and the orthologous mouse SNP (C1473G; Arg447Pro) have been associated with 80% lower function of the TPH2, reduced 5-HTP synthesis rate and serotonin levels, and with depression in one small elderly American population with treatment-resistant depression,[41,443,457] thus linking low TPH2 function to depression aetiology. The rare 441His variant was, however, not found in other populations with depression, anxiety disorders, late-onset depression or treatment-resistant depression.[452,458-463] Several other rare non-synonymous SNPs in *TPH2* have been reported, including the exon 6 Pro206Ser (rs17110563), the rare Ser variant of which has been associated with reduced serotonin production and bipolar disorder.[42]

TPH2 intron polymorphisms

Polymorphisms in intron 5 of *TPH2* have repeatedly shown evidence of association with mood-related disorders. The rs1386494 SNP, situated in intron 5 at position 19.918 of the gene (and haplotypes containing it) was first associated with depression and suicide[464,465]; the common C allele was more common in cases than in controls and the rare T allele was considered protective. A recent study of depression, focusing on intron 5, replicated the association between the C allele of 1386494 and depression by observing an association with a

C-G-G-haplotype composed of the SNPs rs1386494 and 1386493 plus one novel SNP (g.22879A>G).[456] The CC genotype of the rs1386494 has been shown to be rare in female patients with panic disorder[466] and possibly to be related to weaker response to antidepressants. The latter relationship with antidepressant response was more pronounced for an adjacent intron 5 SNP (rs10879346) and also for an SNP in intron 8 (rs1487278).[467] A haplotype encompassing exon 7-9 has been associated with bipolar disorder[468] and SNPs in intron 1 and 4 have been associated with autism.[469]

TPH2 promoter polymorphisms
The promoter of *TPH2* contains two polymorphisms that are in high LD and may be functional: the rs4570625 (G-703T or G-844T, positioned 703 bp upstream of exon 1 and 844 bp upstream of the start codon) and the rs11178997 (T-473A). Carriers of the rare -703T allele of rs4570625 have repeatedly been shown to have higher amygdala reactivity to emotional stimuli compared to carriers of the GG genotype.[470,471] Although the above-mentioned study by Zhou et al.[453] showed no evidence of association for either of these two promoter polymorphisms with depression or anxiety, and studies of possible association between the promoter region and panic disorder and suicide were negative,[462,472] another study showed haplotypes containing the T allele of rs4570625 to be associated with anxious-fearful personality disorders.[473] In contrast, the rare TT genotype has been associated with *low* levels of anxiety-related personality traits,[474] an observation that may be related to enhanced emotional processing in T-allele carriers of both negatively and positively valenced emotional stimuli.[475]

The A allele of rs11178997 has been shown to be under-represented in depression, and has been associated with bipolar disorder.[42,452] Carriers of the G-T or the T-A haplotype of the rs4570625-rs11178997-combination have been reported to display increased risk for bipolar disorder.[476]

One study of the functional consequences of these two promoter polymorphisms has shown haplotype constructs containing the T allele of the rs4570625 to display lower promoter activity.[477] However, this study attributes the lower promoter activity for this allele to the adjacent A allele of the rs11178997, the mechanism being that the transcription-enhancing transcription factor POU3F, the motif of which contains both the rs4570625 and the rs11178997, displays lower binding affinity when the motif contains the A allele of rs11178997. Another study also suggested the A allele of the rs11178997 to give rise to reduced promoter activity, but showed also the G allele of the rs4570625 to be associated with reduced activity.[478]

The alleles of the rs4570625 and the rs11178997 that have been reported to be associated with low amygdala reactivity and low risk for bipolar disorder, respectively, *i.e.* the G and the T alleles, have both been associated with ADHD[479,480] and possibly with OCD.[481]

In paper I and II, the combined influence of the 5-HTTLPR and the *TPH2* rs4570625 promoter polymorphisms on amygdala responsiveness to emotional stimuli, and on the amygdala activity in placebo responders, was investigated in subjects with social phobia. The S and T alleles were associated with increased amygdala reactivity to angry faces, whereas the LL and GG genotypes were associated with placebo-induced attenuation of amygdala activity during public speaking.

Polymorphisms in *HTR3A* & *HTR3B*

The three genes encoding the 5-HT3 subunits A-C have been found to be expressed in the brain. The genes encoding 5-HT3A and 5-HT3B, *HTR3A* and *HTR3B*, are clustered on chromosome 11q23, whereas the genes *HTR3C-E* are clustered on chromosome 3q27.[482,483]

Polymorphisms in *HTR3A* and *HTR3B* have been associated with depression and bipolar disorder and with depression- and anxiety-related traits.[220,484,485] In *HTR3A*, the common C allele of the C178T (or C-42T, rs1062613) polymorphism, encoding an amino acid exchange, Pro16Ser,[485,486] has been associated with enhanced amygdala reactivity to emotional faces[487] and with increased anxiety- and aggression-related traits.[220] The common Tyr allele of the exon 5 amino acid substitution polymorphism of *HTR3B*, Tyr129Ser (rs1176744, 386A>C), has been associated with depression in women, and this is also the case for haplotypes containing the G allele of the adjacent intron 4 polymorphism, rs1176746. The rs1176746 G allele may also be associated with bipolar disorder in men.[485] The Ser allele (the rare allele, which is also the ancestral) of Tyr129Ser seems to have a large impact on receptor function, leading to a sevenfold increase in the mean open time of the ion channel as well as an increase in the maximal response to serotonin.[488]

Polymorphisms in *GATA2*

GATA2 is located on chromosome 3q21, and has acquired its name due to the binding of the protein to GATA sequences in regulatory regions of target genes. Polymorphisms in *GATA2* are expected to affect the differentiation of serotonergic neurons, since deletion of this gene in animals leads to loss of rostral serotonergic innervation.[489]

> In paper IV, several polymorphisms in serotonin-related genes were investigated in women suffering from PMDD. The T allele of rs1386494 and the A allele of the rs11178997 in *TPH2* were associated with PMDD, as were the G alleles of the intron 4 rs1176746 in *HTR3B* and the 3'UTR rs2713594 in *GATA2*.

SEROTONIN & BDNF

Brain-derived neurotrophic factor: introduction

The BDNF protein

Brain-derived neurotrophic factor (BDNF) belongs to the family of neurotrophins and is a crucial factor for neuronal differentiation, proliferation and survival, and for the guidance of axons to their targets in the developing brain.[490,491] It is also involved in activity-dependent synaptic plasticity including dendritic growth, the complexity of dendritic arbors and longterm potentiation (LTP) (a model of synaptic plasticity) in the adult brain.[492-494]

BDNF is produced and secreted as a long pro-BDNF, which is then proteolytically cleaved to the mature 153-aa-long BDNF.[495] The mature BDNF protein acts by binding to the TrkB (tryptomyosin-related kinase B) receptor, encoded by *NTRK2* (chromosome 9q22-23), which in turn influences expression of target genes. Mature BDNF mediates hippocampal LTP via TrkB.[496-498] BDNF-TrkB transmission is required also for hippocampus-dependent learning of fear.

The pro-domain controls synaptic localisation and dendritic trafficking, for which the interaction between the pro-domain and sortilin is important. The long BDNF protein acts on

p75[NTR], the activation of which appears to suppress dendritic growth, increase NMDA-dependent longterm depression (LTD) and facilitate apoptosis.[499-501] p75[NTR] is widely expressed during development; in adulthood it has, however, been suggested to be restricted to cholinergic neurons.[502,503]

Plasmin activation by tPA (tissue plasminogen activator) seems to be critical for the regulation of the balance between pro-BDNF and mature BDNF as it is necessary for the hippocampal conversion of proBDNF to mature BDNF; knock-outs of tPA thus show reduced activation of the TrkB pathway and reduced LTP.[504]

The BDNF gene

The gene encoding BDNF, *BDNF*, is situated on chromosome 11p13-14. The structure of *BDNF* is complex, including use of alternative splice sites and promoters; seven promoters and untranslated 5'exons have thus been found, each forming a unique transcript together with the common coding 3' exon.[505,506] Different transcripts seem to be expressed in different brain regions and cell types[507] and to be involved in different functions. Due to its involvement in activity-dependent synapse development and LTP, promoter number four is the most intensively investigated one.[502]

BDNF holds an SNP (196G/A, rs60760775, previously named rs6265) resulting in an amino acid substitution from valine to methionine at codon 66 (Val66Met) in the pro-domain of the protein. The rare Met allele has a 19% frequency in Caucasians (44% in Asians) and is associated with a reduced efficiency of intracellular pro-BDNF trafficking, pro-BDNF-sortilin interaction and dendritic trafficking, and also with reduced activity-dependent secretion of BDNF.[508-510] The polymorphism only exists in humans but the constructed Met-knock-in mouse shows great similarities with the heterozygous knock-outs with respect to the reduced dendritic arbor complexity of dentate gyrus neurons and reduced activity-dependent secretion.[511]

BDNF involvement in depression, anxiety and antidepressant action

The implication of BDNF in depression is based on the notion that BDNF promotes several forms of hippocampal plasticity and that antidepressants reverse stress-induced reductions in BDNF.[497,512-516] The Met allele of the Val66Met polymorphism is also associated with reduced hippocampal volume[517,518] and with reduced performance on memory tasks and hippocampal processing during episodic memory encoding.[509,519-521] In animal models, reduced BDNF function has been associated with increased anxiety-related traits, aggression and hyperphagia,[511,522] but BDNF-TrkB transmission has also been shown to be pro-depressive by enhancing the depressogenic or anxiogenic effects of social defeat stress on social avoidance.[523]

Antidepressant response has been suggested to be mediated by an upregulation of BDNF-TrkB transmission. BDNF plasma levels have been reported to be reduced in depressed subjects[524-528] and in animal models of depression,[516,529] and to be increased as a result of antidepressant treatment in human plasma,[524,525,528] in human brains as assessed post-mortem,[530,531] and in brains of animals[532-536]; this effect has been suggested to be necessary and sufficient for SRI response.[537,538] BDNF itself may also display antidepressant effects in animal models of depression.[539,540] The BDNF protein crosses the blood-brain barrier[541] and plasma levels of BDNF are similar to those in the CSF,[542] suggesting that plasma levels reflect central concentrations. The antidepressant-induced increase in BDNF is probably mediated by increased extracellular serotonin levels since it is blocked by serotonin depletion[543] and since agonists of the 5-HT2A and 5-HT6 receptors also increase BDNF expression.[544-546] Moreover,

serotonin administration increases BDNF mRNA in raphe, an effect that seems to be mediated by somatodendritic 5-HT1A receptors.[547]

In animal models, the timing of SRI-induced BDNF upregulation may concur with that of antidepressant effect,[533,548,549] suggesting BDNF to be involved in the delayed response displayed by SRIs. As discussed above, several theories regarding the delayed antidepressant response are based on the notion that neurotrophic factors induce hippocampal plasticity, which may be required for restoration of network function and ultimately mood.[384-387,550]

Associations have been reported between the Val allele of the Val66Met and psychiatric conditions including bipolar disorder[551,552] and obsessive compulsive disorder (OCD).[553] The Val allele has also been associated with childhood-onset depression[554] and with the anxiety-related trait neuroticism (as measured by NEO)[555,556]. However, also the Met allele has been associated with anxiety-related traits and with geriatric depression,[557,558] and the Met allele has also been shown to interact with environmental factors in increasing the risk for depression. Two of the studies showing such an interactive effect between the Met allele and environmental exposure on the risk for depression investigated childhood maltreatment and childhood experience on childhood and adult depression, respectively [559,560], and a third study examined the depressogenic effects of SLEs in Corean elderly subjects.[510] The Met allele may be associated with enhanced antidepressant response.[561]

> In paper V, the Met allele of the *BDNF* Val66Met polymorphism, in combination with the S allele of the 5-HTTLPR, was shown to be associated with increased exposure to stressful life events.

