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GENETIC STUDIES OF DEPRESSIVE SYMPTOMS

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Nyss var allt lekande och gott och öppet milliarder mil utåt. Och nu sveper stormen över vajande granar och uppkäftiga tallar. Och spåren, som min pojke gick i dag, äro redan översnöade och borta. Det är icke lätt att sätta varaktiga spår efter sig på jordytan. Jag kan icke hjälpa att jag ser på det hela med dystrare ögon nu än förut, då solen sken.

/AE.

ABSTRACT

This thesis is focused on depressive symptoms particularly in late life. Depressive symptoms are associated with serious negative outcomes a greater level of disability and with excess morbidity and mortality. This thesis includes a series of studies that explore the importance of genetic components on current and past depressive symptoms in a large sub-sample of the Swedish Twin Registry. More specifically gender differences, genetic risk factors, and the role of some genetic variants of candidate genes in the serotonergic system were investigated.

The relative importance of genetic and environmental variance components for depressive symptoms was investigated by means of a twin design. The sex specific heritability estimates (h^2) for depressive symptoms were 14% for males and 29% for females and 23% when constrained to be equal across the sexes. The prevalence of the dichotomous depressed mood variable was 16% for men and 24% for women with corresponding heritability estimates of 7% for males and 49% for females in the full model and 33% in the best fitting (AE) model constrained to be equal across sexes. These results show that depressive symptoms and depressed mood in the elderly are heritable, with an indication of a higher heritability for women than men.

The novel real-time sequencing method, Pyrosequencing™ was evaluated for genotyping of single nucleotide polymorphisms (SNPs) and the first large-scale effort at genotyping using this method was performed. Pyrosequencing genotypes were validated through duplicate analysis of 1022 genotypes against the TaqMan® 5'-nuclease assays. The Pyrosequencing method was highly efficient, robust and accurate in the analysis of SNPs.

The serotonergic system is profoundly associated with mood disorders and there are several serotonergic functions that could contribute to symptoms of depression. Associations between depressed mood and polymorphic variants in the *HTR2A* gene and the *SLC6A4* gene in a sample of 1592 twins were investigated. An increased risk (OR = 2.4) for depressed mood in males associated with a genetic variant of the *HTR2A* gene was found. These findings might be indicative of a gender difference in the genetic composition of depressive symptoms. Monoamine oxidases deaminate biogenic amines like serotonin. The two different forms of the enzyme, MAOA and MAOB are both encoded by genes on the X chromosome. A linkage disequilibrium (LD) map was created by use of nine SNPs to determine the LD structure of the MAO locus. The locus holds two distinct LD blocks, one for each gene, with very few haplotype variants. There was an association with haplotype variants of the *MAOA* gene and trbc-MAO activity. However, there was no association between either, *MAOA* or *MAOB* with depressive symptoms, but an interesting additive effect was observed in females.

Thus, this thesis has demonstrated that genetic variance is important for variation in depressive symptoms and that this variation appears to differ for men and women. Variants of the *HTR2A* receptor are associated with depressed mood in men but not women. Haplotypes of the *MAOA* gene are associated with levels of trbc-MAO activity but there was no clear association between variants of the MAO locus with depressive symptoms. Nonetheless, an increased number of symptoms were found with increasing numbers of alleles of certain haplotypes.

LIST OF PUBLICATIONS

This thesis is based on the following articles and they will be referred to in the text by their roman numerals:

I. **Gender Differences in Heritability of Depressive Symptoms in the Elderly.** M. Jansson, M. Gatz, S. Berg, B. Johansson, B. Malmberg, G.E. McLearn, M. Schalling, N.L. Pedersen. *Psychological Medicine* 2004 *In press*.

II. **Large-Scale Genotyping of Single Nucleotid Polymorphisms by Pyrosequencing™ and Validation Against 5'Nuclease (TaqMan®) Assay.** L. Nordfors, M. Jansson, G. Sandberg, C. Lavebratt, S. Sengul, M. Schalling, P. Arner. *Human Mutation*, 2002, 19:395-401.

III. **Association Between Depressed Mood in the Elderly and a 5-HTR2A Gene Variant.** M. Jansson, M. Gatz, S. Berg, B. Johansson, B. Malmberg, G.E. McClearn, M. Schalling, N.L. Pedersen. *American Journal of Medical Genetics, Part B*, 2003, 120B: 79-84.

IV. **MAO locus haplotypes, serum activity and depressive symptoms.** M. Jansson, S. McCarthy, P. Sullivan, P. Dickman, B. Andersson, L. Oreland, M. Schalling, N.L. Pedersen. *Manuscript*. 2004.

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LIST OF ABBREVIATIONS

5-HIAA	5-hydroxyindolacetic acid
5-HT	5-hydroxyl tryptamine (Serotonin)
HTR2A	Serotonin receptor 2A gene
SLC6A4	Serotonin transporter gene
CES-D	Center for Epidemiological Studies – Depression scale
CNS	Central Nervous System
CSF	Cerebrospinal fluid
DNA	Deoxyribonucleic acid
DRN	Dorsal raphe nucleus
DZ	Dizygotic
h^2	Heritability
Kb	Kilobase (10^3 nucleotides)
LD	Linkage disequlibrium
MAO	Monoamine oxidase
MAOA	Monoamine oxidase A gene
MAOB	Monoamine oxidase B gene
MZ	Monozygotic
PCR	Polymerase Chain Reaction
RNA	Ribonucleic acid
SNP	Single Nucleotide Polymorphism
trbc	Thrombocyte

A BRIEF NOTE ON *MATTER*...

Life is matter. Matter in motion is all that exists and mind and consciousness are merely special configurations of that matter. The rational consequence of this view is the logic of evolution. Sociobiology basically represents an extension of Darwinism from organisms to human behavior. This view of complex behaviors as matter evoked the controversy about nature and nurture in the early 1970's, referred to as the sociobiology wars. The central battleground was at Harvard's Museum of Comparative Zoology and the two main combatants were Edward O. Wilson and Richard C. Lewontin. The two professors perhaps had subtle divergent views on the details of human behavior and its evolution, but the academic dispute had much deeper consequences for the political scene at the time, especially so for the political ideologues on the left together with the social scientists that shared a view of the human mind as being a blank slate from start; that human behavioral development was absolutely free from genetic influences, a mind unaffected by genetic factors or biological processes that might predispose social behavior, especially, to develop in one direction or another. When Edward O. Wilson in 1975 published his book "*Sociobiology*" the controversy reached a climax and it clearly revealed the widespread view of the human mind as a mind that was fully developed by learning, experience, and by the contingencies of history. Now the dispute is settled and biology with its admiral E.O. Wilson has prevailed. Today we know that genetics are of profound importance to all human characteristics.

Fifty years ago the molecular structure of DNA was described by Watson and Crick (Watson et al. 1953) – Three years ago a draft of the human genome was completed (Lander et al. 2001). One of the effects of Watson and Crick's finding was the dawn of a new academic specialty – Molecular biology. Today the split between the two disciplines of biology is so pervasive that many universities have separate departments for molecular biology and the traditional kind. Molecular biology has proceeded in its own way but has taken a long walk back into the traditional field of biology, first by its necessary investigations into cell biology and then onto the organismic level for the need of higher order models. Molecular biology has past the level of the genome in terms of truly major new discoveries and proceeded into the deep sea of proteomics. Proteomics relates to the question of how, after elementary transcription, translation and formation of the proteins, genes are turned on and off. They appear and then do certain things, in part due to context, location, and pre-existing proteins. It takes one or two hundred thousand kinds of proteins to form a cell. The adult human body is made up of approximately 10^{14} cells, all containing the life-sustaining information to keep the complex organism working. How exactly do they come together? Most molecular biologists are now focusing on that area. Once we understand more about the diversity in the genetic code, will we then be able to perceive the strategies that genes have as they create proteins and as the proteins assemble cells? What pathways of evolution have been followed in the course of making adaptations to the environment?

This thesis is an attempt to unravel the genetic properties underlying a common complex human phenotype, depressive symptoms, by means of one of nature's own spectacular events, twinning, and to explore some of the exciting possibilities with new molecular techniques.

INTRODUCTION

GENETICS

Humans have for a long time sensed the concepts of inheritance, mostly from observations of animal and plant breeding, but surely also from familial traits within their own species. In 1866 the Augustinian monk Gregor Mendel concluded that heredity is based on individual factors that are independent of each other. Transmission of these factors to the next generation, i.e. the distribution of different characteristics in the offspring followed predictable proportions and each factor was responsible for a specific characteristic. The term "*gene*" for such a heritable factor was introduced in 1909 by the Danish biologist Wilhelm Johansen. A gene constitutes a single unity of hereditary material that corresponds to a segment of DNA that codes for the synthesis of a single polypeptide chain, i.e. a protein.

As described by Watson and Crick (Watson et al. 1953). DNA is made up of molecules called nucleosides, which constitutes three important molecular units, one base, one sugar and a phosphate. The only variation is in the four different bases, which can be adenine, guanine, cytosine or thymine, commonly referred to by their "letters" i.e. the abbreviations A, G, C and T (Fig 1.). A phosphate –sugar backbone, connects the nucleosides to each other forming a chain, or strand of nucleotides. The DNA-molecule contains two strands of nucleotides. Each strand is then connected to each other by base pairing, A to T and G to C, making them complementary and forming a double helix (Fig 1.). Today we know that the human genome contains some 3,3 billion "letters" (bases) of genetic code, as a draft sequence of the human genome was presented in June 2001 (Lander et al. 2001).

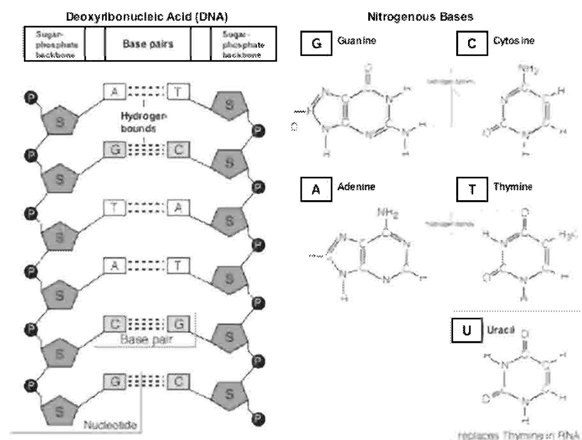

Figure 1. Deoxyribonucleic Acid (DNA). (Picture modified from the National Human Genome Research Institute (NHGRI) by artist Darryl Leja).

Genes are located on chromosomes. Chromosomes are individual, paired bodies consisting of DNA and special proteins and they are located in the cell nucleus. Humans carry 46 chromosomes (Ford et al. 1956; Levan 1956), in 23 pairs, and we inherit 23 from the mother and 23 from the father. Each parent contributes 22

autosomes and one sex chromosome to the next generation. The autosomal chromosomes are numbered from 1 to 22 according to size and similarity. The two sex chromosomes are denoted X and Y. An individual carrying a pair of X chromosomes will become female and an individual carrying one X and one Y chromosome will become a male (Fig 2.). One chromosome in each pair is randomly passed on to the next generation a phenomenon called random segregation.

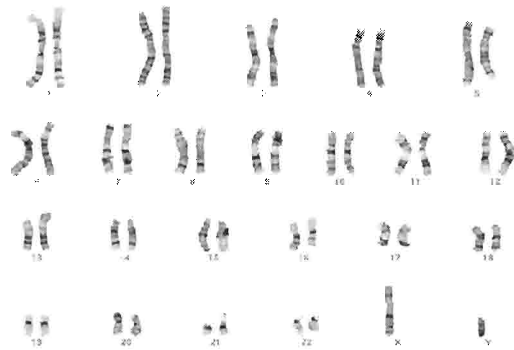

Figure 2. Human male chromosomes

Genes are arranged linearly along each chromosome. Each gene has a defined position, a gene locus, and an individual structure and function that may or may not be known. Humans and all other higher organisms have genes structured into continuous sections of coding sequences, called exons, and non-coding sequences, called introns. Genes vary widely with respect to their overall size (a few thousand to over a million base pairs) number and size of exon/introns, and regulatory DNA sequences that determine their state of activity/expression pattern. The majority of genes are turned of in differentiated and specialized cells and more than 90% of the 3,3 billion base pairs in our genome do not carry any actively protein coding information (Strachan 1996).