BDNF and the serotonergic system

BDNF and its receptor TrkB are expressed in serotonergic neurons within the raphe nuclei.[562-564] BDNF seems to influence the plasticity of these nerve cells. BDNF thus protects serotonergic neurons from neurotoxic damage[565,566] and BDNF administration increases the expression of serotonergic markers[567,568] and serotonin axon density.[569] In line with this, BDNF deficiency introduced by genetic manipulation leads to a reduced density of serotonergic axons[522] – a reduction that increases with age – and to reduced serotonin brain content,[570] a reduced number of 5-HT2A receptors[571] and possibly reduced 5-HT1A function.[572] Although BDNF hence seems to improve the plasticity of serotonergic neurons, the presence of the protein does however not seem to be required for the survival and maturation of serotonergic neurons.[569]

BDNF also seems to promote serotonin transporter function. Reduced levels of BDNF thus imply compromised transporter function, a lack of increase in transporter function with age, and increased extracellular serotonin levels.[573,574] In line with this, BDNF exposure seems to increase serotonin transporter function via TrkB,[575,576] as judged by a reduced increase in extracellular serotonin levels after intracerebral infusion of the transmitter, reduced baseline serotonin extracellular levels and a reduced activity-dependent increase in serotonin.[575] Heterozygous *BDNF* knock-outs show reduced SRI-induced elevation of extracellular serotonin in hippocampus but not in frontal regions and raphe,[574] whereas intrahippocampal BDNF administration leads to an augmented increase in extracellular serotonin after SRI treatment.[577] However, since the reduction in serotonin brain content observed in *5-HTT* knock-outs is potentiated by *BDNF* deletion, the BDNF protein appears to have effects on serotonergic function that are independent of its effect on the serotonin transporter[570] (see above).

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The effect of *BDNF* polymorphisms on serotonin transporter availability was investigated in paper III. Several SNPs were associated with transporter availability, including the Val66Met polymorphism. Carriers of the Val/Val genotype, associated with enhanced BDNF secretion, thus displayed increased transporter availability in most brain regions.

SEROTONIN & SEX STEROIDS

There is a large prevalence difference for men and women for several disorders that respond to SRIs, women displaying at least a twofold prevalence for depression and anxiety disorders.[578] Moreover, during periods of hormonal fluctuations in women, depressive symptoms are common, as illustrated by conditions such as perimenopausal dysphoria, postpartum depression, oral contraceptive-induced dysphoria and PMDD.

The serotonergic system interacts with sex steroids and appears to dampen the effects that sex steroids exert on behaviour. With respect to aggression and sexual drive, androgen and serotonin exerts opposite effects. Serotonin depletion thus results in elevated aggression and sexual drive, *i.e.* behaviours that are increased by sex steroids,[579,580] whereas androgen administration has been shown to decrease amygdalar serotonin release in rats.[581] In line with the dampening effects of serotonin, one of the most common side effects of SRI treatment is decreased libido. In addition there is indicative support for the notion that some conditions that respond to chronic SRI treatment, such as bulimia nervosa and OCD, also respond to anti-androgenic drugs.[582-585] The notion that serotonin dampens the effects of sex steroids is also supported by the instant and strong response that women with PMDD show to SRI treatment. Both acute and chronic estrogen administration to ovariectomized animals increases serotonin levels, tentatively by upregulation of synthesising enzymes and downregulation of MAOA.[586-593]

Acute estrogen treatment increases the expression and function of the serotonin transporter,[594,595] whereas subchronic estrogen treatment during one to four weeks decreases transporter expression and function in both ovariectomized females and castrated males.[592,593,596] Conversely, even longer treatment periods (five months) have been shown to increase serotonin transporter expression.[593]

Estrogen administration has also been shown to upregulate frontal 5-HT2A receptors as well as to down-regulate 5-HT1A receptor function in limbic regions and raphe.[588,594,597-606] The estrogen receptor subtype β (see below) is co-localized with serotonin[607,608] and appears to be closely involved in the regulation of the serotonin system, *e.g.* in mediating the facilitating effects of acute estrogen on the serotonin transporter, serotonin-synthesising enzymes and serotonergic receptors.[595,609] Also progesterone appears to affect serotonergic transmission,[610] and serotonin may inhibit progesterone-induced aggression.[611]

PAPERS I–V

For thorough presentations of methods and results, and for a detailed discussion of the findings, the reader is referred to the enclosed papers and manuscripts. Below will be given a brief summary of the main finding of papers I-V; moreover, a number of important aspects will be commented.

GENOTYPE OVER DIAGNOSIS IN AMYGDALA RESPONSIVENESS: AFFECTIVE PROCESSING IN SOCIAL ANXIETY DISORDER

Paper I is a study of genetic effects on amygdala reactivity to angry faces in healthy subjects (*n*=18) and in subjects with social phobia (*n*=34). Carriers of the S allele of the 5-HTTLPR in the promoter region of the gene encoding the serotonin transporter, and/or the T allele of the *TPH2* G-703T promoter polymorphism (rs4570625), were shown to exhibit increased amygdala responsiveness. The reactivity of the amygdala was however not significantly larger in subjects with social phobia than in controls. The serotonin-related polymorphisms were hence stronger predictors of amygdala reactivity than the diagnosis of social phobia.

Two-locus effects
In our study, the combined effect of the two polymorphisms on amygdala reactivity to angry faces appears to be synergistic. Other studies also find the reactivity to emotional stimuli, as measured both by the early posterior negativity peak of event-related potentials and by functional magnetic resonance imaging, to be highest for carriers of both the S (or L_G of the 5-HTTLPR$_{rs25531}$) and T alleles, but only to the extent expected if the polymorphisms acted independently of each other in an additive manner.[475,612] However, since neuroimaging studies usually are performed on small samples, there are usually only few subjects carrying each two-locus genotype, and these studies are hence not very suitable for interaction analysis. Further studies will be required to clarify if the S allele of 5-HTTLPR and the T allele of *TPH2* G-703T display a synergistic interaction on amygdala responsiveness or not.

The influence of genes and of diagnosis
Subjects with social phobia did display a slightly larger increase than controls in amygdala activity when angry faces were presented. However, this non-significant difference was partly due to the fact that controls carrying LL and GG genotypes did not show enhanced activation to angry faces compared to neutral, thus displaying negative reactivation scores. Since other studies have shown amygdala reactivity to be increased in subjects with social phobia,[167] the lack of a significant difference in this study may be due to low statistical power. However, differences in amygdala reactivity observed between cases and controls have been suggested to be influenced by temporal aspects of the amygdala response; it has thus been shown that the response of the amygdala in patients with social phobia is delayed as compared to that of controls, possibly due to initial focus on the self rather than on the task.[613-617] Another property of perception in social phobia, that may have influenced the results, is that subjects with social phobia also display sustained amygdala activity regardless of whether an emotional face is attended to or not.[618]

This study showed the *TPH2* and 5-HTTLPR polymorphisms to influence amygdala reactivity to a greater extent than did diagnosis of social phobia. In analogy with this finding, a study of ADHD found the G and T alleles of the *TPH2* polymorphisms rs4570625 and rs11178997 to explain variation in neural correlates of a response inhibition task, while diagnosis did not.[619] The influence of the genetic variation on brain processing patterns was hence larger than that of diagnosis. ADHD had previously been associated with both the

neural correlates of response inhibition and with the G and T alleles of rs4570625 and rs11178997.[480]

Serotonergic transmission & amygdala reactivity

Although the relationships between (i) the 5-HTTLPR and depression & anxiety, (ii) the 5-HTTLPR and amygdala reactivity, (iii) the 5-HTTLPR and serotonin transporter availability, (iv) depression & anxiety and amygdala reactivity, (v) depression & anxiety and serotonin transporter availability and (vi) amygdala reactivity and amygdalar serotonin transporter availability are all reasonably well in line with each other (see SEROTONIN), it remains to establish whether enhanced serotonergic transmission leads to increased or reduced amygdala reactivity. Carriers of the S allele, as well as subjects with depression or anxious mood appear to have low levels of the serotonin transporter, suggesting that serotonin levels may be increased. But the effect of few serotonin transporters may also have been exerted during development, possibly leading to a compromised serotonergic system including reduced serotonergic innervation.[262,357] In the same vein, low serotonin transporter availability in patients with depression may reflect a lower density of serotonergic neurons (see below).

In line with the first view, *i.e.* that enhanced amygdala reactivity, in S carriers, is due to an elevated serotonergic transmission, reduced 5-HT1A autoreceptor density is reported to explain more than 40% of the variation in increased amygdala reactivity to emotional stimuli,[620] hence linking a weak negative feedback, tentatively leading to an increased serotonergic influence on the amygdala, to increased amygdala reactivity. On the other hand, in line with the second view, *i.e.* that enhanced amygdala reactivity is due to a reduced serotonergic transmission, serotonin has been shown to reduce amygdala excitation: Electrical stimulation of the raphe thus inhibits the activity of neurons in the rat amygdala, an effect that is blocked by serotonin depletion and restored by 5-HTP administration, thus indicating serotonergic mediation.[621] In addition, serotonin has been shown to inhibit neurons of the lateral amygdala by exciting GABAergic neurons, which in turn inhibits excitatory input from frontal regions to the amygdala,[622,623] GABA A agonists mimicking the effect of serotonin. Current data on whether the effect of the *TPH2* rs4570625 polymorphism on serotonergic transmission is enhancing or reducing also remain inconclusive.[477,478]

Arguing against a view where the quantity of serotonergic transmission is closely related to amygdala reactivity, both tryptophan depletion[438,624,625] and acute SSRI treatment[626] may elevate amygdala reactivity, the former presumably by decreasing serotonin levels and the latter by increasing them. It thus seems that the amygdala may react to endogenous changes in serotonin availability. The amygdala also reacts to exogenous change such as unpredictability; a recent study revealed elevated amygdala engagement when an unexpected tone was presented after a set of predictable tones in both mice and men,[627] an uncertainty-induced amygdala activation that also affected the subsequent sensitivity to negativity. The 5-HTTLPR and *TPH2* polymorphisms may then, possibly, have an influence on the flexibility of the serotonergic system during change.