If a coding sequence of DNA is changed/damaged (a mutation), such as a single nucleotide base exchange, in a germ cell the mutation will be randomly passed on to the next generation. The genetic alteration, or mutation, can be any kind of sequence change such as the above-described point mutation or a deletion, insertion or a repeat expansion. These mutations will be passed on to the next generation in either a dominant fashion, that is only one chromosome of a pair needs to carry the mutation in order to cause a disease phenotype. It can also be inherited in a recessive manner, which means that both chromosomes in a pair need to carry the mutation to cause a disease phenotype. There are also X-linked disorders that usually only manifest in males, since males are hemizygous for almost all genes on the X chromosome. If a mutation causes an alteration of the coded product (protein) that is essential to the function of the cell and thereby causes a disease or character change in the organism, it will if not lethal, be passed on the next generation according to Mendel's law of inheritance. Diseases inherited in this Mendelian manner are often referred to as monogenic, i.e. one mutation (in one gene) one disease.

Most Mendelian monogenic disorders display a strong correlation between the mutation (genotype) and the disorder (phenotype); this means that there is an almost complete (100%) penetrance. Mutations in a specific gene are normally both necessary and sufficient for the phenotype to be expressed. However, there are examples where a deleterious mutation in one of several genes is necessary to cause disease, but a mutation in any of those genes is on the other hand sufficient to cause the disorder. There are examples of both allelic and genetic heterogeneity. Allelic heterogeneity is perhaps best exemplified in cystic fibrosis, where more than a 1000 disease causing mutations is known in the large cystic fibrosis gene (Cystic fibrosis mutation database, http://www.genet.sickkids.on.ca/cftr). An example of genetic heterogeneity is retinitis pigmentosa where more than 20 mutations in different genes are known to cause the disease (Farrar et al. 2002). For monogenic disorders with a high penetrance and with a known family history, it is often possible to determine the mode of inheritance by looking at the family pedigree.

There are now more than 4000 diseases described, and less than 15% of them have no known associated gene (Perez-Iratxeta et al. 2002). More than half of all the phenotypes described exhibit an autosomal dominant inheritance with examples such as Huntington's disease. Examples of recessive disorders are cystic fibrosis and Tay-Sachs disease. Due to the fact that males only carry one X chromosome (hemizygous), recessive X-linked disorders typically affect males. A classic example is the manifestation of hemophilia A in some royal families of Europe but also color blindness in males.

There are however exceptions to the Mendelian segregation of monogenic disorders. There are three known mechanisms that give rise to a non-Mendelian pattern of inheritance. A first example are disorders inherited in the mitochondrial genome (a small genome localized in the mitochondrion and separated from the cell nucleus and maternally inherited). A second mechanism is by uniparental disomy, a special case where both chromosomes in a pair are inherited from the same parent. Partly related, in terms of disorders to uniparental disomy, is the third mechanism, referred to as genomic imprinting. Genomic imprinting reflects a parent-of-origin-specific gene expression of certain genes. The genome contains defined regions where only the maternal or paternal gene copy is expressed. Prader-Willi syndrome and Angelman syndrome are two distinct developmental disorders that involve alterations of genomic imprinting.

COMPLEX GENETICS AND QUANTITATIVE TRAITS

The majority of all diseases where individuals are classified as affected or unaffected do not display a clear or simple Mendelian inheritance and they do not cluster in a consistent pattern in families. Disorders like type II diabetes, cardiovascular disease and depression manifest a complex pattern reflecting a multitude of risk factors of both environmental and genetic origin. They are often referred to as polygenic, i.e. several genes act (additively or non-additively) to manifest the disorder. These multifactorial diseases often display a considerable comorbidity such as, obesity and cardiovascular disease (Must et al. 1999) or Alzheimer's disease and depression (Alexopoulos et al. 1993).

Many traits can be measured on a continuum, such as cognitive abilities, blood pressure, or depressive symptoms. Diseases, however, are typically considered to be dichotomous outcomes (affected or non-affected), although in some cases one can apply a semi-continuum of mild to severe affectation. When a trait is essentially a categorical outcome, the quantitative geneticist assumes that there is an underlying liability to develop the disorder that is continuously and normally distributed in the population. Only those individuals who exceed a certain threshold in liability manifest as affected. Relatives of affected individuals have an increase in their mean genetic liability compared to the population mean (Fig 3.).

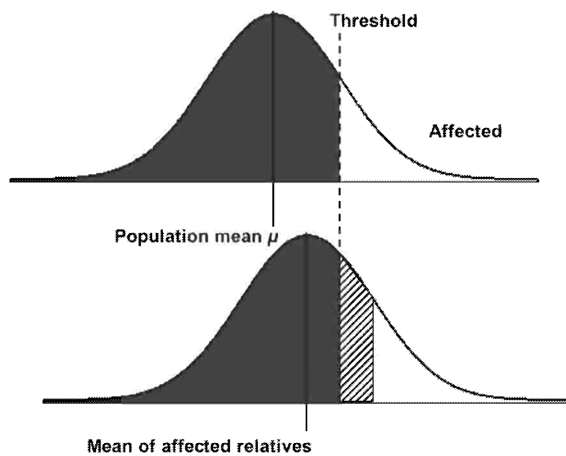

Figure 3. Liability threshold model.

In the simplest quantitative genetic model, any complex phenotype (P) is the result of a combination of genes (G) and environments (E):

$$P = G + E \qquad \textbf{Equation 1}$$

The variation in complex phenotypes (V_p) is thus a function of genetic variance (V_g) and environmental variance (V_e):

$$V_p = V_g + V_e \qquad \textbf{Equation 2}$$

The genetic contribution to variation in a trait (V_g) comprises both additive genetic variance (V_a) and dominance variance (V_d). The heritability (h^2) of a trait is the proportion of the total phenotypic variance that can be attributed to additive genetic effects and is referred to as the narrow definition of h^2. The total genetic contribution to a trait is commonly used and it is referred to as the broad definition of h^2:

$$h^2 = \frac{V_g}{V_p} \qquad \textbf{Equation 3}$$

If the genetic contribution to a trait is purely additive then broad sense and narrow sense h^2 will necessarily be the same.

Similarly, environmental variance (V_e) can be divided into common or shared environmental variance (V_{ce}) and non-shared environmental variance (V_{ns}). Shared environmental variance refers to environmental variation contributing to the similarity among family members whereas non-shared environmental variation contributes to differences within families. The proportion of phenotypic variance (V_p) attributed to shared environment (V_{ce}/V_p) is referred to as c^2.

Familial aggregation in a trait might be due to either h^2 or c^2, or both if any of them is greater than 0. It is important to note that both terms (h^2 and c^2) are population estimates that refer to the proportion of variance accounted for in a specific population at a given time. Neither h^2 nor c^2 has a simple explanation at the individual level. An h^2 of 0.5 for a trait, e.g. IQ, does not imply that 50% of an individual's IQ score from that population is determined by genes, but rather that 50% of the variation (individual differences) in IQ in that particular population is due to genetic differences among the members of that population. Variance attributed by h^2 and c^2 varies between populations for a number of traits.

The three key methods used to study quantitative psychiatric genetics involve families, adoptees and twins. Family studies generate information on the extent of familial aggregation while adoption and twin studies allow effects of shared environment and shared genes to be estimated.

FINDING THE RIGHT GENES

It is not as straight forward to find the causative genetic variants behind complex phenotypes as it is with monogenic disorders. The mixed effects and interactions of multiple genes and environments usually cause huge heterogeneity in the disorder or trait. In addition to heterogeneity there are other complicating factors such as gene-gene or gene-environment interactions (Bateson 1909; Cordell 2002).

For rare monogenic disorders, positional cloning has been a very successful approach to find causative genes; cloning of the cystic fibrosis gene in 1989 serves as a good example of this (Kerem et al. 1989; Riordan et al. 1989; Rommens et al. 1989). While hundreds of genes causing monogenic disorders have been cloned and characterized through linkage followed by positional cloning often in large disease burdened families, the approach has unfortunately not been as successful when it comes to complex disorders (Altmuller et al. 2001).

Another approach to map disease-causing genes, or variants of genes, is through association studies that can be performed either as a candidate gene approach or as a candidate region approach (often based on linkage results). The major difference compared to linkage studies is that association studies are based on an *a priori* disease or biological hypothesis and are therefore generally used for a higher genetic resolution. They are also typically performed in large unrelated case-control settings instead of family data. Association studies have become a very popular tool in trying to map genes for complex diseases, much because the approach under the right circumstances can be far more successful than linkage studies (Risch et al. 1996).

In an association study, disease markers (e.g. SNPs) are typically selected in the hypothesized disease gene or gene region and then genotyped in a case-control setting or population sample. The genotype frequencies are then compared between the two

groups of affected and unaffected individuals and significant differences will indicate a causative effect of the variant. The choice of marker is of great importance for the interpretation of the results. A SNP associated with increased risk for disease can either be a direct effect of the variant, e.g. if it causes a vital amino acid substitution affecting the protein function, or the SNP can be in linkage disequilibrium (LD) with one or more other variants affecting the protein by function or expression.

In genomic regions with high LD, haplotypes (LD blocks) can be used for associations and potentially also for disease gene mapping. Measures of LD vary widely in the human genome both due to population history and recombination frequencies in different parts of the genome. There is a fair correlation between LD measures and physical distance in the human genome, but it is not absolute (Dunning et al. 2000; Taillon-Miller et al. 2000; Reich et al. 2001a; Stephens et al. 2001a; Phillips et al. 2003). The only way to ascertain the true haplotypes of a genomic region is to sequence the complete region in every study subject although this is not yet, a realistic approach for most studies. Although a haplotype map project is under way, there is no global harmonization of LD measures; every study has to make its own interpretations of their LD findings based on the prior knowledge of the genomic region, distance between markers, ethnic background of the study population etc.

It has been suggested that common variants will cause common diseases (Reich et al. 2001b), and common genetic variants will most likely have a long history in the human genome reflecting an insignificant selective pressure against them. Another hypothesis is that multiple rare variants displaying a large degree of allelic heterogeneity will be responsible for most common diseases (Pritchard 2001; Weiss et al. 2002). If the rare variants are the major genetic risk factors for common diseases then the use of LD maps and haplotypes to find these causative variants becomes more cumbersome. There are several successful examples where risk alleles, both common and rare, have been characterized in complex diseases. The *NOD2* gene in Crohn's disease, where both a linkage study and an association study based on a biological hypothesis were successful in finding the risk variants, is one example (Hugot et al. 2001). In this case there was strong LD in the region of interest and several markers could be used for equally good association results (Rioux et al. 2001). Other examples are *APOE* in Alzheimer's disease (Saunders et al. 1993), *PPAR-γ* in type-2 diabetes (Altshuler et al. 2000a) and *CKR5* in HIV infection (Dean et al. 1996).

Despite the advantages gained in association studies there are some risks that are necessary to keep in mind. Just because a genetic variant has been associated with a particular disease, this does not definitely prove that the variant is causative for the disease, but merely shows that the variant is over represented among disease cases compared to the controls. It is therefore of utmost importance to have controls matched for each and every character except for the disease with the patients in a case-control setting. It is also important to be aware of possible secondary effects or confounders. For example, low birth weight may be associated with a higher risk for cardiovascular disease later in life. However, it is not necessarily so that a low birth weight actually leads to cardiovascular disease later in life. The effect may be seen because a low birth weight renders reduced physical stature compared to equal aged and thereby less physical activity (due to less success in sports). The lack of exercise in turn increases the risk for cardiovascular disease. Considerable concern in association

studies has been focused on chance findings, false associations commonly referred to as type I errors. When large numbers of variants are being investigated (multiple testing) in limited sized samples, significant associations will definitely be found as an effect of the chosen level of significance. Methods to compensate for multiple testing, like Bonferroni correction, make it more or less impossible to find any associations in studies where several variants are tested, since small effect sizes are generally to be expected for causative mutations in a complex disorder. Replication studies are therefore needed before association data are accepted as fact.

A NATURAL EXPERIMENT - TWIN STUDIES

Twinning is an unusual event in nature, but occurs in approximately 1 in 80 births in humans. Twinning can occur from a single fertilized egg, giving rise to monozygotic twins (MZ) or from two different eggs rendering dizygotic twins (DZ). MZ twins arise by separation at a very early stage of development and they always have a common placenta, but they may be in one common or two separate amniotic cavities. DZ twins on the other hand, always have their own amniotic cavity and they can either share a placenta or have separate ones. Of all twin births roughly one-third are MZ twins, one-third same-sex DZ twins and one-third opposite-sex DZ twins.

Twinning is an excellent natural experiment for estimating effects of genetic and environmental influences on complex human traits (Martin et al. 1997; MacGregor et al. 2000). Since MZ twins are the product of a single fertilization they will be genetically identical clones and necessarily be concordant for any genetically determined characteristics, with very few exceptions. This is true regardless of the mode of inheritance or number of genes involved. Fraternal, or DZ twins share half their genes on average, the same as any pair of sibs. Assuming that both MZ and DZ twin pairs are equally influenced by environmental effects, any greater similarities for a genetically influenced trait should on average show a higher concordance (similarity) in MZ than DZ twins.

Expanding on Similarities

Intra-pair twin resemblance is often reported as a concordance rate. There are two measures of concordance, pairwise- and probandwise concordance, where probandwise concordance is the preferred measure (McGue 1992). Pairwise concordance is calculated as the number of pairs where both are affected divided by the total number of pairs. Probandwise concordance is calculated as the number of affected twins divided by the total number of co-twins.

The proportion of variance in a trait attributed to genetic factors (h^2), shared environment (c^2) and non-shared environment (e^2) can be estimated from intra-pair correlations. Since DZ twins on average share 50% of their segregating genes and MZ twins necessarily share 100% of their genes, DZ twins have a genotypic correlation of 0,5 (on average) and MZ twins a genotypic correlation of 1. Therefore the DZ-pair correlation, in terms of sources of variation, will be:

$$r_{DZ} = \tfrac{1}{2}h^2 + c^2 \qquad \textbf{Equation 4}$$

And the MZ-pair correlation:

$$r_{MZ} = h^2 + c^2 \qquad \textbf{Equation 5}$$

Falconer (Falconer 1965) proposed a simple algebraic formula to resolve h^2:

$$h^2 = 2(r_{MZ} - r_{DZ}) \qquad \textbf{Equation 6}$$

This means that the proportion of variance attributed to genetic factors (h^2) in a quantitative trait can be estimated roughly by doubling the difference between MZ and DZ correlations. This estimate is the broad sense heritability, which is a statistic that describes the proportion of phenotypic variation that can be attributed to all genetic influences. Consequently, shared environment (c^2) and non-shared environment (e^2) can also be estimated from intra-pair correlations:

$$c^2 = 2r_{DZ} - r_{MZ} \qquad \textbf{Equation 7}$$

$$e^2 = 1 - h^2 + c^2 \qquad \textbf{Equation 8}$$

If identical and fraternal twin correlations are the same, h^2 is estimated as zero, whereas the estimate of the c^2 will be approximately the same as the average twin correlation. If identical twins correlate 1.0 and fraternal twins correlate .50, a heritability of 100 percent is implied. That is, genetic differences among individuals completely account for their phenotypic differences. So, the question of whether or not a trait is heritable is a question about the role that differences in genes play in the phenotypic differences between individuals.

Path analysis with model fitting

As discussed above the phenotypic variance (V_p) is a function of genetic (h^2), shared environmental (c^2) and non-shared environmental (e^2) factors. The sources of V_p can be illustrated in a path diagram representing a linear model, (Fig 4.) in which the path coefficients h, c and e represents the regression coefficients (beta weights) in a multiple regression model.

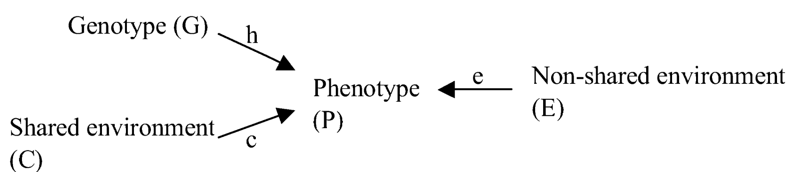

Figure 4. Individual path diagram.

Applying a path model to a pairs of MZ and DZ twins will display the coefficients of importance for resemblance in the pairs. The genetic correlation (r_g) is set to 1 for MZ twins and 0,5 for DZ twins and based on the equal environment assumption the shared environmental correlation (r_c) is set to 1 in both instances. The rules of path analysis can then be applied to derive the same coefficients as noted in equations 4 and 5 above, e.g. that the correlation for MZ twins is h * 1 * h plus c * 1 * c whereas the resemblance of DZ pairs is h * 0.5 * h plus c * 1 * c, which may be reduced to 0.5 h^2+c^2 (Fig 5.).

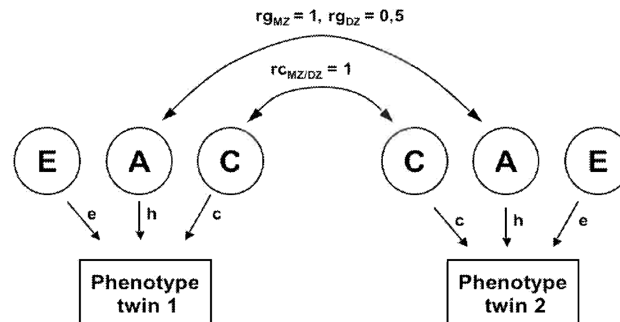

Figure 5. Path diagram for pair of twins

One purpose of fitting statistical models based on the resemblance between relatives for a particular trait is to partition the variation in the phenotype in a testable manner that allows assessment of goodness of fit. Estimating parameters in the simple way described above, from e.g. concordances or differences in intrapair correlations does not allow for such model-based stringency.

Rather than using concordances to partition the phenotypic variation in a trait, it is practicable to use iterative methods to find the best fitting model and estimates of variance components. Iterative programs such as Mx, (Neale 1999) will return the best fitting model according to the lowest χ^2-value or the maximum log likelihood value, given appropriate starting values and model expectations. This procedure, called structural equation modeling, evaluates expectations concerning familial similarity with observed measures of similarity, such as variance-covariance matrices and it will generate confidence statistics for all the estimated parameters.

Nested models, with fewer estimated parameters, can subsequently be compared to the "full" model, where all parameters are estimated, by their fit statistics. Significant differences between two models will then indicate which model should be preferred. At least two groups of twins are typically used for these analyses: MZ twins and same sex DZ twin pairs.

Gender differences in twin studies

To estimate differences in heritability between males and females four groups of twins are needed in the analysis – Male MZs and DZs together with female MZs and DZs.

A second series of models can be used to evaluate other aspects of gender differences (Neale et al. 1989). By including opposite sexed pairs one can evaluate not only whether the magnitude of genetic and environmental effects is equal in men and women, but also whether different genes influence liability to the disorder in males and females. When genetic and environmental parameters are constrained to be equal it is possible to test for gender differences in the relative importance of genetic and environmental effects for the phenotype. Subsequently, one fit models for sex-specific genetic (r_g) and familial-environmental (r_c) effects. The genetic correlation (r_g) for opposite-sexed DZ twin pairs can be estimated instead of being fixed to 0.5, and in a separate model the shared environmental correlation (r_c) can be estimated rather than being fixed to 1. The estimation of r_g and r_c in separate models allows for detection of

qualitative differences, i.e. different genes or shared environments in men compared to women.

DEPRESSIVE SYMPTOMS

Major depression is expected to be the second most important global disease after ischemic heart disease in disability-adjusted life years (DALYs) by year 2020 (Murray et al. 1996). Unfortunately, the etiologies of mood disorders remain largely unknown. It seems fairly clear that health problems or stressful events may contribute to precipitating depression (Murrell et al. 1992), but it is also apparent that genetic factors increase the vulnerability to depressed mood. Depression is the most common emotional disorder among older adults (Blazer 1980) and it significantly decreases quality of life in the elderly population (Blazer 2003). Among older adults, not only major depression but also subsyndromal depressed mood has consequences for mortality and morbidity (Burvill et al. 1994; Gallo et al. 1997).

Measurements and sub types of depression

There is no universal agreement among clinicians and clinical investigators as to what exactly constitutes a clinically significant depression and what its components are. But there are several commonly used scales for both clinical diagnoses and population screening.

Major depression is diagnosed in adherence to the Diagnostic and Statistical Manual, Fourth edition (DSM-IV), when one or both of the two core symptoms, depressed mood and lack of interest, are present together with four or more of the following symptoms for at least two weeks: 1) Feelings of worthlessness or inappropriate guilt, 2) diminished ability to concentrate or make decisions, 3) fatigue, 4) psychomotor agitation or retardation, 5) insomnia or hypersomnia, 6) significant increase or decrease in weight or appetite, 7) recurrent thoughts of death or suicidal ideation (APA 1994).

Subsyndromal depression is present according to DSM-IV criteria when one of the core symptoms listed for major depression is present along with one to three additional symptoms (APA, DSM-IV 1994). A common technique to assess less severe depression, not meeting the criteria for major depression, is to apply a score of 16 or above on the Center for Epidemiologic Studies Depression Scale (CES-D) (Radloff 1977; Lewinsohn et al. 1997). This scale summarizes the frequency of 20 depressive symptoms that may have been experienced during the past week. The CES-D scale is commonly used in population screenings and CES-D scores are quite stable over time (Fiske 2002). It has further proven to have good psychometric properties in elderly Swedish community samples (Gatz et al. 1993).

Epidemiology of depressive symptoms

High levels of depressive symptoms are common among the elderly in western societies, with clinically significant levels reported in 10 to 20% (Blazer et al. 1980; Gurland et al. 1983; Lindesay et al. 1989; Snowdon 1990; Haynie et al. 2001; Hybels et al. 2001). Slightly higher proportions have been reported from southern Europe (Amaducci et al. 1998; Zunzunegui et al. 1998; Fuhrer et al. 1999; Minicuci et al. 2002). Despite this, high scores of depressive symptoms surely reflect a poor health status. Cultural differences may influence reporting and perception of depressive symptoms;

the prevalence rates are considerably higher compared to major depression that range from 1% to 4% in elderly community samples (Blazer 1980; Blazer et al. 1987b; Beekman et al. 1999; Steffens et al. 2000). Depressive symptoms are not more frequent in late life compared to mid-life (Murrell et al. 1983; Charles et al. 2001) but effects of higher mortality and problems in finding cases in late life might bias the frequencies (Mirowsky 2000). The oldest old display higher frequencies of depressive symptoms but this is explained by the higher proportion of women, age related physical disabilities, cognitive impairment and lower socioeconomic status (Meller et al. 1996; Blazer 2000).

A large number of studies have reported higher prevalences of both depressive symptoms and clinical depression in women than in men. If this sex difference in prevalence is not an effect of biased ascertainment, it is possible that the higher prevalence in women reflects a true gender difference in genetic and environmental influences on liability to depressive symptoms.