Different responses to change in S and LL carriers have thus been observed in several studies; healthy S allele carriers display increased amygdala activation at rest after increased exposure to stress, possibly increased activation of the amygdala after tryptophan depletion, and increased depressive/anxious symptoms and reduced motivation after tryptophan depletion. In contrast, healthy carriers of the LL genotype display reduced amygdala rest activation after increased stress exposure and increased motivation after tryptophan depletion.[433,438,439,442]

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A LINK BETWEEN SEROTONIN-RELATED GENE POLYMORPHISMS, AMYGDALA ACTIVITY
AND PLACEBO-INDUCED RELIEF FROM SOCIAL ANXIETY

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Paper II is a study of the influence of serotonin-related genetic variation on placebo response and its neural correlates. Amygdala activity during public speaking was measured before and after chronic placebo treatment. The placebo response rate in this study was 40% (responders: $n=10$; non-responders: $n=15$). Responders displayed reduced public speaking-induced amygdala activity after placebo treatment, and this reduction was more pronounced in carriers of the LL and GG genotypes of the 5-HTTLPR and the *TPH2* rs4570625 promoter polymorphisms, respectively. The *TPH2* polymorphism was also significantly associated with placebo response; out of nine placebo responders (with genotype information), eight were homozygous for the G allele of the *TPH2* polymorphism.

The 5-HTTLPR and the TPH2 polymorphisms and treatment response

Notably, the LL genotype of the 5-HTTLPR has also been associated with superior antidepressant response to repetitive transcranial magnetic stimulation,[628] to light therapy and sleep deprivation.[629,630] The response to chronic SRI treatment has also been shown to be superior for L carriers of the 5-HTTLR in meta-studies of depression and for subjects with social phobia.[631-635] Contrary to the enhanced placebo response in *TPH2* GG carriers, however, one study investigated the *TPH2* polymorphism in relation to antidepressant response and showed indicative evidence of an enhanced response for T allele carriers.[467]

Similarities between placebo and active treatments

In line with our observations, several of the changes in brain activity induced by pharmacological and psychotherapeutic treatment of depression and anxiety disorders, including reduced amygdala activity,[36,69,186,187] have previously been observed also after successful placebo treatment.[213,215,216] One explanation for this would be that all active treatments act by means of a final common pathway, including reduced reactivity of the amygdala, and another that amygdala hyperreactivity is a consequence of the disorder, which hence may be reduced whenever the symptoms are disappearing, regardless of the reason for this improvement.

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GENETIC VARIATION IN BDNF IS ASSOCIATED WITH SEROTONIN TRANSPORTER BUT NOT
5-HT1A RECEPTOR AVAILABILITY IN HUMANS

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Paper III is a study of serotonin-related proteins in the brain, as measured by positron emission tomography (PET) and single photon emission computed tomography (SPECT). Binding of the radioligands [^{11}C]MADAM ($n=25$) and ^{123}I-β-CIT ($n=18$) to the serotonin transporter, and of the radioligand [^{11}C]WAY ($n=53$) to the 5-HT1A receptor was hence measured and related to variation in the gene encoding BDNF. Several SNPs were shown to be associated with serotonin transporter availability as

measured by [¹¹C]MADAM binding potential, including the Val66Met polymorphism; men carrying the Val/Val genotype, associated with enhanced BDNF secretion, thus displayed increased transporter availability in most brain regions. This finding was partly replicated in an independent sample where serotonin transporter had been measured using the ¹²³I-β-CIT ligand. There was no difference between *BDNF* genotypes in [¹¹C]WAY binding potential.

How BDNF may increase serotonin transporter availability
Since the Val allele of the *BDNF* Val66Met polymorphism is associated with an increased activity-dependent secretion of BDNF, the observed association suggests enhanced BDNF function to be associated with enhanced transporter function. Such a relationship could be due either to a direct influence of BDNF on the expression of the gene encoding the serotonin transporter – implicating a relationship between enhanced BDNF function and reduced extracellular serotonin levels – or to a plastic effect of BDNF on serotonergic neurons[565,566] – probably implicating a relationship between enhanced BDNF function and increased serotonergic output. In line with our finding, heterozygous *BDNF* knock-outs display reduced transporter function; moreover, BDNF administration leads to a direct enhancement of transporter function.[573-575,577]

The interaction between BDNF and the serotonergic system in relation to depression
The notion that BDNF enhances serotonin transporter function also appears to be in line with the relationships both between BDNF and depression and between the serotonin transporter and depression. Both brain serotonin transporter availability[308-311,313,314] and BDNF plasma levels appear to be reduced in depression. Treatment-induced elevations of BDNF levels may also be involved in the mechanism of action of antidepressants.[524,525,528,530-540] Although results on the relationship of the *BDNF* Val66Met polymorphism with depression diverge,[554,557] the Met allele, which previously has been associated with reduced BDNF secretion,[508-510] and which is associated with reduced serotonin transporter availability in men in this study, has been associated with a dysfunctional stress system[636] and with reduced hippocampus volume and function,[509,517-521] both of which are traits that may be related to depression. The same allele has also been associated with the depressogenic effect of SLEs.[510,559,560]

The lack of influence of BDNF variation on 5-HT1A density
There was no association between variation in *BDNF* and 5-HT1A density, either in the raphe, or in other regions of the brain. Since 5-HT1A receptors are situated on serotonergic neurons in the raphe, this finding may suggest that the potential effect that *BDNF* may have on the plasticity of serotonergic neurons does not have an impact on neuron number in the raphe, and hence that the influence of variation in *BDNF* on serotonin transporter availability is not related to the density of serotonergic neurons in raphe. The density of 5-HT1A receptors may however be affected by many other factors, rendering the power of finding an influence of *BDNF* variation low. One of these factors is stress. Both BDNF and the serotonergic system are influenced by stress. For example, the gene encoding the 5-HT1A receptor, *HTR1A*, is a direct target of the glucocorticoid receptor (GCR).[334,637]

> **A STUDY OF 22 SEROTONIN-RELATED GENES REVEALS ASSOCIATION BETWEEN PREMENSTRUAL DYSPHORIA AND GENES ENCODING THE GATA2 TRANSCRIPTION FACTOR, THE 5-HT3B RECEPTOR SUBUNIT AND TRYPTOPHAN HYDROXYLASE 2**

Paper IV is a case-control association study of PMDD. The evidence supporting serotonergic transmission to be involved in PMDD is strong. For example, tryptophan depletion aggravates the symptoms of PMDD, and women with this disorder respond instantly to SRI treatment (but not to non-serotonergic antidepressants) by displaying markedly reduced irritability; moreover, they may also show reduced symptoms after administration of serotonin releasers or tryptophan. In paper IV, 58 polymorphisms in 22 serotonin-related genes were investigated in women suffering from PMDD (two groups n_1=293, n_2=57) and in controls (n=825). In *TPH2*, the T allele of rs1386494 and the A allele of the rs11178997 were associated with PMDD, as were the G allele of the intron 4 rs1176746 SNP in *HTR3B* and the G allele of the 3'UTR rs2713594 in *GATA2*.

Association studies and function of the polymorphisms associated with PMDD
The C allele of the intron 5 rs1386494 polymorphism in *TPH2* has previously been associated with depression and suicide, whereas the T allele has been associated with panic disorder.[464-466] The A allele of the *TPH2* promoter polymorphism (rs11178997) has been associated with bipolar disorder but may be protective against depression.[452] The *HTR3B* G allele has been associated with depression, and a polymorphism in the gene encoding the A subunit of the 5-HT3 receptor has been associated with anxiety- and anger-related traits, as well as with amygdala reactivity.[220] GATA2 is involved in the development and differentiation of serotonergic neurons and variation in *GATA2* is hence expected to affect serotonergic transmission.

The A allele of the rs11178997 has been associated with reduced *TPH2* promoter activity.[477,478] The G allele of the *HTR3B* rs1176746 is in LD with the Tyr allele of the Tyr129Ser (rs1176744) in exon 5, and a haplotype containing the G-Tyr alleles has been associated with depression in women.[485] The Ser allele of the Tyr129Ser has been associated with a sevenfold enhanced 5-HT3 receptor function,[488] hence suggesting the G allele of the rs1176746 to be related to a reduced function of the 5-HT3 receptor.

The relationship between serotonergic transmission and mood & anxiety disorders
The antidepressant and anxiety-reducing effect of chronic SRI treatment is often believed to be due to an enhancement of serotonergic transmission. However, since the effects of acute and chronic antidepressant treatment on several phenotypes, including anxiety, are largely opposite, and since acute SRI administration clearly increases serotonergic output, this view prompts reconsideration.

Acute SRI treatment thus increases anxiety in anxious subjects, the recognition of fear, the processing of anxiety-related stimuli and amygdala reactivity to emotional faces in healthy subjects and fear conditioning in animals,[105,106,370,626,638-640] whereas chronic SRI treatment is antidepressant, anxiety-reducing, reduces amygdala reactivity to emotional stimuli, and reduces fear conditioning in animals.[36,186-188,190,370] Although extracellular serotonin levels are increased also after chronic antidepressant treatment, it is possible that the high serotonin levels induce

adaptations of the serotonergic system, possibly leading to a reduced serotonergic output in spite of increased extracellular levels. The recent finding that jugular 5-HIAA levels are high in depressed subjects and in subjects with panic disorder[302,303] – an increase that is normalized by antidepressant treatment – supports the notion that depression and/or anxiety disorders are related to enhanced serotonergic transmission. The increased TPH2 levels observed in depressed suicides may also support this notion. On the other hand, there is a large body of data that support the notion that serotonergic transmission is reduced in depression and anxiety, including the association between loss-of-function *TPH2* polymorphisms and depression,[41,42] depression relapse after tryptophan depletion and a reduced prolactin response to serotonin-releasing agents in depressive and anxious subjects.[6,7,265-269,280] The fact that some side effects related to low serotonergic transmission are present also after chronic SRI treatment also contradicts a view where chronic SRI treatment is associated with a reduced serotonergic output.

Although the relationship between depression and anxiety disorders, on the one hand, and serotonergic transmission, on the other, thus is difficult to elucidate, the influence of serotonergic transmission on PMDD seems to be more clear-cut. A large body of evidence strongly suggests PMDD to be related to a deficiency in serotonergic transmission. A disorder that is due to a deficiency in serotonergic transmission would be expected to be accompanied by abnormal levels of serotonin-related biological markers; examples of reported differences between women with PMDD and controls are thus reduced 5-HTP levels during phases of PMD symptoms[279] and reduced density of the serotonin transporter in platelets in women with PMDD.[290] Symptoms of a disorder related to low serotonergic function would also be expected to increase by serotonin or tryptophan depletion; animals do display irritability when serotonin is depleted, and tryptophan depletion and serotonin receptor antagonists do induce an aggravation of PMD symptoms, in particular irritability.[378,641] A disorder that is due to a deficiency in serotonergic transmission would also be expected to be ameliorated by drugs that increase serotonin levels; both acute SRI treatment and serotonin-releasing agents reduce PMD symptoms,[642,643] as do tryptophan and a TPH co-factor (pyridoxine).[101,102,288,644] Notably, amongst all SRI indications, PMDD is the one displaying the most rapid response to these drugs, as well as the largest effect size,[99,101,102] especially for the irritability-related symptoms. The findings of the present study, *i.e.* that alleles that have been linked to a reduced function of the TPH2 and of the 5-HT3 receptor are associated with PMDD, are hence perfectly in line with a large number of other observations supporting the view that PMDD is related to deficiencies in serotonergic transmission.

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Paper V

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> **POSSIBLE EFFECTS OF INTERACTIONS BETWEEN THE SEROTONIN TRANSPORTER POLYMORPHISM 5-HTTLPR, THE BDNF VAL66MET POLYMORPHISM AND ANXIETY-RELATED PERSONALITY TRAITS ON CONTROLLABLE STRESSFUL LIFE EVENTS**

Paper V is a study of the inter-relationship between controllable stressful life events (SLEs), current and past depression, anxiety-related personality traits and the 5-HTTLPR and *BDNF* Val66Met polymorphisms. Previous studies have shown an interaction between the S allele of the 5-HTTLPR, the Met allele of the Val66Met and SLEs to increase the risk for depression, a finding that we, however, could not replicate. However, the 5-HTTLPR and the *BDNF* polymorphism did show an interactive effect

on exposure to controllable SLEs in men, carriers of SS and Met-containing genotypes displaying the highest number of SLEs. There was an interactive effect also between the 5-HTTLPR and anxiety-related personality traits on reports of controllable SLEs, such that men with the SS genotype had experienced *less* SLEs if they had high anxiety-related traits and that men with the LL genotype had experienced *more* SLEs if they scored high on anxiety-related traits. The previously observed synergistic interaction between SLEs and the 5-HTTLPR on risk for depression was only observed in men with low anxiety-related traits.

Interactions between stress & serotonin

An increased stress sensitivity in S carriers of the 5-HTTLPR has previously been shown by an increased startle response,[418] by an increased HPA axis response to stress,[645] by increased fear conditioning,[419] and by the synergistic interaction between the S allele and SLEs, or childhood maltreatment, on depression risk.[86,420] This increased stress sensitivity in S allele carriers has been proposed to be mediated by the amygdala, which shows enhanced reactivity and rest activity in S carriers. The enhanced rest activity of the amygdala, observed in S carriers, has been reported to be further increased by the exposure to SLEs,[38,433] whereas LL carriers have been reported to display a negative correlation between the number of SLEs and amygdala rest activity. The present study suggests that the correlation between anxiety-related personality traits and controllable SLEs also depends on 5-HTTLPR genotype; SS carriers thus display a negative correlation between the number of controllable SLEs reported for the last year and anxiety-related personality traits, while this correlation is positive in carriers of the LL genotype. Whether or not causality is involved in these relationships remains uncertain. High anxiety-related traits in S-allele carriers could lead to an active avoidance of controllable SLEs (harm avoidance), or, the interaction could reflect that LL carriers develop increased anxiety-related traits as a consequence of controllable SLEs, whereas carriers of the SS genotype somehow are protected against such an influence.

In this study, the interaction between the S allele and controllable SLEs, which previously has been demonstrated by several researchers,[86,90,420,422] was observed only in men with low anxiety-related personality traits. One possible explanation for this observation may be that self-reported depression, as well as self-reported SLEs, may be more robust and reliable, and less influenced by personality traits, in subjects with low anxiety-related traits than in those displaying high anxiety-related traits.[646] The notion that stressful events interact with a polymorphism that affects serotonergic transmission is not surprising given the extensive body of data that suggest serotonin to affect the stress response[362,478] and stress to affect the serotonergic system.[321,647-655] The interaction that other groups have shown between the 5-HTTLPR and SLEs could reflect an enhanced effect of stress on the serotonergic system in S carriers, or an effect of the S allele on the serotonergic system, which in turn affects the stress response.

Interactions between stress, serotonin and BDNF

Three studies have reported a three-way interaction between environmental exposure and the S and Met alleles on depression.[510,559,560] Two of the studies on this subject investigated the influence of childhood maltreatment and childhood experience on childhood and adult depression, respectively,[559,560] and the third study examined the depressogenic effects of SLEs in Corean elderly subjects.[510] The notion that a polymorphism in *BDNF* interacts with the 5-HTTLPR-SLE interaction observed for depression is also not surprising, given that the *BDNF*

Met allele has been associated with dysregulation of the HPA axis,[636] and that BDNF largely interacts with the serotonergic system (see paper III and the SEROTONIN & BDNF section). In addition, overexpression of glucocorticoid receptors has been shown to increase BDNF levels and to reduce the depressogenic-like effects of SLEs in mice.

Our study did not include an assessment of current depression by means of diagnostic interview. By using standard cut-offs on the depression scale,[656] we could however not replicate the finding of an interaction between SLE exposure in adulthood, the 5-HTTLPR and the Val66Met polymorphism on depression risk.[510] Previous studies of the relationship between the *BDNF* Val66Met polymorphism and depression- and anxiety-related phenotypes are however inconclusive. A study aiming at investigating the neural correlates of emotional processing has thus found the reduced connectivity between the amygdala and the ACC, observed in 5-HTTLPR S carriers,[202] to be present only in carriers of the *BDNF* Val/Val genotype, possibly suggesting the *BDNF* Met allele to be protective against 5-HTTLPR-S-induced effects on emotional processing and anxiety-related traits.[657] In addition, association studies of the *BDNF* polymorphism with respect to depression- and anxiety-related phenotypes report both the Val and Met alleles to be associated with such traits.[554-558]

We did observe an interaction between the 5-HTTLPR and the *BDNF* polymorphism on the number of controllable SLEs, male carriers of the SS genotype and Met allele reporting the highest numbers of controllable SLEs. Since this interaction was present also when all subjects that could be depressed were excluded, it is not likely that life events were perceived as stressful because of ongoing depression in these individuals.

Overlapping heritability between SLEs and depression has been suggested to be a confounding factor in studies aiming at elucidating the relationship between SLEs and depression.[89] The notion that the same genotypes that have been reported to interact with SLEs in increasing the risk for depression, also increase the risk for exposure to SLEs, may influence the interpretation of the former finding and may also explain why we did not observe the 5-HTTLPR-*BDNF*-SLE-interaction for depression in our study. It may also be considered as support for shared heritability between depression and SLEs.

The inter-relationship between factors that have been related to depression
Although there is evidence that serotonin, stress, hippocampal atrophy and BDNF may be involved in depression pathophysiology, it is worth noting that neither cortisol administration, nor hippocampal lesions, nor neoneurogenesis inhibition, nor serotonin depletion, nor BDNF inhibition consistently give rise to depression in healthy individuals.[265,273,538,658] [6,115,128,131,659] It would be interesting to know whether the same subgroup of depressed patients that displays dysregulation of the HPA axis also has reduced hippocampal volumes[660] and if these subjects are those who drive the interaction between the 5-HTTLPR S allele and SLEs. A normalization of the hippocampal volume after remission has been correlated with a normalized HPA axis regulation, and the volume reduction of the hippocampus is larger in depressive subjects who have experienced SLEs or trauma.[134,137] Although the S allele has been associated to an enhanced HPA axis response to stress,[645] the interaction between the 5-HTTLPR and SLEs has not been related to either HPA axis regulation or hippocampal volume.

Sex differences related to stress, serotonin and BDNF
We only observed genetic effects in men. Although the interactive effect between SLEs and the 5-HTTLPR on depression has previously been demonstrated for both men and women, factors that affect this association may be different in men and women. Both the behavioural and the

molecular response to stress may differ in men and women. In animals, exposure to stressful events appears to facilitate learning in males and impair performance in females, a difference that is prevented by perinatal testosterone administration to females or inhibition of the action of androgens during prenatal development in males.[335,661-663] Rearing environment also affects male and female animals differently; whereas females display more dendritic sprouting than males when reared in enriched environments, the relationship is the opposite when the animals are reared in normal householdings.[664] In addition, the serotonergic systems of men and women may display differences that can influence the interaction.[665 666 667-669 670] The finding that the interaction between variation in the genes encoding the serotonin transporter and BDNF was present only in men is consistent with animal studies suggesting the interactive effects between the genes encoding the serotonin transporter and BDNF to be reduced by female gender or estrogen administration.[570]

INFLUENCE OF SEX STEROID–RELATED GENETIC VARIATION ON PERSONALITY, AUTISM AND TRANSSEXUALISM

TRAITS

AUTISM

Autism characteristics

Autism and autism spectrum disorder (ASD)
Autism, first described in 1943, is a disorder characterized by social and language impairments as well as restricted, repetitive behaviours and interests. Autism spectrum disorder also includes Asperger syndrome, pervasive developmental disorder (PDD), child disintegrative disorder, Rett syndrome and PDD not otherwise specified (PDD-NOS). The level of cognitive function in autism spans from severe mental retardation to a superior IQ and even savant skills[C]. According to DSM-IV, mental retardation is characterized by an IQ under 70 as well as by adaptation impairments during childhood. The proportion of autistic individuals who meet the criteria for mental retardation has been reported to be in between 25 and 70%.[671] The prevalence of autism and ASD may be as high as 0.5% and 1%, respectively[672,673] and autism and ASD are at least four times more common in men than in women.[674-676]

Heritability of autism
Autism aetiology has a large heritable component. The heritability thus is approximately 80%,[10,677,678] with highest heritability estimates obtained for a broad phenotype. The difference in concordance between MZ and DZ twins is large; the concordance is over 80% in MZ and under 10% in DZ twins,[679,680] and the risk for autism when a sibling is affected is increased 25-75 times.[681,682] The three aspects, *i.e.* social function, language and repetitive and or restrictive domains, are all highly heritable but show low covariation; distinct genetic influences have been identified for all three components.[678]

Syndromic forms of autism
Approximately 5% of autism cases co-occur with known genetic syndromes, which usually are accompanied by facial dysmorphia and sometimes other somatic phenotypes. The actual number may be higher since new syndromes are detected continuously.[683] Two examples of such syndromes are the Fragile X syndrome, caused by mutations of the *fMR1* gene located on chromosome Xq27, and Rett syndrome, caused by mutations of the *MECP2* gene located on chromosome Xq28. The *fMR1* mutations results in failure of the production of the fragile X mental retardation protein (FMRP), which is required for normal development and transcriptional repression. Mutations, the large majority of which are *de novo* mutations (*i.e.* that appear in the parental germ line), in *MECP2* cause Rett syndrome, a disorder that exclusively affects girls. *MECP2* encodes the methyl CpG binding protein 2, which is involved in transcriptional repression and chromatin remodelling. The chromosomal regions 15q13 and 22q13 are also implicated in syndromic forms of autism.[684-686]

Theories of autism

Deficits in autism include impairments in comlex processing,[687] executive function,[688] central coherence[689] and joint attention.[690] Subjects with autism display impairments in theory of mind (TOM) (also called mentalizing or empathizing), which is the ability to attribute mental states to self and others with the role of making sense of their thoughts and behaviour, and the capacity to show emotional reactions appropriate to the mental states of others.[691] The reaction to irrelevant stimuli appears to be increased and the filtering of incoming information – with the role both of protecting the brain against information overload and of letting the information that is most relevant to get more attention – appears to be impaired. Abnormal brain growth, especially of frontal white matter, and reduced frontal activity when performing the above-mentioned tasks as well as reduced functional connectivity between brain regions has also been reported.[3,692-710] Several overlapping theories have been proposed for why and how cognition is different in subjects with autism and ASD.