Etiology of depressive symptoms

Numerous twin studies of affective disorders have indicated that the concordance for MZ twins differs markedly from that of DZ twins. In a meta-analysis of major depression, five twin studies with an average age ranging from 34 to 53 revealed an overall heritability estimate of 37% (Sullivan et al. 2000). The highest heritability was found in a sample of Swedes who were on average older than the other samples (Kendler et al. 1995a). There was almost no effect of shared environment, but a substantial effect of the unique environment (Sullivan et al. 2000). That genetic factors are involved in the etiology of depressive symptoms among the elderly has been implicated in three previous twin studies (Gatz et al. 1992; McGue et al. 1997; Carmelli et al. 2000). These studies of depressive symptoms in community samples of older adults have reported somewhat lower heritabilities than those for affective disorders; 18% to 25%, with a small influence of shared environment but a major effect of unique environment (Gatz et al. 1992; McGue et al. 1997; Carmelli et al. 2000). When a clinical cut-off was applied in the Gatz et al. study, differences between heritability estimates for major depression and for depressive symptoms from self-report scales was diminished. In that study, which included twins aged 60 and older from the Swedish Adoption/Twin Study of Aging (SATSA), concordance rates for scoring 16 or higher on the CES-D depression scale were 33% in MZ and 12% in DZ pairs (Gatz 1992). Concordances for affective illness found in a Swedish clinical sample were 45% and 19% for MZ and DZ pairs respectively (Kendler et al. 1993).

There have been several studies that have addressed the issues of gender differences in major depression using a twin study design (Bertelsen et al. 1977; Kendler et al. 1995a; McGuffin et al. 1996; Bierut et al. 1999; Kendler et al. 2001) and virtually all studies indicate differences between genders. The largest of these studies found a higher heritability for major depression in women than men as well as an indication of separate genes acting on the liability to major depression (Kendler et al. 2001).

The biological patterns of depression in late life are strongly associated with medical illness such as dementia, cardiovascular disease, Parkinson's disease, head injury etc. The comorbidity of depression with these disorders has lead to a growing interest in analyzing specific genetic markers for late-life depression. One example of

such an attempt is the *APOE* gene, where the ε4 allele is strongly associated with Alzheimer's disease, although no association has been found with depression (Blazer et al. 2002). Genes associated with vascular lesions in the CNS have also been investigated with depression, such as a genetic variant of the *MTHFR* (Methylene Tetrahydrofolate Reductase) gene that has also been associated with major depression in the elderly (Hickie et al. 2001). Depression is one of the initial symptoms for cerebral autosomal dominant arteriopathy with infarcts and leukoencephalopathy (CADASIL), which is caused by a very rare mutation in the Notch 3 gene. These findings suggest that there are polymorphisms and mutations that cause a genetic predisposition to depression in the elderly (Desmond et al. 1999; Krishnan 2002). Furthermore, major depression and even subsyndromal depression assessed through the CES-D scale can be effectively treated with antidepressant medication, predominantly targeting serotonin in the CNS (Snaith 1987). As a result, there has been considerable interest directed toward serotonergic activity in mood disorders.

THE SEROTONERGIC SYSTEM AND THE MOOD HYPOTHESIS

Serotonin – A substance originally derived from serum, has been known as a neurotransmitter in the human brain since the mid 1950's (Brodie et al. 1955).

The monoamine serotonin or 5-hydroxytryptamine (5-HT) has been intensely studied due to its demonstrated involvement in mammalian behaviors and several lines of evidence support a role for the serotonergic system in the pathogenesis and treatment of several psychiatric disorders, among them the spectrum of mood disorders.

The prefrontal cortex is involved in a large number of behavioral traits and controls, hierarchically the activity of subcortical structures (Fuster 1997; Miller et al. 2001). Modulatory inputs to the prefrontal cortex arise from the brainstem, e.g. the raphae nuclei (Azmitia et al. 1978) where large fraction of the serotonergic cells are located (Rubenstein 1998).

Synthesis and catabolism

Serotonin is widely distributed in the human body, in fact only 1-2% of the total body serotonin is found in the brain. But 5-HT cannot pass the blood-brain barrier and therefore brain cells must be capable of synthesizing their own 5-HT. The synthesis of 5-HT is based on the amino acid tryptophan, which is hydroxylated at the 5' position by the enzyme trytophanhydroxylase (TH) to form 5-hydroxytryptophan (5-HTP) and then decarboxylated by amino-acid decarboxylase (AADC) to 5-HT (Fig 6.). The synthesis of 5-HT is affected by the availability of tryptophan. Thus, a limited intake of tryptophan results in a lowered synthesis of serotonin (Gessa et al. 1974).

Figure 6. 5-HT synthesis and catabolism.

The major mechanism for the termination of synaptic signaling and maintaining transmitter homeostasis at the nerve terminals of released 5-HT is performed by re-uptake into the presynaptic intracellular space through the serotonin transporter molecule (5-HTT) (Fig 7.). Serotonin is then, if not restored in new vesicles, degraded by monoamine oxidases A and B to 5-hydroxyindoleacetataldehyde and further oxidized by aldehyde dehydrogense to 5-hydroxyindolacetic acid (5-HIAA) in the mammalian brain (Fig 6 and Fig 7).

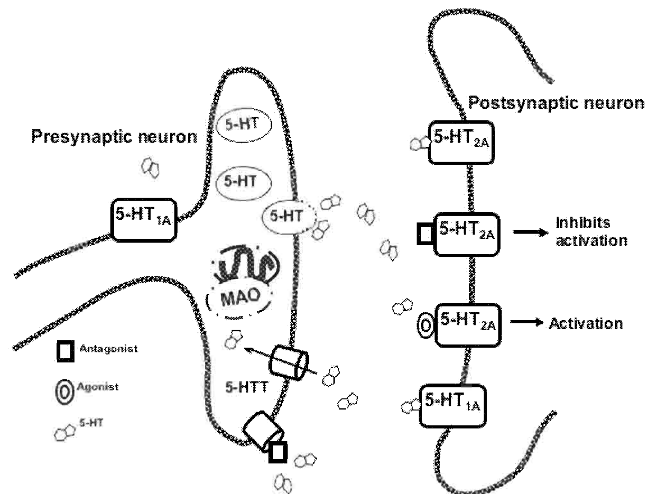

Figure 7. Synaptic localization of 5-HT.

CNS serotonergic projections

Serotonergic cells in the adult human CNS are located in the raphe nuclei, and largely restricted to the basal plate of the pons and medulla of the brainstem (Rubenstein 1998). The serotonergic neurons in the raphe nuclei sends efferent projections innervating a wide distribution of receiving areas throughout the nervous system, from the spinal cord to the cortex (Dahlström et al. 1964). One major cluster of the raphe nucleus is the dorsal raphe nucleus (DRN) that produces axonal projections to mid- and forebrain regions (Molliver 1987).

Forebrain serotonin is derived nearly entirely from neurons located in the dorsal and median raphe nucleus (DRN) of the midbrain (Anden et al. 1966; Ungerstedt 1971). Prominent forebrain terminal regions include the hypothalamus, cortex, septum, hippocampus, amygdala and the striatum. The frontal cortex and striatum appears to be primarily innervated by neurons in the DRN (Azmitia et al. 1978; Molliver 1987). Furthermore, serotonergic neurons are highly bifurcated, indicating that they affect several regions of the CNS simultaneously (Fig. 8).

Serotonin containing neurons in the midbrain directly innervate corticotropin-releasing hormone (CRH) containing cells located in paraventricular nucleus (PVN) of the hypothalamus. Serotonergic input into the PVN mediate the release of CRH, leading to the release of adrenocorticotropin, which triggers glucocorticoid secretion from the adrenal cortex. 5-HT_{1A} and 5-HT_{2A} receptors are the main receptors

mediating the serotonergic stimulation of the hypothalamic-pituitary-adrenal axis. In turn, both CRH and glucocorticoids have multiple and complex effects on the serotonergic neurons. Therefore, these two systems are interwoven and communicate closely. The intimate relationship between serotonin and the hypothalamic-pituitary-adrenal axis may be of great importance in normal physiology such as circadian rhythm and stress, as well as pathophysiological disorders such as depression, anxiety, eating disorders, and chronic fatigue (Dinan 1996a; Dinan 1996b).

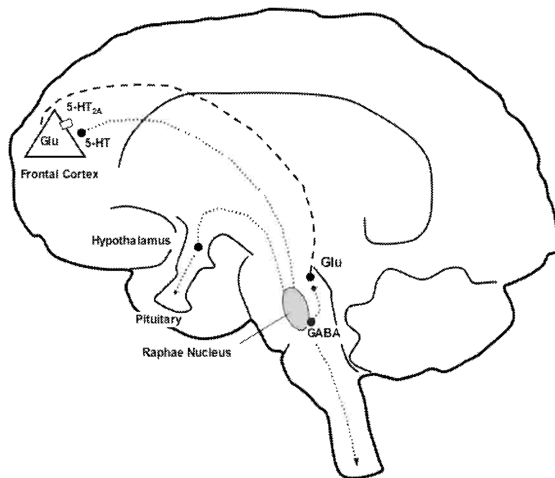

Figure 8. Serotonergic projections in the human brain.

Regulation

DRN, one major serotonergic nucleus, receives input from several brain regions such as the prefrontal cortex (PFC), hypothalamus and the brainstem which modulates its function. The electrophysiological activity (firing rate) of serotonergic neurons in the DRN is also subject to inhibitory feedback regulation trough presynaptic 5-HT_{1A} autoreceptors activated by transmitter release (Wang et al. 1977; Bunin et al. 1998; Celada et al. 2001).

In the mPFC-DRN circuit the activity of cortical pyramidal cells and serotonergic cells in the DRN is regulated by complex interaction between activating 5- HT_{2A} and inhibiting 5- HT_{1A} receptor activity. The mPFC receiving excitatory input from DRN, activates through 5-HT_{2A} receptors which enhance the release of glutamate, which acts to increase impulse flow from pyramidal neurons projecting to the DRN resulting in an increased 5-HT activity and 5-HT release in mPFC (Martin-Ruiz et al. 2001). Activation of inhibitory 5-HT_{1A} receptors on pyramidal cells in the mPFC decreases 5-HT release, likely by descending inputs to the DRN (Celada et al. 2001). The firing-suppressant action on DRN 5-HT neurons is probably accounted for by an action on local GABAnergic elements inhibiting 5-HT neurons (Liu et al. 2000).

The Serotonin Transporter

The 5-HT transporter gene (*SLC6A4*) located on chromosome 17q11.1-q12 codes for a 630 amino acid long protein with 12 transmembrane domains. The transporter protein is driven by the ionic and electrical gradient over the cell membrane

(Ramamoorthy et al. 1993a; Ramamoorthy et al. 1993b). The 5-HTT protein regulates the local presynaptic re-uptake of 5-HT and thereby the synaptic effect of 5-HT. 5-HTT is also the main target for modern antidepressant drugs (i.e. SSRIs) but is also affected by drugs like cocaine and amphetamine (Ramamoorthy et al. 1993a).

The *SLC6A4* gene has been extensively studied in association studies with psychiatric traits such as major depression (Ogilvie et al. 1996). Two polymorphisms are commonly used, a variable number of tandem repeats (VNTR) polymorphism (in the literature often referred to as 5-HTTLPR) located in the promotor region that affects the level of gene expression (Heils et al. 1996; Lesch et al. 1996), and a second VNTR within intron 2 of the *SLC6A4* gene suggested to have an enhancer effect on the transcription (Fiskerstrand et al. 1999). These two VNTR polymorphisms have both been shown to differentially affect gene expression; it is also possible that there are other additional polymorphisms in LD with these two, affecting transcription of this gene (Veenstra-VanderWeele et al. 2000).

The short allele of 5-HTTLPR has been associated both with a reduced *SLC6A4* gene expression and with neuroticism and affective disorders (Collier et al. 1996; Lesch et al. 1996). A vast number of other psychiatric phenotypes have also been investigated with regard to the 5-HTTLPR (Glatt et al. 2002). Depressed patients homozygous for the long 5-HTTLPR allele have been shown to respond better to SSRIs treatment (Catalano 2001). Elderly depressed patients have an increased platelet activation associated with the 5-HTTLPR polymorphism. This group also has significantly higher platelet factor IV and β-trombogolbulin levels, suggesting that some depressed patients may be at greater biological risk for comorbidity with ischemic heart disease (Whyte et al. 2001).s

It is also important to note that 5-HTTLPR allele frequencies vary between different ethnic groups (Gelernter et al. 1999) indicating a risk for population stratification in association studies. A meta-analysis of bipolar disorder and the 5-HTTLPR variant was unable to show any significant associative evidence (Craddock et al. 2001). Likewise, a meta-analysis between seasonal affective disorder and the gene variant could not show any significant association (Johansson et al. 2003). There is a general view that the 5-HTTLPR variant shows stronger associative evidence with personality traits related to affective disorders, than with narrow clinical diagnoses of affective disorders (Ueno 2003).