The Central coherence theory suggests an exaggerated focus on details at the expense of a limited ability to generalize and to see the bigger picture, to be the core issue in autism. The increased focusing on details is described as a cognitive style, since it gives rise to both talents and impairments.[689] It has been proposed to be due to poor connectivity between different brain regions.[711] It may also be related to the increased risk for autism in males, since males and subjects with smaller 2D:4D (indicative of larger prenatal androgen exposure) have been shown to display increased performance on tasks that require focus on details. Another autism theory is the Complex information processing or Executive function theory, which suggests fronto-parietal developmental impairments to result in problems with *e.g.* flexibility and planning, thus causing social difficulties and repetitive behaviours.[687]

The systemizing-empathizing theory for psychological sex differences[712] has been extended to the Extreme male brain theory for autism.[675,713] This theory is based on the similarities between sex differences, on the one hand, and autism case-control differences, on the other, with respect to empathizing and systemizing behaviours, and also on the differential prevalence for autism observed for men and women. On average, women are better at empathizing tasks such as emotion recognition, while men are better at systemizing tasks such as the embedded figures task.[703,714-717] Similarly, subjects with autism have a delayed development of TOM and joint attention, both related to empathizing, and they also display restricted interests and obsessions that are often related to systemizing, *e.g.* regarding lawful systems and machines. Such findings are the basis of the hypothesis that increased testosterone exposure during prenatal development may increase the risk for autism and ASD.[675,713]

> In paper VII, the association between polymorphisms in the androgen receptor and ASD is investigated, showing evidence for an influence of an allele that increases androgen receptor function on autism spectrum disorder in women.

Genetics of autism

The genetic background of autism appears to be largely heterogenetic. Linkage analyses have revealed peaks on several chromosomes, and larger genetic aberrations have also been found on many chromosomes.[718] In addition, several rare genetic variants have been shown to be associated with autism; some have been exclusive for autism, while others have been associated with different autism spectrum diagnoses, as well as with other diagnoses and some have also been found in healthy parents.[29-32,719,720]

Rare variants including cytogenic abnormalities, *e.g.* deletions at 15q11-13 and 22q11-13, as well as copy number variations (CNVs), have been found to be common in autism and ASD[684,685,718,721-726]; these include several *de novo* deletions (appearing in the parental germ line) that may be causal.[718] Together with the syndromic forms of autism and the known rare mutations, these genetic aberrations probably explain around 20% of autism cases.[718,726-728] Notably, the notion that *de novo* mutations are common in autism is in line both with the large difference in concordance between MZ and DZ twins and with the increased autism incidence with increased parental age.[729] Rare genetic variants associated with autism have also been found in the genes encoding neurexins and neuroligins, interaction between which controls synapse formation,[29,30,33,730-738] and in the gene encoding the β3 subunit of the gamma-aminobutyric acid (GABA) A receptor (GABRB3).[739]

Although several associations between common alleles and autism have been reported, none has been consistently replicated across studies and is considered to be established [727]. The fact that no common gene variants have been confirmed as risk factors for autism may be due to a large genetic heterogeneity, including the possibility that rare *de novo* variants are more important than previously expected.

TRANSSEXUALISM

Transsexualism is characterized by a gender identity in conflict with the assigned sex, and a strong identification with the opposite sex. The prevalence of transsexualism ranges from 1:3.000 to 1:100.000.[740-742] Both aberrations in early sexual differentiation and psychosocial factors have been proposed as possible aetiological factors.[743] A large familial co-occurrence of transsexualism suggests a hereditary component[744-746]; the heritability has thus been considered to be over 50%.[747]

Differences between transsexuals and controls have been observed in volume and structure of different brain regions,[748,749] and the brains of male-to-female transsexuals have been reported to respond to the same odours as do female brains.[750] Female-to-male transsexualism has been associated with disorders accompanied by hyperandrogenemia, such as polycystic ovary syndrome and congenital adrenal hyperplasia,[751,752] and also with testosterone aromatase insufficiency.[753] It is hence not far-fetched to suggest that the early organizational effects of sex steroids may be involved in the aetiology of transsexualism (see SEX STEROIDS). In DSM-IV, the term gender identity disorder (GID) replaced the term transsexualism.[754]

> **The results of paper VIII suggest that sex steroid-related genetic variation influences the risk for transsexualism.**

INTRODUCTION TO SEX STEROIDS

Estrogens and androgens pass the blood-brain barrier. Synthesising enzymes, co-activators and receptors are expressed in brain regions such as the hypothalamus, the hippocampus, the limbic system and the PFC.[755,756]

Testosterone is produced from the precursor cholesterol, and acts both via androgen receptors (ARs) and, through the conversion by aromatase to estrogen, via estrogen receptors (ERs) of the subtypes α and β. The AR and ERs are ligand-activated transcription factors. When the ligand binds to the receptor, they dissociate from chaperones, dimerize and migrate to the nucleus. The receptor-dimer then binds to its motifs in the promoter regions of target genes and, together with co-activators, initiates the expression of these. The promoter regions of the target genes contain estrogen response elements (ERE; two palindromic half sites 5'AGGTCA3' with three nucleotides in between) and androgen response elements (ARE; 5'-AGAAGA and TGTACA-3' with three nucleotides in between). The ERα and ERβ receptor subtypes have similar affinity for the most potent estrogen, *i.e.* estradiol, as well as for the ERE of target genes. Estrogens and androgens can thus act via receptors, but also via membrane-bound steroid receptors or via direct, protein synthesis-independent mechanisms, on ion channels or second messenger systems.[757]

The ERα receptor is expressed primarily in the amygdala, hypothalamus and PFC, whereas the ERβ is more abundant in hippocampus, entorhinal cortex and brainstem.[758] ERs of the α type seem to be closely related to several functions related to cognition, including dendritic spine growth and the mediation of the effect of estrogens on neuroprotection and memory.[759-762] The effect of estrogen on cognition is however not unambiguous, since estrogen, in estrous cycle phases characterized by high estrogen levels, appears to interfere with the spatial ability of rats, an influence that is prevented by inhibition of the ERα.[759] The ERβ receptor is involved in serotonergic function (see SEROTONIN & SEX STEROIDS above); ERβ knock-out animals thus display altered serotonergic function and morphological and neural abnormalities including neuronal deficits.[763]

The differential prevalence in men and women for several psychiatric disorders suggests that sex steroids may be involved in the aetiology of these conditions. Whereas autism, alcoholism and ADHD are disorders that are more prevalent in men,[674-676] serotonin-related disorders such as depression and anxiety disorders are more common in women (see above). During development, males are exposed to large amounts of testosterone at different stages: in the second trimester *in utero*, perinatally and during puberty, and there are also hormonal differences in *e.g.* levels of androgens and estrogens between men and women later in life that may underlie differences in the prevalence for different conditions.

PRENATAL ANDROGEN EXPOSURE

Effects of testosterone *in utero* are believed to primarily be mediated by estrogen receptors (ERs), via the conversion of testosterone to estrogen by aromatase (cyp19). Androgen receptor (AR) function, however, also appears to be important,[764] *e.g.* for the expression of aromatase in the hypothalamus during embryonic development.[765]

Androgen exposure during prenatal development may be estimated retrospectively using the second to fourth digit ratio (2D:4D), which is negatively correlated with prenatal androgen

exposure.[766-768] Women exposed to androgens during prenatal development, due to male co-twins, thus display smaller 2D:4D,[769] and women with polycystic ovary syndrome (PCOS) and congenital adrenal hyperplasia (CAH), disorder characterized by increased prenatal androgen levels, also display a 2D:4D indicative of increased androgen exposure *in utero*.[770-772]

Adult testosterone levels may not be a good measure for prenatal androgen exposure, since men with effective ARs may display compensatory reductions in testosterone levels as adults. In fact, prostate cancer has been associated with high AR expression and low levels of testosterone[773] and an allele associated with high AR activity may be associated with reduced testosterone levels in men.[774,775] In addition, men with low 2D:4D (indicating high prenatal androgen exposure) may display lower adult testosterone levels,[776,777] although several studies do not find this relationship.[778-782] A relationship between high AR function and a compensatory reduction in testosterone levels does however not seem to be valid for women.[778,780]

An influence of prenatal androgen exposure on behaviours displaying differences between males and females, such as aggression and sexual behaviour, gains support from numerous findings: (i) Whereas female rodents exposed to testosterone during prenatal development develop adult male sexual behaviour,[783,784] anti-androgen treatment of males during the same period counteracts male-specific behaviour in the adult animal.[785] (ii) Women who tentatively have been exposed to increased prenatal androgens, by having male co-twins, may display enhanced aggressive and sensation-seeking behaviour.[786,787] (iii) Women with CAH, a condition accompanied by elevated foetal testosterone,[771,772] display increased aggression as well as interests typically preferred by males.[788-790] In line with these findings, female animals exposed to androgens during early development, as well as women with male co-twins, display masculinized neural connectivity and brain lateralisation.[791,792]

The notion that prenatal androgen exposure affects behaviour is also supported by indicative evidence of a relationship between prenatal androgen exposure and autistic traits. The extreme male brain theory of autism states that increased prenatal masculinization of the brain increases the risk for autistic traits such as increased systemizing and narrow interests and decreased empathizing and social behaviours.[675,713] This theory was first based on similarities between sex differences and differences between subjects with autism and controls, as indicated in the AUTISM section. Interestingly, 2D:4D has been associated with performance on spatial tasks that display both gender differences and differential performance in subjects with autism and healthy controls.[780,781] In favour of the extreme male brain hypothesis of autism, a small 2D:4D, indicative of increased prenatal androgen exposure, has been associated with autism and autistic traits in both men and women[793,794]; moreover, parents of autistic children display intermediate 2D:4D. In children, there is also a positive correlation between prenatal androgen exposure and restricted interests, and a negative correlation between prenatal androgens, on the one hand, and social relationships, as well as the frequency of affective statements in intentional propositions (I believe, I think), on the other.[795-798] In addition, women with autism display more androgen-related traits, including differences in sexual identity or orientation, dysmennoré, PCOS, acne and epilepsy,[799] and girls with CAH display autism-like traits to a higher extent than their unaffected sisters.[800] Prenatal masculinization of the brain has also been proposed to increase the risk for tic disorders, as suggested by the finding of an association between tics and gender dysphoria in women.[801]

THE MENSTRUAL CYCLE

The follicular phase is the phase during which follicles in the ovary mature. During this phase, estrogen levels are increasing slowly, reaching their maximum level just before ovulation. During ovulation, estrogen levels decrease markedly. After ovulation, the luteal phase begins by the initiation of the formation of the corpus luteum. Estrogen levels display a mild increase, followed by a subsequent decrease. Progesterone levels, having been low before the luteal phase, display a marked increase, followed by a marked decrease. The luteal phase ends with regression of the corpus luteum and menstruation.

PMDD is related to the fluctuations of the sex steroids estrogen and progesterone over the menstrual cycle. This notion is supported by: (i) gonadotrophin-releasing hormone (GnRH) analogues can prevent symptoms by disrupting hormonal cyclicity, (ii) ovariectomy also abolishes PMDD, and (iii) after depletion of endogenous sex hormones, administration of both progesterone and estrogen can induce PMD symptoms, but only in women who previously experienced PMD symptoms, indicating an increased vulnerability to the effects of hormonal fluctuations in these women.[802] Whether progesterone or estrogen is most important for the pathophysiology of PMDD is unknown. Milder premenstrual symptoms, including both sad and irritable mood and somatic symptoms are common also in women without PMDD. Irritability and aggression are observed also in female non-human animals, who display aggression and irritable behaviours during the metestrous and diestrous phases of the estrous cycle, *i.e.* the phases when the animal is sexually non-receptive.

SEX STEROID-RELATED GENES

The AR gene

The AR gene (*AR*), situated on chromosome Xq11-12, holds three polymorphisms in exon 1, encoding the region of the protein involved in transcriptional activation of downstream genes.[803] Two of these are repeat polymorphisms; the CAG repeat encodes a polyglutamine stretch and the GGN repeat encodes a polyglycine stretch. Situated between these two repeats is the *Stu*I SNP (rs6152, G1733A), so called because of its recognition by the restriction enzyme *Stu*I. The rs6152 does not affect the amino acid sequence.

Shorter CAG repeats are associated with a higher activity of the AR as a transcription factor since they increase the interaction between the AR and co-activators.[804-811] Shorter CAG repeats are also associated with a reduced 2D:4D, indicating an elevated prenatal androgen exposure. The 2D:4D is in fact largely genetically determined, displaying a heritability of 80%.[769,812]

In accordance with the increased AR activity displayed by shorter CAG repeats, the short CAG repeat has been associated with somatic phenotypes related to increased androgenic activity, such as PCOS and prostate cancer.[813-817] Both short and long repeats have been associated with cognitive impairment,[818,819] and short *AR* CAG repeats have shown modest association with psychiatric traits including depression.[817,820] A recent study showed large effects of short CAG alleles on violent crime.[821]

The functional consequences of the GGN repeat remain uncertain. Three studies indicate a positive correlation between repeat length and protein amount or activity.[808,822,823] One of these also shows longer repeats to have higher activity per protein molecule, and that the largest difference in activity is between the two most common repeat lengths, *i.e.* 23 and 24.[822] In contrast, another study shows the most common 23-repeat allele to have highest transactivating

capacity,[824] and a fifth study showed that shorter repeat lengths are associated with a larger protein amount.[825]

Some association studies of the GGN repeat in men have indicated that a shorter repeat length is associated with higher androgenic activity, as reflected by an increased risk for prostate cancer[826,827] (although a meta-study was negative[828]), increased risk for baldness[829,830] and increased fertility.[831] Other studies, however, show a positive correlation between the length of the GGN repeat and fertility and prostate cancer,[824,832] indicating a reduced androgen function for the short repeat. One study indicates that shorter GGN repeats are associated with increased androgen function in women, as illustrated by a smaller risk for endometrial cancer (androgens displaying a negative effect on cell proliferation in the endometrium).[833] The common G allele of the rs6152 polymorphism has been associated with baldness,[829,834] indicating an enhancing influence on the AR.

In paper VII, the relationship between genetic variation in the *AR* and autism was examined. A short CAG repeat length was associated with increased risk for autism spectrum disorder in women, a finding that is in line with the theory that the risk for autism is increased by prenatal androgen exposure, since shorter CAG repeat lengths are associated with increased AR function.

The ERβ gene

The ERβ gene (*ESR2*), situated on chromosome 14q22-24, holds a CA repeat in intron 5. Although the functional effect of this polymorphism remains uncertain, longer repeats have been associated with increased bone mineral density,[835] whereas shorter repeats have been associated with higher androgen levels and depression.[820,836,837]. These findings may suggest that longer repeats are associated with enhanced estrogenic function.

The aromatase gene

The aromatase enzyme catalyses the conversion of testosterone to estrogen. Longer repeat lengths of the tetra-nucleotide TTTA polymorphism, situated in intron four of the aromatase gene, have been associated with breast[838] and endometrial cancer,[839] indicating that long repeats enhance estrogenic activity. Polymorphisms in the same gene have also been associated with estrogen levels.[840,841]

In paper VIII, the *AR* CAG repeat, the *ESR2* repeat and the aromatase repeat were investigated in relation to male-to-female transsexualism. Long *AR* CAG repeat lengths were more common in subjects with transsexualism. Subjects carrying short CAG repeats displayed a very small risk for transsexualism if they also were carriers of short *ESR2* or aromatase alleles.

PAPERS VI–VIII

For thorough presentations of methods and results, and for a detailed discussion of the findings, the reader is referred to the enclosed papers and manuscripts. Below will be given a brief summary of the main finding of papers VI-III; moreover, a number of important aspects will be commented.

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INFLUENCE OF ANDROGEN RECEPTOR REPEAT POLYMORPHISMS ON PERSONALITY TRAITS IN MEN

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In paper VI, the relationship between the androgen receptor gene (*AR*) CAG and GGN repeats, encoding poly-glutamine and poly-glycine stretches, and personality traits, as measured by the KSP and TCI scales, is investigated. Shorter CAG repeat lengths were associated with more extraversion (as measured by KSP) only when the GGN repeat length was long, a finding that was replicated in an independent sample. The association was found to be significant for both subscales, *i.e.* impulsiveness and monotony avoidance. A tendency for increased neuroticism, including the subscales somatic anxiety and muscular tension, in carriers of the short CAG allele, was also observed, although this association did not survive correction for multiple testing and only showed a trend for significance in the second sample. The short CAG repeat was also associated with self-forgetfulness and spiritual acceptance, both of which are subscales of the self-transcendence subscale of the TCI. This finding almost reached significance in the second sample.

Sex steroid-related genetic variation and the personality traits extraversion and self-transcendence
In line with our results, another sex steroid-related polymorphism, which previously had been associated with increased testosterone and estrogen levels, was recently related to both self-transcendence and the extraversion-related subscale novelty seeking.[842,843] As in our study, the allele that was associated with high scores on novelty seeking was also associated with high self-transcendence. The self-transcendence susbscale has previously been associated with CAG repeat length.[844]

Effects of androgens on extraversion-related behaviour
In men, adult testosterone levels have been correlated with extraversion-related behaviours, including novelty seeking, sensation-seeking behaviours and propensity to engage in aggressive behaviours in response to provocation or threat.[845-847] Testosterone administration has been shown to mildly increase manic and punishing behaviours in men,[848,849] and also to increase the response of the amygdala to conscious threat.[850,851]

Androgens also exert early organizational effects.[852] Notably, brain regions that differ in size between men and women are typically those rich in sex steroid receptors.[153,853-856] In men, but not in women, prenatal exposure to androgens, as estimated by the 2D:4D, has been associated with the propensity to engage in aggressive behaviours,[857] thus suggesting an organizational effect of androgen exposure on related traits.

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POSSIBLE ASSOCIATION BETWEEN THE ANDROGEN RECEPTOR GENE AND AUTISM SPECTRUM DISORDER

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The extreme male brain theory suggests that the risk for autism is increased by enhanced prenatal androgen exposure. This theory is based on the higher prevalence for autism in

men than in women, the similarities between sex differences, on the one hand, and differences between subjects with autism spectrum disorder (ASD) and controls, on the other, and also on the association of autism with androgen-related disorders in women. In paper VII, the possible relationship between androgen receptor gene (*AR*) polymorphisms and ASD is examined using case-control (*n*=267 cases and *n*=617 controls) and family-based (*n*=118 families) association analyses. A short CAG repeat length, which previously has been linked to increased AR activity, was associated with increased risk for ASD in women, a finding that is in line with the extreme male brain theory of autism. This relationship between short CAG repeats and ASD was observed in the case-control study. Exploration of the family-based sample showed over-transmission of one specific short, quite rare allele to female offspring. The GGN repeat displayed an increased transmission of short alleles to female offspring and an increased transmission of long alleles to male offspring.

Possible influence of androgen exposure on brain development
Autism is a genetic disorder that displays several signs of neurodevelopmental abnormalities. In line with an influence of prenatal factors, such as early androgen exposure, on autism aetiology, the abnormal growth of frontal brain regions during the first years of life, that is observed in autism, is believed to be compensatory to some other primary pathology present at birth.[3,695,710,858] A possible long-distance under-connectivity and local over-connectivity, caused by abnormal brain development, has been suggested to account for the social difficulties and narrow interest characterizing this condition.[692,695,698,699,709,711,859,860] These aspects of the disorder have also been proposed to be influenced by enhanced prenatal androgen exposure.[675,713]

Possible influence of prenatal androgen exposure on synaptic plasticity
Sex steroids appear to affect synapse formation in adult animals. Estrogens thus enhance synaptic plasticity via estrogen receptors[861] and the reversal of the castration-induced reduction of synaptic contacts and dendritic spines by testosterone and non-aromatizable testosterone suggests ARs to mediate a part of the synaptogenic action of androgens in adult animals.[862-864] Also prenatal androgens appear to affect plasticity, as illustrated by an association between the 2D:4D and hippocampal structure in women.[865] In addition, males and females display differences in dendritic organization. These differences may be prevented by prenatal androgen administration to females.[866] An inhibiting effect of testosterone on synapse formation *in utero* has been suggested by the finding that the increased number of afferent synapses on some neurons in females is reduced to the level of that of males by prenatal androgenisation.[867]

The possibility that the effect of genetic variation in the *AR* on autism is mediated by an effect on plasticity is supported by the fact that several studies have found mutations in genes encoding neuroligins and neurexins in individuals with autism and ASD.[29,30,33,732-736] The interaction between neuroligins and neurexins leads to adhesion between axons and dendrites and subsequent synapse formation, enabling memories to be formed.[730,731]

Sex differences in association studies
In this paper, as well as in paper III and V, the genetic effects are observed only in women or only in men. Although the phenotypes studied in this paper and in papers III and V, *i.e.* autism, serotonin transporter density and depression and SLE reportings, all display sex differences, it is worth noting that the absence of sex differences with respect to a certain

behaviour does not imply that differences[I] in brain processing or in the mechanism by which the behaviour is controlled, are absent.[868] Selection may have affected mechanisms in men and women differently. Interestingly, it has been suggested that some differences observed on the neural level are compensatory to sex steroid-induced developmental differences between men and women, and that these compensatory effects may have the role of preventing large sexual dimorphism in behaviour.[869]

<p align="center">**Paper VIII**</p>

<p align="center">**SEX STEROID-RELATED GENES AND MALE-TO-FEMALE TRANSSEXUALISM**</p>

Animal experiments suggest interactions between testosterone, estrogens, the aromatase that converts testosterone to estradiol, androgen receptors (ARs) and estrogen receptors (ERs) to be necessary for the sexual differentiation of the brain. In paper VIII, the *AR* CAG repeat, an intron 5 CA repeat in the gene encoding ERβ (*ESR2*), and an intron 4 tetra-nucleotide repeat in the aromatase gene, are investigated for association with male-to-female transsexualism (*n*=29 cases and *n*=229 controls). A long CAG repeat, indicative of reduced AR activity, was associated with increased risk for transsexualism, but only when the repeat lengths of the ERβ and aromatase polymorphisms were short, indicating low estrogenic function. Longer ERβ repeat lengths and longer aromatase repeat lengths are associated with an increased risk for transsexualism, but only when the *AR* CAG repeat length is short.