Serotonin receptors

Serotonin receptors represent a quite heterogeneous group of molecules with several subtypes, but a substantial understanding of their structure and functionality has emerged and facilitated their classification. There are seven serotonergic receptors types known today, 5-HT1 – 5-HT7, and at least 14 distinct receptor subtypes (Hoyer et al. 1994). All serotonin receptors belong to the large family of receptors interacting with G-proteins, except the 5-HT3, which is a ligand-gated ion-channel. The serotonin receptors belonging to the G-protein coupled receptor superfamily are characterized by the presence of seven transmembrane domains and the ability to modualte G-protein-dependent intracellular signaling. The receptors are further grouped into sub-families according to second-messenger system and amino acid sequence homology.

The 5-HT_{2A} receptors are located centrally in the cortex and basal ganglia. They have been implicated peripherally in tissues to mediate contractile responses of many

vascular, urinary, gastrointestinal and uterine smooth muscle preparations, platelet aggregation and increased capillary permeability. The gene coding for 5-HT_{2A} (previously termed 5-HT_2), *HTR2A* is located on human chromosome 13q14-q21. The 5-HT_{2A} receptor is linked to the phosphoinositol hydrolysis signal transduction system via G-proteins. Stimulation of central 5-HT_{2A} receptors may mediate the effects of hallucinogens such as lysergic acid diethylamide (LSD) in humans. Pharmacologically these receptors are important and are affected by a wide variety of pharmacologic agents.

5-HT_{2A} receptor binding decreases dramatically in a variety of brain regions, such as the anterior cingulated, optical cortex and hippocampus, through mid-life, but with a less decrease from mid-life to late-life. In normal healthy subjects the decrease from young adulthood, age 20, to mid-life, age 50, is approximately 70% (Sheline et al. 2002).

The *HTR2A* gene harbors quite a lot of genetic variation, but there are three SNPs, the -1438G/A, 102T/C and the 45His/Tyr that have been more frequently studied. The –1438G/A and the 102T/C variants have been suggested to be in complete LD with each other (Ranade et al. 2003). Both variants have been associated with affective disorders and drug response (Masellis et al. 2000). The 102T/C variant has been associated with schizophrenia in a meta-analysis of more than 3000 individuals (Williams et al. 1997). The same variant investigated for bipolar disorder did not show any association (Craddock et al. 2001). The amino acid substitution 45His/Tyr, reported to affect intracellular calcium response, has recently been associated with short-term memory (de Quervain et al. 2003).

It has been suggested that the *HTR2A* gene might be, at least partially, maternally imprinted (Kato et al. 1996a). Involvement of parent-of-origin effects has been investigated with results of both maternal and paternal effects (McMahon et al. 1995; Lin et al. 1997; Kornberg et al. 2000) and non-replicated results (Kato et al. 1996b; Murphy et al. 2001). It has also been suggested that 5-HT2A gene exhibits a polymorphic imprinting in the human adult brain (Bunzel et al. 1998; Pastinen et al. 2004), with some individuals showing mono-allelic expression while others bi-allelic expression.

MAO A and B

Monoamine oxidase A and B are two important enzymes in catalyzing the oxidative deamination of both exogenous and endogenous monoamines. Important endogenous amines include neurotransmitters like dopamine, 5-HT, noradrenaline and adrenaline. MAOA and MAOB are encoded by two separate but adjacently located genes, approximately 20kb of non-coding DNA apart in a tail-to-tail orientation on the short arm on chromosome X (Xp11.23-3). Both genes are encoded by 15 exons and have a similar exon-intron structure, but with a large difference in intron size. The *MAOA* gene spans approximately 65kb whereas *MAOB* is almost double in size, 115kb. Both enzymes are flavoproteins with flavine adenine nucleotide (FAD) as a co-enzyme covalently bound to the C-terminal region and both are located in the outer mitochondrial membrane. They have a molecular weight of approximately 65kDa and display a 70% amino acid sequence homology with each other (Shih et al. 1999). MAOA and MAOB differ in substrate specificity, inhibitor specificity and tissue distribution. MAOA has a higher specificity for serotonin and norepinephrine

while MAOB has a higher specificity for β-phenylethylamine (β-PEA) and benzylamine (Waldmeier 1987). Clorgyline selectively inhibits MAOA while Deprenyl selectively inhibits MAOB. The two enzymes are present in most tissues throughout the human body, with the highest expression levels in brain and liver. There is also tissue specific expression for one or the other of the two, e.g. placenta mainly expresses MAOA and platelets and lymphocytes only express MAOB (Shih et al. 1990). In the human brain, MAOA is predominantly present in chatecholaminergic neurons and MAOB is mainly present in serotonergic neurons, histaminergic neurons and astrocytes (Saura et al. 1996).

Platelet MAOB has the same amino acid sequence as brain MAOB (Chen et al. 1993), however there is no clear correlation between brain and platelet, or trombocytic MAOB (trbc-MAO) activity (Winblad et al. 1979; Adolfsson et al. 1980; Young et al. 1986). Trbc-MAO activity is characterized by a quite large inter-individual variability but the enzyme activity is stable over time, with a suggested increase in activity after age 40 (Fowler et al. 1997). Also, women have a 10-20% higher trbc-MAO activity (Murphy et al. 1976; Bridge et al. 1985). Several environmental factors are known to strongly affect trbc-MAO activity, one of the most common and important factors is smoking that is known to reduce the enzyme activity by approximately 15-30%. Notably, also brain MAO activity is affected by smoking (Berlin et al. 2001). Other important factors are certain medications that also alter the enzyme activity.

In both family and twin studies it has been shown that there is a high degree of heritability in trbc-MAO activity (Reveley et al. 1983; Oxenstierna et al. 1986). A twin study performed in the Swedish twin registry showed a heritability of 77% in both genders (Pedersen et al. 1993).

Very few genetic variants have been reported in the two MAO genes and except for a couple of repeat polymorphisms that are present in all populations, a majority of the SNPs found are only present in specific populations. As an example, only one SNP in the MAOB gene, a variant in intron 13, has been found in Africans, Caucasians as well as Asians, but with very different population frequencies is.

Human behaviors influenced by the serotonergic system

Serotonin has been observed to affect a wide range of physiological systems, such as arteriolar vasomotor responses, respiration and thermoregulation but also a number of behavioral traits such as sleep-wake cycles, appetite, aggression, sexual behavior, pain sensitivity and learning. In addition, a broad range of impulse-control disorders and personality traits has been associated with 5-HT function, including substance abuse, gambling, obsessive control and attention-deficit disorder (Lucki 1998). Dysregulation of 5-HT transmission has been argued to be involved in a range of psychiatric disorders such as schizophrenia, eating disorders, anxiety disorders and depression. Abnormalities in serotonergic neurotransmission have been reported to occur at several critical points in 5-HT synthesis and transmission.

Studies on human subjects have investigated the association of CFS 5-HIAA levels with depression and other behavioral characteristics. It has been shown that depressed patients that attempted suicide displayed reduced levels of CSF 5-HIAA (Asberg et al. 1976; Brown et al. 1986; Tuinier et al. 1995; Asberg 1997).

The importance of MAO in the catabolism of neurotransmitters suggests a role in human behavioral traits and psychiatric disorders. *MAOA* has, among others, been associated with substance abuse, Parkinson's disease and mood disorders (Lim et al. 1994; Lin et al. 2000). Likewise, has *MAOB* been associated with both depression and Parkinson's disease (Kurth et al. 1993; Ho et al. 1995; Costa et al. 1997). There have been both positive and negative finding, and even some contradictory associations.

There are a number of compounds that have a profound effect on the serotonergic system and directed development of drugs affecting this system has been observed to be efficient in the treatment of mood disorders. This suggests that an unbalanced serotonergic control might be of importance for the development of mood disorders and related phenotypes. There are several different classes of anti-depressive medications; like tricyclic antidepressants (TCAs), serotonin selective reuptake inhibitors (SSRIs), monoamine oxidase inhibitors (MAOIs) and a range of selective receptor blockers.

SNPs AND THE DEVELOPMENT OF MOLECULAR TECHNIQUES

The human genome project (HUGO) was launched in 1990 as a 15-year plan, managed with two major revisions in 1993 and 1998, to deliver a draft sequence of the human genome in February 2001, years ahead of the original plan (Collins et al. 1998; Lander et al. 2001). As part of the publication of the draft human genome sequence, 1,42 million SNPs were reported and already by June the same year that number had grown to 1,69 million SNPs available in dbSNP at NCBI (http://www.ncbi.nlm.nih.gov/SNP) and ENSEMBL (http://www.ensembl.org). Today more than 4 million SNPs have been mapped and deposited in public and private databases. The SNPs designated "rs" (for reference SNP) in dbSNP represent a non-redundant set, since many SNPs have been found by more than one strategy. On average, two unrelated individuals will differ at about 1 base in every 1000 of the 3 billion or so bases in their genome, this also implies that any individual will have about 3 million SNPs (Kruglyak 1997; Taillon-Miller et al. 1998; Marth et al. 1999). The SNP Consortium (TSC) formed by the Wellcome Trust and a number of companies funded academic efforts to sequence genomic DNA to find SNPs (Altshuler et al. 2000b; Mullikin et al. 2000). The combined effect of all searches for genetic variation in the human genome offers a major resource and the possibility of a more detailed genetic analysis of complex human disorders and traits. There are also issues regarding the information quality of SNPs in the databases. For example, almost most nothing is known about the frequency in different populations.

As the number of identified and mapped SNPs increase, there is a growing need for robust and efficient SNP genotyping technologies to allow screening of large patient materials and populations. In medical genetics the need to investigate the human genome and to decipher the molecular background of complex diseases has accelerated the development of enhanced, high throughput methodologies. New molecular techniques to score these variants have virtually flooded the field of molecular biology. This has made large-scale and high throughput screenings and association studies possible even for moderately sized laboratories. There are now more variants than subjects to study in some projects.

Today there are many different techniques established for SNP scoring, be it by a hybridization method like the Illumina BeadArray system (Oliphant et al. 2002), mass spectrometry like MALDI-TOF (Pieles et al. 1993), TaqMan Liquid Phase hybridization (Lee et al. 1993), Invader Assay (Lyamichev et al. 2003), Affymetrix (Fan et al. 2000) or any other, the choice is usually just a matter of means to achieve the same goal.

One of the first new SNP scoring methods that arrived was Pyrosequencing, developed at the Royal Institute for Technology in Stockholm (Ronaghi et al. 1996). Pyrosequencing differs from many of the other techniques in that it dose not only score variants, but it actually performs a real-time sequencing reaction. The method utilizes four enzymes, DNA polymerase, ATP sulfurylase, firefly luciferase and the nucleotide degrading enzyme apyrase to perform the sequencing reaction. It represents a real-time sequencing method in that it registers the sequence as it reads it. This method is well adapted for SNP analysis, without the need for gel electrophoresis or use of restriction enzymes for specific variants.

AIMS OF THE STUDY

The general aim of the work presented in this thesis has been to investigate the genetic influence on depressive symptoms in the elderly using twin methods and a sequencing based SNP strategy for exploreing complex genetic traits, and specifically to:

Ascertain how much of the variation in depressive symptoms, assessed by the CES-D scale can be explained by genetic factors, the heritability, by means of a twin design.

Evaluate a novel real-time sequencing based technique, Pyosequencing™ in comparison to the 5'-nuclease (TaqMan®) assay, for large-scale SNP genotyping.

Investigate whether two likely candidate genes in the serotonergic system, promotor located SNPs in the postsynaptic serotonin 2A receptor gene (*HTR2A*) and the serotonin transporter gene (*SLC6A4*), influence depressive symptoms.

Investigate the MAO locus on the X chromosome by novel sequencing of promotor regions in search for genetic variants and by use of a SNP based LD map to determine the genetic structure of the locus. To evaluate haplotype effects on depressive symptoms as well as trombocytic MAO activity.

MATERIALS AND METHODS

Participants

All participants in **paper I to IV** were part of the Swedish twin registry (STR), except the subjects in **paper II** that were Swedish individuals collected at Huddinge Hospital (HS) and in **paper IV** healthy blood donors from the Uppsala region in addition to the twin participants. The specific samples in each paper are described in the following:

In **paper I** a sample of 1918 individuals in 959 twin pairs aged 50 years and older and in **paper III** a total of 1592 individuals were taken from three longitudinal twin studies of aging. All three studies have previously been described in detail and are sub-samples of the population based STR (Lichtenstein et al. 2002): The OCTO-twin study with up to four longitudinal occasions(McClearn et al. 1997), the Swedish Adoption / Twin Study of Aging (SATSA) with up to six longitudinal occasions (Pedersen et al. 1991) and the Gender study with two interview occasions (Gold et al. 2002). All participants are Caucasians and born in Sweden. For all three sub-samples, individuals participated in at least one in-person session in which a blood sample was drawn and depression questionnaires were administered or other information about current or previous depressive episodes was gathered. The mean age of the sample was 73.4 years. In **paper I** there were 123 female MZ, 90 male MZ, 109 same sex male DZ, 207 same sex female DZ and 430 unlike sex DZ twin pairs.

In **paper IV** the participants were a subset of those included in Paper I and III, participating in (SATSA) with up to six measurement occasions (Pedersen et al. 1991). All individuals who participated in an in-person testing session where questionnaires were administered and a blood sample was drawn were selected. The mean age of the sample was 61,3 years at the time of testing. Twenty two percent of the participants were current smokers; 35% of the males and 15% of the females.

Zygosity determination for like-sexed pairs was initially based on responses to the question, "During childhood, were you and your twin partner as like as 'two peas in a pod' or not more alike than siblings in general?" If both individuals of a pair responded "alike as two peas in a pod" they were classified as monozygotic (MZ), if both responded, "not alike" they were classified as dizygotic (DZ). For all SATSA pairs and those OCTO-Twin pairs not classifiable by this technique, which has proven to be more than 95% accurate in a number of studies (Lichtenstein et al. 2002), zygosity was determined by serological analyses and comparisons of up to 10 DNA markers.

Blood samples were available from 1511 participants in **paper III**. For MZ twins who had no DNA but whose partner had DNA, we extrapolated the genotypes from the co-twin with DNA available to the co-twin without DNA, since MZ-twins should be genetically identical. Out of the 1511 participants who were genotyped for the *HTR2A* promotor polymorphism and the 1498 participants who were genotyped for the *SLC6A4* exon 1B polymorphism, 81 and 86 MZ co-twins were extrapolated, respectively. This procedure yielded a total sample of 1592 participants with *HTR2A* genotypes and 1584 participants with *SLC6A4* genotypes in **paper III**.

In **Paper IV**, DNA samples were available from 574 twins. Thrombocytic-MAO activity measures were available from 565 twins. Only single-responding individuals and one individual from each complete pair were used for the analyses of trbc-MAO activity in this study (N=340). For preliminary screening of the promotor and first exon and intron regions for novel variants in **paper IV**, 94 Swedish males were randomly selected from a larger sample set collected among healthy blood donors in the Uppsala region to study MAO regulation. All were between the ages of 20 to 40 years and non-smokers.

In the evaluation of the Pyrosequencing™ method for large-scale SNP genotyping in **paper II** a total of 1022 Swedish individuals were genotyped for six SNPs: A004B36, stSG15820, stSG4025, stSG30264, stSG15903 and WI-16905. For the comparison between the Pyrosequencing™ and the 5' nuclease (TaqMan®) assay, the same 1022 individuals were also genotyped for the SNP stSG30264 by the 5'-nuclease (TaqMan™) assay. The samples used for the pooling experiment were the same as in **paper III**.

Genotyping by Pyrosequencing

The Pyrosequencing™ technology is based on detection of real-time pyrophosphate (PPi) which is released as a result of nucleotide incorporation in a sequencing-by-synthesis reaction (Ronaghi et al. 1996). The PCR product containing the SNP to be analyzed is immobilized onto the solid support at the biotinylated end. The double stranded PCR product is then separated by melting in a weak alkali solution and commonly only the bound strand is used for sequencing (Fig. 9).

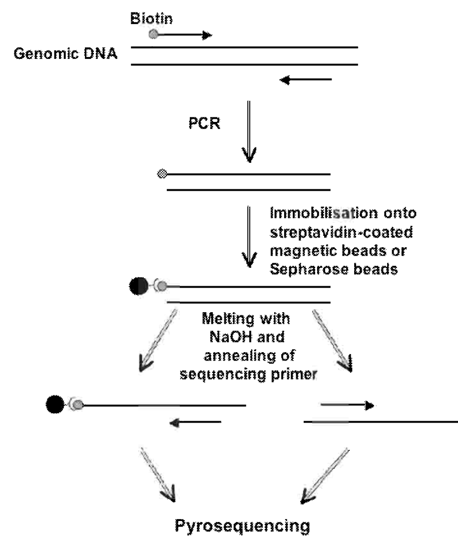

Figure 9. Pyrosequencing preparation.

After sequence-primer hybridization, the template is incubated with a four-enzyme mixture of DNA polymerase, ATP sulfurylase, firefly luciferase and apyrase, after which deoxynucleotides are added iteratively. The following enzymatic reactions proceed as described in Fig. 10. During DNA synthesis, pyrophosphate is released in proportion to the number of nucleotides being incorporated. ATP sulfurylase

converts the generated PPi to ATP, which in turn is converted to light in the luciferase-catalyzed reaction. Real-time signals, representing the DNA sequence, are presented in a pyrogram, with peak heights corresponding to the number of identical residues incorporated, as shown in Fig. 10.

In **paper II, III and IV** all pyrosequencing reactions was performed on a PSQ96™ Instrument from Pyrosequencing AB (Uppsala, Sweden) as described (Ronaghi et al. 1996). In **paper II and III** the biotinylated PCR product was immobilized onto streptavidine-coated super paramagnetic beads (Dynabeads® M280-Streptavidine, Dynal, Oslo, Norway) and in **paper IV** the biotinylated PCR product was immobilized onto streptavidine-coated sepharose beads and DNA.

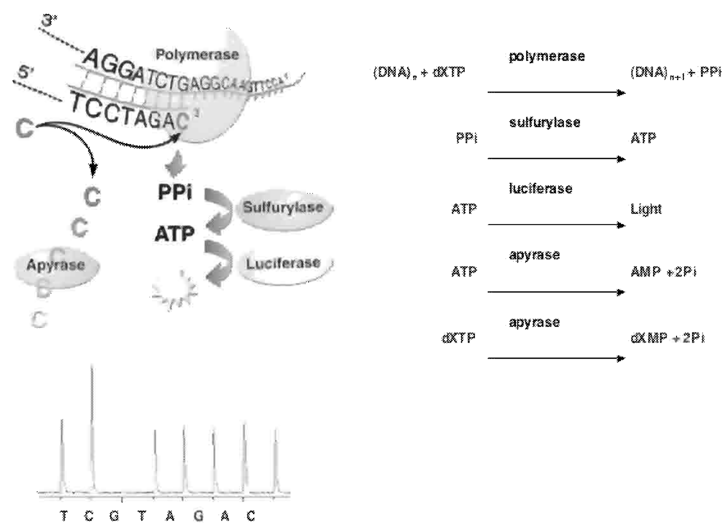

Figure 10. Pyrosequencing reaction.

In short, the sequencing primer was hybridized to a minimum of 1 pmole of immobilized single-stranded DNA template. A four enzyme mixture of DNA polymerase, ATP sulfurylase, firefly luciferase and the nucleotide-degrading enzyme apyrase, the substrate luciferin and the four separate deoxynucleotides were loaded into a reagent cartridge (PSQ96™ SNP Reagent Kit, Pyrosequencing AB). The cartridge and the 96 well plate containing the template was inserted into the PSQ96™ instrument. Following dispensation of the enzyme reagents and the substrate into the microtiter wells to a reaction volume of 50 µl, each different nucleotide was dispensed sequentially by "ink-jet" technology, every 65 seconds. For SNP detection the dispensation order was preprogrammed and selected automatically for each SNP to be analyzed, using the SNP Entry module of the SNP Analysis Software (Pyrosequencing AB). The repeated cycles of nucleotide delivery resulted in pyrophosphate (PPi) release for every nucleotide incorporated. The pyrophosphate was converted to ATP by ATP sulfurylase and light generated from the subsequent reaction, catalyzed by luciferase, was recorded by a CCD camera. Unincorporated nucleotides were degraded by apyrase between cycles. Pre-synthesized DNA templates, corresponding to homozygotes and heterozygotes for the analyzed SNPs, as well as template and primer oligonucleotides was obtained from Pyrosequencing AB and used as positive controls.

The output from the CCD unit was continuously monitored as a pyrogram and the pyrosequencing software analyzed the results from the completed sequencing reactions.

The SNPs analyzed in **paper II**, were A004B36 [T/C], stSG15820 [T/C], stSG4025 [C/T], stSG30264 [A/G], stSG15903 [A/C] and WI-16905 [C/T], all of which map uniquely to human chromosome 10. Sequences of all six SNP PCR primer pairs were obtained from dbSNP at the National Center for Biotechnology Information (NCBI) and either the forward or the reverse primer in each primer pair was biotinylated. Sequencing primers with a typical primer length of 18-24 bases were placed within 5 bases of the SNP.

In **paper III** a total of 1511 subjects were genotyped for the *HTR2A* promotor polymorphism and 1498 subjects were genotyped for the *SLC6A4* exon1B polymorphism. Sequences of all PCR primers, *SLC6A4* exon1B (-925 T/G) [accession: U79746] and *HTR2A* promotor (-1438 G/A) [accession: S78723], were obtained from dbSNP at the National Center for Biotechnology Information (NCBI) and the forward *HTR2A* primer and the reverse *SLC6A4* primer in each primer pair was biotinylated. Sequencing primers with a length of 15 and 18 bases were placed within one base of the SNP.

In **paper IV**, either the forward or the reverse primer in each primer pair was biotinylated. Sequencing primers with a length of 14 to 18 bases were placed within one base of the SNPs.

5'-Nuclease (Taq Man®) assay

For allelic discrimination, the two allele-specific probes were each labeled in the 5' end with different fluorescent reporter dyes (FAM and VIC), in addition to a fluorescent quencher dye (TAMRA) in the 3' end. The probes were designed using the Primer Express™ program and synthesized by PE Biosystems.

The 5' nuclease (TaqMan®) assay was undertaken as previously reported (Lee et al. 1993). In short, a labeled probe is hybridized to the template between the primers during a PCR reaction. The probe carries a fluorescent reporter molecule at the 5' end and a quencher molecule (usually TAMRA) in the 3' end. Upon cleavage by Taq polymerase, the reporter dye will fluoresce as it is no longer quenched and the intensity of the emitted light is measured by a fluorimeter. In genotyping by allelic discrimination, two probes complementary to the two SNP alleles are used. They are labeled with different reporter molecules (in our experiment we used FAM and VIC). Hybridization conditions are designed to discriminate between the matched and the mismatched probe. If only one allele is present (i.e. a homozygote) only a FAM or a VIC-specific signal will be detected. Detection of both signals indicates heterozygosity.

Amplification and sequence detection were both performed in the Perkin Elmer ABI PRISM™7700 Sequence Detection System. The alleles were analyzed and determined using the graphical view from the software Sequencing Detection System (PE Biosystems). Pre-synthesized DNA template containing the stSG30264 SNP was used as positive controls.