Influence of sex steroid-related genes on masculinization
In our study, dysfunctional masculinization when the *AR* CAG repeat length is short appears to require the presence of aromatase and *ESR2* alleles that are associated with increased estrogenic function (see Figure 6). The association between longer CAG repeat lengths in the *AR* and transsexualism was recently replicated in a much larger sample (*n*=101), showing the mean CAG length in transsexuals to be larger than that in controls.[870] In our sample, the majority of long CAG repeats are more common in transsexuals, but the mean length difference did not reach significance, probably due to the low number of transsexuals. Notably, recent sequencing revealed that the cut-off for the short and long categories of the CAG repeat in this study is the same as for the personality and autism papers. The 18-repeat length in paper VIII is actually 21 repeats long.
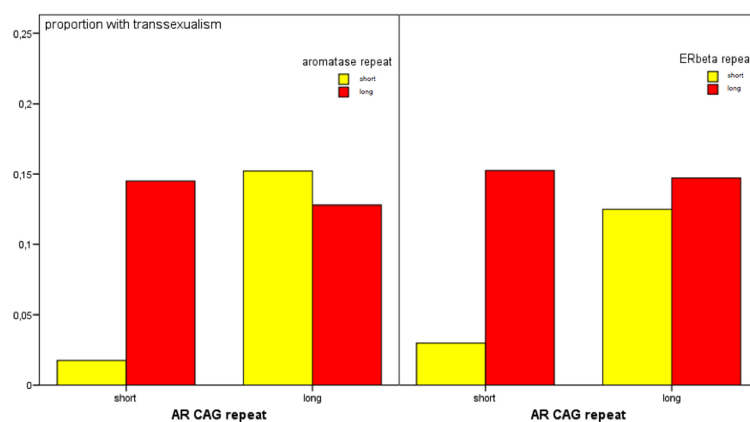

Figure 6. The allele category distribution for paper VIII.

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ON THE DETECTION OF TWO-LOCUS GENE-GENE EFFECTS

Paper IX

DETECTING TWO-LOCUS GENE-GENE EFFECTS USING MONOTONISATION OF THE PENETRANCE MATRIX

In paper IX, a new method for assessing two-locus effects that display a monotone pattern, such as synergistic gene-gene interactions or additive effects, is introduced. The test is 10 percent units more powerful at finding monotone two-locus effect patterns than unrestricted tests.

Different types of two-locus effects
When searching for two-locus effects amongst a number of polymorphisms, the number of tests is dramatically increased compared to the search for single-locus effects only, implicating a reduced power, *i.e.* a reduced probability of finding an effect that exists. The number of possible patterns for a penetrance matrix representing the nine two-locus effects is high. Detected effect patterns may sometimes be discarded because they are considered unlikely. For example, when genotype *A/A* displays the largest phenotypic value in the single-locus analysis for locus A and genotype *B/B* in the single-locus analysis of locus B, a two-locus effect pattern showing a small increase in risk for the *a/a-B/b*-two-locus genotype may be considered to be unlikely and uninteresting.

The power of tests restricted to monotone effect patterns
The power of a single-locus test of finding effects that are monotone, *i.e.* where the phenotypic value of the heterozygote is not outside the interval defined by the phenotypic value for the two homozygotes, can be increased by treating the genotype as a covariate in a linear regression analysis (the middle value representing the heterozygous genotype). Similarly, a two-locus test can be restricted to search only for monotone effect patterns in a two-locus penetrance matrix, thereby increasing the power of finding such effects. In paper IX, the need for tests with increased power is met by the introduction of a test that displays 10 percent units enhanced power by being restricted to search only for monotone two-locus effects. It is however worth noting that such a test would not detect largely non-monotone effects.

Bakgrund

Både neurotransmittorn serotonin och könshormonerna östrogen och testosteron är viktiga för hjärnans funktion. Serotonin är bland annat kopplat till stämningsläge och ångest, vilket illustreras av att läkemedel som agerar på det serotonerga systemet genom att hämma serotonintransportören, och därmed serotonin-återupptaget från synapsen, utövar antidepressiv och ångestlindrande effekt. I hjärnan agerar neurotransmittorerna genom att binda till receptorer på andra neuron än dem som de kommer från, vilket leder till att signalen vidarebefordras. En aktiv serotonintransportör gör att serotonin får utöva effekt under kortare tid på sådana receptorer, d.v.s. att signalen minskar. Blockeras återupptaget fås därmed en förstärkt signal.

Könshormoners roll för stämningsläge illustreras av att de flesta depressions- och ångest-relaterade sjukdomar är vanligare hos kvinnor än hos män, och att sänkt stämningsläge är vanligt under perioder då hormonnivåerna är i förändring. Könshormoner är också viktiga för hjärnans tidiga utveckling, och härigenom sannolikt av betydelse t.ex. för könsidentitet.

Neurotrofa faktorer ökar hjärnans plasticitet, det vill säga till vilken grad som neuronens kopplingar till varandra kan ändras vid t.ex. inlärning. En sådan faktor är brain-derived neurotrophic factor (BDNF). Just denna neurotrofa faktor verkar vara nära kopplad till det serotonerga systemet.

DNA är en molekyl som finns i varje cells kärna i form av 23 stycken kromosom-par. En gen är en bit DNA som kodar för ett protein. Dessa proteiner kan vara t.ex. receptorer, transportörer, eller enzymer som är nödvändiga för syntesen av neurotransmittorer. DNA-sekvensen består av fyra olika kemiska substanser som förkortas A, C, G och T. Även om människors DNA-sekvens är identisk till minst 99.9% så finns det viss variation. På vissa positioner, eller *loci*, i genomet bär olika människor således på olika varianter. Dessa loci är alltså polymorfa och kallas därför *polymorfismer*. En människa kan ha varianten, eller *allelen*, T på en speciell position, där en annan har allelen C, och det kan leda till att proteinmängden eller proteinfunktionen påverkas.

Syfte

I denna avhandling undersöks (i) vilket eventuellt inflytande variation i serotonin-relaterade gener har på ångestrelaterad hjärnaktivitet, premenstruell dysfori, nedstämdhet och personlighetsdrag och (ii) vilket eventuellt inflytande variation i könshormonsrelaterade gener har på personlighetsdrag, autism och transsexualism. Alla dessa drag eller *fenotyper* är till viss del ärftliga.

Resultat

Amygdala är en hjärndel som tidigare visats reagera mer än normalt på emotionella stimuli, som t.ex. bilder av arga ansikten, hos människor som lider av depression eller ångestsjukdom. Dessa förändringar normaliseras av antidepressiv behandling. När vi undersökte polymorfismer i gener som kodar för serotonintransportören och för ett enzym som är nödvändigt för serotoninsyntes i förhållande till aktivering av amygdala, hos patienter med social fobi och hos friska kontroller, visade det sig att polymorfismerna hade ett starkare inflytande på den aktivering av amygdala som induceras av arga ansikten än vad diagnosen social fobi hade. Samma polymorfismers relation till aktivering av amygdala före och efter placebo-behandling undersöktes också hos individer med social fobi som fick ligga och hålla tal för en grupp

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människor samtidigt som deras hjärnaktivitet mättes. Resultatet av denna studie var att de varianter som *inte* var associerade med ökad amygdala-reaktivitet vid presentation av arga ansikten däremot var associerade med huruvida dessa patienter hade svarat på behandling med placebo, och framförallt med hur mycket aktiviteten hos amygdala sjönk under placebobehandlingen.

Vi har också mätt antalet serotonintransportörer i hjärnan, och visat att mängden transportörer hos män är relaterad till variationer i den gen som kodar för den neurotrofa faktorn BDNF. Detta samband kan vara av betydelse för tolkningen av resultat som visar att interaktioner mellan BDNF och det serotonerga systemet påverkar emotionell reglering. Vi visar också att polymorfismer i generna som kodar för serotonintransportören och BDNF verkar samverka i att öka risken för att utsättas för stress, vilket i sin tur skulle kunna öka risken för depression.

I en annan studie undersöks sambandet mellan flera seroronin-relaterade gener och premenstruell dysfori, en allvarlig form av premenstruellt syndrom. Fyra olika genvarianter visade sig vara vanligare hos kvinnor med premenstruell dysfori än hos dem utan. Funktionella studier visar att åtminstone två av dessa varianter leder till en minskad serotonerg transmission, vilket är i linje med att premenstruell dysfori lindras av behandling som förstärker den serotonerga aktiviteten och förvärras av att man stänger av bildningen av serotonin.

Under prenatal utveckling påverkas hjärnan av testosteron. Manliga foster utsätts för mer testosteron än kvinnliga. Autism är mycket vanligare hos män än hos kvinnor, och vissa beteenden som är vanligare hos män än hos kvinnor (på gruppnivå) är också vanligare hos personer med autism än hos kontroller. I tre studier undersöktes den möjliga betydelsen av polymorfismer i gener vars produkt skulle kunna påverka hur stort inflytande könshormoner får på hjärnans utveckling.

Varianter som leder till en ökad aktivitet av den receptor som testosteron agerar via var 30% vanligare hos kvinnor med autism än hos kontroller. Samma variant var också associerad med vissa personlighetsdrag hos män. Varianter som ger minskad aktivitet av samma receptor var relaterade till en ökad risk för transsexualism i XY-individer.

För båda de sista resultaten så var effekten bara tydlig då en speciell variant var närvarande på en annan position i antingen samma gen eller i andra könshormonsrelaterade gener. Fenomenet att en polymorfisms effekt är olika beroende på vilken variant som personen bär på ett annan locus kallas gen-gen-interaktion. Styrkan hos ett statistiskt test för att hitta två-locus-effekter, inklusive vissa sorters gen-gen interaktioner, kan ökas genom att man sätter vissa restriktioner på två-locus-effekternas mönster. En metod som använder sådana restriktioner introduceras i det sista arbetet.

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Methods for assessing genotypes

Amplification
Before genotyping and sequencing, the relevant sequences of DNA need to be amplified. This is done by polymerase chain reaction (PCR). After denaturation or separation of the two DNA strands from each other at 95°C, the two primers anneal to the their target sequences on one DNA strand each at approximately 60°C, a step that is followed by elongation at 72°C, catalysed by a DNA polymerase, leading to an identical copy of the original DNA sequence. In the next cycle, this doubled amount of DNA is again doubled, leading to an exponential increase in DNA amount. The finding of the Taq polymerase, named after the Thermus aquaticus bacterium from which it was originally isolated, revolutionized DNA amplification since it is thermophilic and hence is unaffected by the high temperatures required for DNA denaturation. Before the Taq polymerase was found, DNA polymerase needed to be added to the PCR reaction after each cycle.

Agarose gel separation
The 5-HTTLPR has been genotyped by means of the length difference between the amplified sequence of the short and long alleles. DNA is negatively charged and thus moves in an electric field. DNA placed on an agarose gel in an electric field moves faster when the fragment is shorter. The 14-repeat (short) and the 16-repeat (long) alleles differ in length by approximately 40 basepairs and the short repeat can hence travel faster on the gel. The gel is supplemented with ethidium bromide, which allows for visualization of the genotypes by ultraviolet transillumination.