Sequencing

Amplification and nested sequencing primers were designed with the CPrimers programme from Genbank entry GI:8671203 containing the promoter, coding exon 1

and flanking intronic sequence of MAOA (~4.964kb, nucleotides 46490-51454) and Genbank entry GI:2440066 spanning the same characterized sequences of MAOB (~4kb nucleotides 35033-39021). Twelve primer pairs necessary for amplification and nested sequencing of overlapping amplicons of the intragenic region and eleven primer pairs for the promoter were designed.

Direct sequencing reactions were performed using DYEnamic ET Dye Terminator Cycle Sequencing Kit (Amersham Biosciences) and separated using a Megabace 1000. Reads were base called with Phred (Ewing et al. 1998), assembled using Phrap and viewed using Consed Version 13 (Gordon et al. 1998). All SNPs were documented and cross validated with dbSNP at NCBI. Failed reads were re-sequenced. We could validate our SNPs using similar grounds as Nickerson, Taylor (Nickerson et al. 1998) and Clark, Weiss et al.(Clark et al. 1998).

Depressive symptoms and depressive state

Depressive symptoms were measured with the Center for Epidemiologic Studies Depression Scale (CES-D), a 20-item self-report scale developed for use in the community and well established for use with older adults (Radloff 1977) (Appendix 1.). The scale has been shown to have minimal overlap with physical illness (Berkman et al. 1986) and very good criterion validity for major depression (Beekman et al. 1995). Respondents scoring 16 or higher on the CES-D scale are considered to have a clinically relevant depressed state. The Swedish translation of the CES-D scale has good psychometric properties in elderly community samples (Gatz et al. 1993).

In **paper III and IV** an average CES-D score was used for each participant who had answered the questionnaire at multiple occasions, or a single CES-D score for those assessed only once. We used a cut-off value of 16 on the average CES-D score from each participant, with a value of 16 or above leading to a classification as "depressed". CES-D scores are quite stable over time (Fiske 2002). In **paper I** a single measurement occasion was used.

In **paper I and II** medication status and use of anti-depressants were also assessed at every interview and self-report occasion in addition to the CES-D score. Medications were reported by their "trade name" and then coded into the ATC-system (Anatomical Therapeutic Chemical classification system). All drugs with the ATC code N06A were classified as anti-depressive. Participants were classified as having a depressed mood if they had ever reported any use of anti-depressive medications (N=118). In **paper III** information through medical records was also used for the OCTO-twin sub-sample, including whether the participant had ever had a diagnosis of minor or major depression. If a participant had a clinical diagnosis of minor (N=63) or major depression (N=37), he or she was also classified as "depressed". There was some inconsistency across sub-samples in how depressed mood was determined as only one of the three sub-samples had information from medical records about diagnosed depression. We found that over two-thirds of those with diagnoses of major or minor depression also reported anti-depressant medication; thus, the different methods were not predominately identifying new individuals, but rather, supporting the identification of the same individuals.

Correlations and Concordances

The log-transformed continuous CES-D measure was corrected for age using a regression procedure (McGue et al. 1984) and intraclass correlations for all five zygosity groups (monozygotic males MZM, monozygotic females MZF, dizygotic males DZM, dizygotic females DZF and dizygotic opposite-sex pairs DZO) were calculated.

Probandwise concordances and tetrachoric correlations were calculated separately for the five different zygosity groups for the dichotomous depressed state variable. Probandwise concordances were defined as the number of affected index siblings of affected individuals divided by the total number of affected individuals. Tetrachoric correlations were calculated from 2x2 contingency tables using SAS software (version 8.02, SAS Institute).

Model fitting

For the categorical depressed state variable, genetic models were fit to the 2x2 contingency tables. The liability to develop a depressed state is assumed to have an underlying continuous normal distribution with a threshold above which the subject develops the condition (Falconer 1965) (Fig. 3.). This liability distribution reflects contributions from both genetic and environmental effects. For the categorical data, thresholds for the underlying liability were allowed to differ between men and women, since there are large differences between the sexes in the population based prevalence of the disorder.

For age-adjusted CES-D measures of depressive symptoms MZ and DZ variance - covariance matrices (Eq. 9) were generated using SAS software (version 8.02, SAS Institute). Genetic models were then fitted to these matrices using the structural equation modeling software Mx (Neale 1994).

$$\begin{bmatrix} Var_{T1} & Co\text{var} \\ Co\text{var} & Var_{T2} \end{bmatrix} \Rightarrow \begin{bmatrix} \sigma_A^2 + \sigma_C^2 + \sigma_E^2 & r_g\sigma_A^2 + r_c\sigma_C^2 \\ r_g\sigma_A^2 + r_c\sigma_C^2 & \sigma_A^2 + \sigma_C^2 + \sigma_E^2 \end{bmatrix}$$

Equation 9. Variance-Covariance matrix.

The full model included additive genetic effects (A), shared environmental effects (C), and non-shared environmental effects (E), which were estimated separately for men and women (Fig. 6). Nested models were then fitted that included only additive genetic and non-shared environmental contributions (AE model) or shared and non-shared environmental contributions (CE model). The fit of these models was assessed by a goodness-of-fit chi-square test. The genetic and environmental parameter estimates and 95% CI were calculated for all models. After genetic and environmental effects were estimated independently for men and women, parameters were constrained to be equal to test for gender differences in the relative importance of genetic and environmental effects for the phenotypes. Subsequently, we fit models for sex-specific genetic (r_g) and familial-environmental (r_c) effects. The genetic correlation (r_g) for opposite-sexed DZ twin pairs was estimated instead of being fixed to 0.5, and in a separate model the shared environmental correlation (r_c) was estimated rather than being fixed at 1 (Fig. 6).

Monoamine Oxidase Activity

Thrombocytic MAO activity was measured from blood in SATSA. Participants were asked to fast beginning at midnight before the test date (the majority of tests were taken in the morning). During the first ten minutes of the test situation, 5 ml samples of blood were drawn into vacutainer tubes with sodium citrate. The red cells were sedimented by centrifugation at 130g for ten minutes. Approximately 1 ml of platelet-rich plasma was collected by suction and the platelet concentration counted on a Coulter Counter® (Dunstable, England) and then frozen at –20 °C until analysis. After thawing, the samples were sonicated four times for ten seconds each and estimation of enzyme activity was performed by incubation at 37 C for four minutes with ^{14}C-β-phenyletylamine as substrate in a final concentration of 50μM (New England Nuclear, Boston, MA) as described previously by (Hallman et al 1987). Assessments of activity were carried out randomly and in duplicate, and the mean values were used.

The samples were evaluated in 21 batches and a standard platelet MAO preparation was estimated twice in each batch. The mean activity of this standard was (±SD) 16,14 ±1,65 nmoles of β-phenyletylamine oxidized /minute/10^{10} platelets. The coefficient of variation for the estimations was 10,2%. The error of estimation for the duplicate standard within each batch was calculated as the square root of the squared sum of the differences divided by 2 times the sample size $(((\sum(diff)^2/(2N))^{1/2})$, were 8,0% of the mean β-phenyletylamine. Thus, the variation in activity of the standard platelet MAO preparation was low within each session. Batch effects were adjusted to the overall sample mean.

Linkage Disequilibrium

Female bi-allelic haplotypes were estimated using an EM algorithm (Sham 1998) and the pair-wise LD measures D' (Lewontin 1964) and Δ^2 (Hill et al. 1968). In the female population we used "PHARE" (by David G Cox, available at http://bioinformatics.org/macroshack/programs/PHARE) to create input files for "PHASE" (Stephens et al. 2001b; Stephens et al. 2003) to construct haplotypes. In males we could extrapolate the haplotypes directly since the MAO locus is located on the X chromosome and males are thereby hemizygous carriers.

Association Analysis

The association between the genotypic information and the dichotomous depressed mood measure in **paper III** was analyzed in a generalized linear model (GLM). GLMs allow for dependence structure that was applicable to our data as both members of twin pairs were included in the analyses. We used a GEE approach with an exchangeable correlation structure where it was assumed that the correlation of the residuals was the same for both MZ and DZ twins (from stratified models the estimated correlations were 0.21 and 0.36 for DZ and MZ twins respectively). Odds ratios (OR) with 95% confidence intervals (CI) were estimated for the effects of high-risk genotypes. All statistical analyses were performed in SAS 8.01 using GENMOD procedure (SAS Institute Inc. Cary NC).

In **paper IV** a linear regression was used to estimate the association between trbc-MAO activity and genotypic information using a generalized estimating equation (GEE) approach and alternating logistic regression (ALR) (Carey 1993) to estimate the

association between depressed state and the genotypic information. All estimates were adjusted for current smoking status. Associations were modeled between each of the outcomes and haplotype constructs. We estimated both dominance and co-dominance models. Explanatory variables in the dominance models were binary (affected/unaffected) whereas in the co-dominance models they were coded as the number of affected alleles (i.e., 0, 1, or 2 for females and 0 or 1 for males). The parameter estimates for the co-dominance models represent the change in the outcome (trbc-MAO activity or odds of being in a depressed state) per affected allele. We also estimated, among females, the effect of homozygote compared to heterozygote carriers. If the co-dominance model is a good fit to the data then these estimates should be similar to the `per allele' estimates from the co-dominance model. All statistical analyses were performed in SAS 8.01 using GENMOD procedure (SAS Institute Inc. Cary NC).

RESULTS

HERITABILITY AND GENDER DIFFERENCES IN DEPRESSIVE SYMPTOMS (PAPER I)

Twin materials are ideal for estimating the importance of genetic and environmental effects for variation in traits and diseases. Twins are matched for age and in most cases even for gender. Identical (monozygotic or MZ) twins share the same genes whereas fraternal (dizygotic or DZ) twins share on average half of their segregating genes.

The genetic and environmental variance components for depressive symptoms were investigated by means of a twin design in **paper I**. A five-group, sex-limitation model with 959 complete twin pairs was used to test not only for differences in the relative importance of genetic and environmental effect by sex, but also for the presence of genetic and environmental effects that are specific to one sex beyond those effects that are common to both. As shown in table 2, the sex specific heritability estimates (h^2) for depressive symptoms were 14% for males and 29% for females (Model I) indicating an approximately twice as large genetic effect in females. When constrained to be equal in males and females, the heritability estimate was 21% in the full model (Model IV). There were no statistically significant differences between the models, but twin pair correlations and concordances support the interpretation of a difference.

	Parameter estimate					
Model	**Males**			**Females**		
CES-D	**A**	**C**	**E**	**A**	**C**	**E**
I: ACE	**14**	**2**	**84**	**29**	**1**	**70**
II:AE	**16**	**0**	**84**	**30**	**0**	**70**
IV: ACE	**21**	**1**	**77**			
V:AE	**23**	**0**	**77**			
Depressive state						
I: ACE	**7**	**4**	**89**	**49**	**0**	**51**
II: AE	**9**	**0**	**91**	**49**	**0**	**51**
IV : ACE	**33**	**0**	**67**			
V : AE	**33**	**0**	**67**			

Table 1. Partitioned variance

The prevalence of the dichotomous depressed state variable was 16% for men and 24% for women. The heritability estimates were 7% for males and 49% for females in the full model (Model I) and a constrained heritability of 33% for the best fitting (AE) model (Model V) as shown in table 2. There were no statistically significant differences in heritability estimates between men and women but the estimates are very similar to heritability estimates for clinical depression (Kendler et al. 1999). These results show that depressive symptoms and depressed state in the elderly

are moderately heritable, with a clear indication of a higher heritability for women than men.

SNP DETECTION USING PYROSEQUENCING (PAPER II)

There are a multitude of molecular techniques available today with different principles and applications that can be used for special genotyping strategies. All techniques have their own specialty and optimal applications. There are several important variables that need to be considered in choosing a particular method, such as familiarity, throughput, price and perhaps most important for a smaller academic laboratory with many different demands, quality and versatility.

One of many new molecular techniques for sequencing and genotyping is the Pyrosequencing™ method that provides several desirable features such as high quality sequencing, frequency estimations in pooled samples, high throughput, simple assay design and robustness.

In **paper II** in this thesis the Pyrosequencing™ method was evaluated as a tool for large-scale genotyping in comparison to the 5'-nuclease (TaqMan®) assay from PE Biosysytems, regarded as a "gold standard" at the time, by simultaneous analysis of one SNP using both methods. The pyrosequencing method was also tested for its signal linearity in a smaller post-PCR pooling design.