Pyrosequencing
Pyrosequencing is a sequencing-by-synthesis method that relies on the luminometric detection of pyrophosphate release upon nucleotide incorporation via an enzyme cascade. Hybridisation of the sequence primer to the target sequence is followed by addition of A, C, G and T in a specific dispensation order to the reaction. Depending on genotype, different nucleotides will hybridise with the target sequence at the site of the polymorphism, and the nucleotide incorporated causes the release of pyrophosphate (PP). ATP sulfurylase converts PP, to ATP, an ATP molecule that drives the luciferase-mediated conversion of luciferin to oxyluciferin, hence generating light in an amount proportional to the amount of ATP. The light is detected by a charge-coupled device camera and visualized as peaks.[871] [872]

Sequenom
Sequenom is a genotyping tool based on multiplex PCR, *i.e.* several sequences can be amplified simultaneously, followed by a single base primer extension reaction per polymorphism and a MALDI TOF mass spectrometry analysis. After the PCR, one extension primer per polymorphism, as well as nucleotides, are added to the PCR product mixture. These primers anneal to their target sequence, extension is initiated and advancement of the extension depends on the allele present, the difference between the alleles being the mass of one nucleotide. The different masses are separated by a MALDI TOF mass spectrometer, thus producing a spectrogram where the different genotypes can be visualized.[873]

Methods for assessing brain activity, serotonin transporter and 5-HT1A availability

Brain activity can be measured using positron emission tomography (PET) with radioactively labelled water ($H_2^{15}O$) as tracer. The binding potential correlates with regional cerebral blood flow (rCBF). Comparisons of two conditions are often used (reactivity) to prevent biases introduced by other factors than those controlled for. Regional CBF can be measured also with single photon emission computed tomography (SPECT) with the radioligands 99mTechnetium-hexamethylpropanolamine oxime (HMPAO) or Technetium-99m-ethyl cysteinate diethylester (99mTc-ECD).

When using PET, the radioactively labelled tracer binds to its target in the brain. When the isotope decays it emits a positron that collides with an electron nearby the location where the tracer binds its target, resulting in release of energy in the form of two gamma rays travelling in the exact opposite direction. The position from where the collision took place can be determined by detection of the two gamma rays around the head, taking the temporal aspect into account. The signal is hence proportional to the number of collisions in a brain region. A short halftime of the isotope is favourable since less time then is required for positrons to leave the tracer molecule and since the temporal resolution of PET is determined by the time needed for detecting a sufficient number of signals. When using radioactively labelled water (shortest halftime of O_2: 2 minutes), signals need to be recorded for 30 minutes to become large enough for reliable detection. Rapid fluctuations in brain activity can hence not be measured using PET.

Several different measures are used for assessing binding between the tracer and its receptor. Binding potential is estimated as B_{max} divided by K_d, where B_{max} is the number of binding sites and K_d is the dissociation constant, determined as the ratio between the dissociation rate constant and the association rate constant.

Using PET, the density or availability of the serotonin transporter can be estimated by the binding potential of the tracer [11C]MADAM ([11C]N,N-dimethyl-2-(2-amino-4-methylphenylthio)benzylamine), which displays high affinity for the transporter. A low-affinity tracer can not measure density since it competes with the endogenous neurotransmitter, resulting in lower binding when endogenous levels are high.

The SPECT radioligand ^{123}I-β-CIT (2-beta-carbomethoxy-3-beta-(4-iodophenyl)-tropane labelled with [123]iodine) is a potent ligand for both dopamine and serotonin reuptake sites and can be used to estimate the availability of both of these proteins, the serotonin and dopamine reuptake sites being separated by means of differences in the time of ^{123}I-β-CIT uptake. For the serotonin transporter, the uptake of the ligand is expected to be maximal at the 1-h recording, and this assessment can be corrected for concomitant dopamine transporter uptake using the later SPECT measurements.

No competition with serotonin has been shown for either of [11C]MADAM or ^{123}I-β-CIT. Manipulation of serotonin levels has however been shown to affect binding of the PET radioligand [11C]DASB (3-amino-4-(2-dimethylaminomethyl-phenyl-sulfanyl)- benzonitrile) to the serotonin transporter. [11C]DASB displays a similar affinity and specificity for the serotonin transporter as [11C]MADAM.[874] Tryptophan depletion has been found to decrease [11C]DASB binding, a finding that does not suggest competition with serotonin, but instead serotonin deficiency-induced internalisation of serotonin transporters.[875,876] Increasing serotonin levels by 5-HTP administration, however, leads to reduced [11C]DASB binding potential, a finding that suggests the [11C]DASB ligand to compete with endogenous serotonin over transporter sites.[327] The density of 5-HT1A receptors in the brain can be estimated by the high-affinity radioligand and 5-HT1A antagonist [11C]WAY100635,[329] which does not appear to display any competition with endogenous serotonin.

FOOTNOTES

A Drugs affect the emotional state of individuals. Examples of this are the following: Ecstacy or MDMA (3,4-methylenedioxy- *N*-methylamphetamine), which turns the serotonin transporter around, thus leading to substantially enhanced serotonin levels, induces euphoria and intimacy and reduces fear. Angeldust or PCP (phencyclidine) may induce psychotic symptoms by inhibiting the glutamatergic NMDA receptor.

B The *p*-value: The output of a test that compares groups with regard to various characteristics (*e.g.* genotype frequency) is accompanied by a significance level, a *p*-value. The *p*-value is the probability that the observed data would differ more from the null hypothesis (H_0) than they do, given that the H_0 is true. For the H_0 to be rejected, this probability needs to be low, since a high probability for the observed deviation from the values expected by H_0 suggests H_0 to be true. Before H_0 is tested, a threshold (α) is set on the significance level. If the test results in a *p*-value smaller than this value, then H_0 can be rejected and the alternative hypothesis (H_1) can be accepted. The evidence for rejecting H_0 is considered to be sufficient when α is 0.05 and the *p*-value obtained by the statistical test thus is smaller than this threshold (the probability that the data differ as much as they do from H_0 is less than 5% if H_0 is true). The value α is the type I error, which is defined as the probability that H_0 is rejected given that H_0 is true. When it is set too high, false positives, *i.e.* rejections of the H_0 in spite of the fact that H_0 is true, will occur with a higher probability. However, if α is set too low, the power of finding true effects, *i.e.* the probability that H_0 is rejected given that it is false, will be compromised meaning that the type II error, β, increases ($P(H_0$ accepted| H_0 false)). The statistical power of a test is defined as 1- β and is hence the probability the H_0 is rejected given that H_0 is false, i.e. the chance of finding a true effect.

C Savant skills: In between 0.5 and 10% of individuals with autism spectrum disorder display unusual abilities, such as extraordinary memory performance,[877,878] such as extraordinary memory performance, the ability to read two pages of text simultaneously, the ability to determine complicated mathematical calculations as fast as computers, and the ability to remember all details of a picture after being presented with it for a short period of time. There is evidence for brain morphology and processing differences in subjects that display savant skills, including smaller corpus callosum and the use of posterior brain regions associated with unconscious, procedural memories, when solving complex tasks, for which others require large activation of frontal regions associated with complex mental processing.

D Studies aiming at measuring emotional perception often use the **Ekman faces**. In the sixties, the psychologist Ekman found, by showing photographs from unfamiliar cultures to an isolated tribe, that facial expressions of the six emotions anger, fear, disgust, happiness, sadness and surprise are not culturally determined, as many anthropologists believed at the time, but universal to human culture and thus biological in origin.[879]

E Animal models for depression and anxiety: Fear conditioning is sometimes used as an animal model for assessing anxiety- or depression- related. Fear conditioning is the association of a neutral stimulus (the conditioned stimulus, CS, *e.g.* a light) with an intrinsically aversive stimulus (the unconditioned stimulus, UCS, *e.g.* a footshock), which generates conditioned fear of the CS. Increased fear response to the CS is interpreted as increased anxiety-like behaviour. Contextual fear conditioning is fear conditioning where the CS is an environment. The lateral amygdala is central in the acquisition of fear conditioning, being the location where the neutral and aversive stimuli converge and hence where alterations in synaptic transmission encode the memory according to Hebb's law, stating that Neurons that fire together wire together, originally described as: *When an axon of cell A is near enough to excite cell B and repeatedly or persistently takes part in firing it, some growth process or metabolic change takes place in one or both cells such that A's efficiency, as one of the cells firing B, is increased.* Other animal models for depression and anxiety are (i) the open field test, where the degree to which rats avoid open spaces is positively correlated with their anxiety level, (ii) the elevated plus-maze, where anxiety is quantified as the avoidance of entering open arms, (iii) the tail suspension test where high depression-related behaviour is considered to be reflected by the lack of efforts to escape the situation, (iv) learned helplessness tests, which measures depression-like behaviour as the degree to which the animal acts helpless although it has the power to change its unpleasant situation.

F Memories are divided into explicit, declarative or conscious memories and implicit or unconscious memories. The explicit memories are further divided into episodic memories, which are memories of episodes (where, why, how), and semantic memories, which are memories of facts. The implicit memories include procedural memories such as motor skills and habits and classical conditioning, memories that we are unaware of during encoding, and which are demonstrated in our behaviour. Explicit memories are developed later in evolution (phylogenetically) and later in the development of the individual (ontogenetically).

The hippocampus is required for the formation of new explicit memories. The illustrative HM case[131] lost his hippocampus when lobotomized for epilepsy, an operation that resulted in anterograde amnesia, meaning he could no longer create any new conscious explicit memories. He could however still form procedural memories; when he practiced on tasks requiring motor skills, he improved without the conscious memory of ever having performed the task. The HM case thus clearly illustrates that encoding or retrieval of conscious memories requires the involvement of the hippocampus, whereas formation of unconscious, implicit memories does not. The HM cases did not display depressive symptoms.

G Tryptophan depletion: Tryptophan is actively transported over the blood-brain barrier. When attempting to deplete tryptophan from the brain, so-called tryptophan depletion, a mixture that is rich in amino acids that compete with tryptophan is administered. The other amino acids thus occupy all available binding sites for transport over the blood-brain barrier and tryptophan can consequently not enter the brain.

ᴴ Increased CSF and jugular venous blood **5-HIAA** levels in depression does not necessarily imply increased serotonin levels, since an increased conversion of serotonin to 5-HIAA (increased levels of MAOA have been reported for depression[880]), which is not balanced by a corresponding increase in serotonin synthesis (reduced serotonin synthesis has been reported for depression[881]) is a possible scenario.

ᴵ Equal **behaviour in two groups**, such as men and women, does not imply that there are no differences on the neural level. Thus, when investigating men and women with superior IQ, men show an increased volume of a part of the visouspatial region of the parietal cortex (also large in the brain of Einstein), whereas there are no differences in the morphology between the brains of women with superior IQ and women with normal IQ.[709] Other sex differences are related to lateralization. Men who have damaged the right PFC, and women who have damaged the left PFC, display impairments in decision-making, but not vice versa.[868]

The notion of equal behaviour or performance in spite of processing differences translates to any group differences. Compensatory and opposing influence from other systems may either prevent behavioural differences or render the power too low to show an effect on behaviour. Examples of this are (i) equal performance on working memory between schizophrenic cases and controls, which, however is accompanied by an exaggerated inefficient PFC activation in the schizophrenic group only, an inter-mediate phenotype that otherwise is associated with decreased WM,[882,883] and (ii) equal performance in predicting the correct response in spite of a reduced prediction error signal in schizophrenics.[884] However, the fact that differences are not seen on the behavioural level may also be related to power.

ABBREVIATIONS

5-HTTLPR	Serotonin transporter (5-HTT) –linked polymorphic region
ACC	Anterior cingulate cortex
AR	Androgen receptor
BDNF	Brain-derived neurotrophic factor
CSF	Cerebrospinal fluid
ER	Estrogen receptor
HPA	hypothalamus pituitary adrenal
LD	Linkage disequilibrium
PFC	Prefrontal cortex
PMDD	Premenstrual dysphoric disorder
SRI	Serotonin reuptake inhibitor
TPH	Tryptophan hydroxylase

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