Six SNPs, A004B36, stSG15820, stSG4025, stSG30264, stSG15903 and WI-16905, at a locus on chromosome 10 were genotyped in a large Swedish sample by pyrosequencing. The SNP stSG30364 was simultaneously analyzed using the 5´-nuclease (TaqMan®) assay for confirmation of the pyrosequencing data. The stSG30264 genotype result for each of the 1022 individual samples was identical using both methods and genotype frequencies were: A/A 0.33; G/G 0.19 and A/G 0.48. Allele frequencies determined by pyrosequencing for the analyzed SNPs were as follows: A004B36 T/C 0.98/0.02; stSG15820 T/C 0.94/0.06; stSG4025 C/T 0.78/0.22; stSG30264 A/G 0.56/0.44; stSG15903 A/C 0.58/0.42 and for WI-16905 C/T 0.89/0.11.

In an initial experiment to test a pooling strategy using Pyrosequencing™, 282 PCR samples were pooled into one single microtiter well. Here, the peak height represented the frequency of a specific base in the PCR sample population. In this case the allele frequency was determined with a total error rate of 1.6 alleles (as determined from peak heights) out of 564, or 0.3%, as compared to individual analysis. In an extended study several pools were generated with up to 1126 samples in one microtiter well. In the largest pool of 1126 individuals, the deviation from the actual genotype was only 1.1% or 24.7 out of 2252 alleles.

We conclude that Pyrosequencing™ is highly efficient and accurate in the analysis of SNPs and represents a more than satisfactory solution to high throughput genotyping of large sample populations.

ASSOCIATION STUDIES OF DEPRESSIVE SYMPTOMS (PAPER III AND IV)

The Serotonin receptor 2A and the Serotonin Transporter

The serotonergic system has been associated with occurrence of depression and there are several serotonergic functions that could contribute to the symptoms of depression. In **paper III** two biologically interesting candidate genes from the serotonergic system were selected to evaluate their role in the etiology of depressive symptoms. An association between depressive symptoms and polymorphic variants in the promotor regions of the *HTR2A* gene and the serotonin transporter (*SLC6A4*) gene was investigated in a sample of 1592 elderly twins. The main findings indicate that the A allele of the –1438 G/A polymorphism in the *HTR2A* gene contributes to the etiology of depressed mood in elderly Swedish males but not in females. There was a significant effect for the A/A genotype with an OR of 2.4 for males and a non-significant OR of 1.4 for the A/G genotype. This difference indicates that there might be an additive effect of the A allele for the risk of depressed mood in older males. These findings, with an allelic variant associated with a higher risk for depressed mood in males and not in females might be indicative of a gender difference in the genetic architecture of the disorder. The *SLC6A4* gene was not associated with depressed mood in this sample.

Genetic Structure of the MAO locus

The MAO locus on the X chromosome (Xp11.23-3) harbors both *MAOA* and *MAOB*. Previous investigations have indicated that there is very little genetic variation within the two MAO genes. In **paper IV** novel polymorphisms were sought by sequencing 9kb of the *MAOA* and *MAOB* genes. Approximately 4.5kb of both the *MAOA* and *MAOB* gene promotors, including the first exon were sequenced from a total of 148 X-chromosomes. Power to reveal SNPs with frequencies greater than 1% and 3% for such a sample size was 77% and 100% respectively. However, no variants were found in the *MAOB* gene, in contrast to the *MAOA* gene where one previously reported variation was confirmed (rs3788863) lying within the first intron as well as two additional variants further down stream with a minor allele frequency greater than 1%. Both the recorded and most distal variants showed complete LD with each other, therefore only one was chosen for further analysis.

In total nine SNPs covering the MAO locus were used to assemble haplotype profiles of the locus. To assemble the haplotype structure of the genomic region we first used males to determine the exact haplotypes present in the sample. In the female population we estimated the pair-wise LD between all pairs of di-allelic loci using both D' (Lewontin 1964) and Δ^2 (Hill and Robertson 1968). The estimated LD map from the female population displayed the same haplotypes, with almost exactly the same frequencies as were found in the male population. The haplotype information revealed a clear structure of the MAO locus with strong LD across the *MAOA* gene that defines a distinct LD block. The *MAOB* gene also displays a block-like structure, although the pattern of LD is not as robust as *MAOA* (Fig. 11). This is perhaps due to the rarity of SNPs in the MAOB locus.

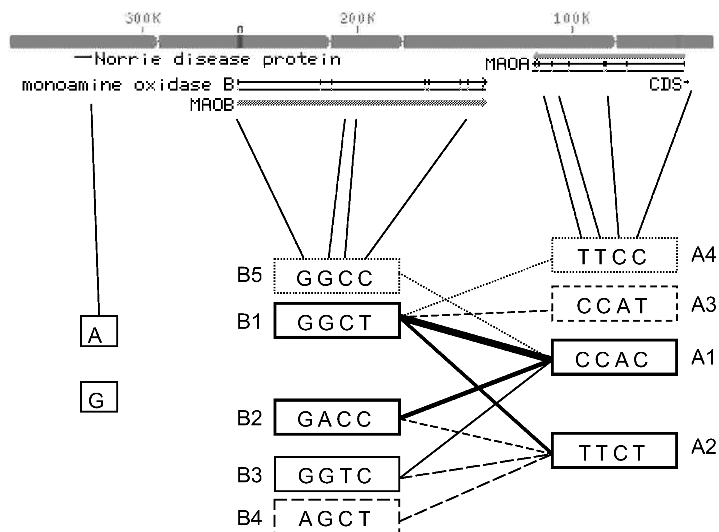

Figure 11. MAO locus

Thrombocytic MAO activity

MAOB is the only form that is expressed in blood cells; its activity can therefore quite easily be measured (trbc-MAO activity) by serological assays. The mechanism behind the genetic control of trbc-MAOB activity is of great interest as there are several reports of associations between trbc-MAOB activity and a number of behavioral traits. There is also a high heritability for trbc-MAOB activity however, little is known about the extent to which genetic variants within the MAO locus contribute to variability in trbc-MAOB activity.

It is well known that females have a higher mean trbc-MAO activity than males. In **paper IV** a clearly significant difference between males and females in mean trbc-MAO activity ($t=4.69$; $p \leq 0{,}0001$) was found. It is also well established that smoking strongly reduces trbc-MAO, as well as brain MAO activity by approximately 15-30%. In this sample, current smokers showed a 23% lower trbc-MAO activity ($t=5{,}86$; $p=<0{,}0001$) than non-smokers. Depressive state was significantly associated with higher trbc-MAO activity ($t=1.96$; $p=0{,}05$). When analyzed by gender, only females with a depressive state had a significantly higher trbc-MAO activity ($t=2{,}02$; $p=0{,}04$).

No single genetic variant was associated with trbc-MAO activity in the total sample. However, in females the C/C and C/T genotypes of rs979605 SNP in the *MAOA* gene were associated with a significant decrease in trbc-MAO activity, (-2,9; CI 95%: -5,2 - -0,6) and (-2,4; CI 95%: -4,7 - -0,1) respectively.

In the association analyses of haplotype profiles from the MAO locus and trbc-MAO activity, perhaps unexpectedly, two MAOA haplotypes, A1 and A3 were found to be significantly associated with a decreased trbc-MAO activity. The A1 and A3 haplotypes share identical alleles at the first three haplotype positions (CCA-) (Fig. 11).

This finding could indicate that regulatory regions affecting *MAOB* expression are located within the *MAOA* haplotypes.

Depressive state

Associations between *MAOA* and *MAOB* locus haplotypes and depressive state were also investigated in **paper IV**. There was no significant association between depressive state and any specific haplotype. But interestingly, in females all *MAOB* haplotypes displayed increasing odds ratios with depressive state in homozygote carriers, i.e. the OR for the homozygotes was greater than that for heterozygotes, indicating an additive genetic effect. Similarly for *MAOA*, female homozygotes displayed a greater odds ratio than heterozygotes. If there is unequal X-chromosome inactivation for this locus in females, the difference in OR that we found may reflect gene-dosage effects and provides some insight into the greater prevalence of depressive state in females than males.

DISCUSSION

Genetic risk factors for complex human traits have proven to be elusive and difficult to identify. There is certainly more than just one gene leading to the occurrence of a complex trait such as depressive symptoms or depressed mood and circumstances like incomplete penetrance and environmental interactions further complicate the possibilities to uncover the genetic risk factors for the trait. In psychiatric and behavioral traits there are few really well accepted genes known to influence a trait, at least well accepted out side the field of psychiatric genetics. Depressive symptoms are no exception, although fewer genetic studies have been performed on this phenotype compared to studies on major depression. But the fact that there are some successful examples where genes have been proven to be "true" genetic risk factors (which generally means that the findings have been replicated in independent studies) for complex human traits, should encourage scientists in the field to pursue the hunt for causative genes/genetic variation. In fact, our biological understanding tells us that there are many possible candidate genes and even excellent biological candidate genes that needs to be investigated in complex phenotypes. However, many candidate genes fail to show replicated associations. But this is perhaps what we shall expect from psychiatric genetics at least as long as limited case-control settings with narrow diagnostic criteria represent the study norm. If the effect sizes of the genetic risk factors are small as would be expected when there are several, perhaps even hundreds of genes involved in a complex trait, and the effect size on average is far smaller than for example the ε4 allele of *APOE* in Alzheimer's disease, then the genes will be very difficult to find but they will likely show up as positive findings in special sample settings due to circumstances such as population structure. A possible way to circumvent this might be to use large population based samples for more common phenotypes or to use broader diagnostic criteria.

Numerous studies demonstrate that genetic factors influence clinical depression in both mid-life and older adult populations (Blazer et al. 1987a; Bierut et al. 1999; Kendler et al. 1999; Blazer 2003). Depressive symptoms, as distinguished from the clinical depressive disorder, also appear to have significant genetic influences. Within this thesis a study was performed to explore the importance of genetic effects on current and past depressive symptoms in a large sub-sample of the Swedish Twin Registry (STR). We emphasize that the purpose was not to replicate the multitude of studies indicating a substantial heritability for major depression or bipolar disorders (Lykouras et al. 1987; Kendler et al. 1995b; Karkowski et al. 1997; Gershon 2000). Therefore, the findings are particularly unique, as they address influences on the "everyday" experiences of feelings of depression that may be more common than we like to believe (Blazer 2003). There is a moderate heritability for depressive symptoms in the elderly, indicating that genetic variation is not of great importance in this population for this trait, but it seems as though genetic variation do have a greater influence in females than in males, even if the difference in influence was not statistically significant. Very large samples are needed to detect any such difference between genders if the effect is not huge. When comparing depressive symptoms with the clinical syndrome, defined as having a CES-D score of 16 or higher, the pattern of higher heritabilities for the trait in females than in males is confirmed. The results also demonstrate that genetic variation is of greater importance for this dichotomous trait

in females than in males. The reason for the differences between the two measures could be explained by the fact that narrow definitions of psychiatric disorders tend to be more familial than broader definitions when evaluated in multiple threshold models (McGuffin et al. 1994).

The heritability estimate only informs us about the amount of variance in the trait that can be explained by genetic variation in the population, assuming the trait is normally distributed in the population and that the underlying genes fit an additive model. There is no information about the number of genes in play; we just assume that the trait is polygenic.

It is a challenge to find the individual causative genes in a complex trait such as depressive symptoms. There are three commonly used methods today to find causative genes in complex traits. These are expression profiles, linkage and association analyses, the last being by far the most common. The motives for performing an association study are mainly two. Most commonly there is a biological hypothesis formed about a gene and its product and a potentially causal effect on the trait under study. The candidate gene may, or may not have any known functional variants that can be used as markers to test the hypothesis. The second reason is usually to replicate other positive findings in an independent sample. It is clear that association studies need to be more thoroughly followed up, not only by replicate studies but by functional studies as well. The disadvantage of association studies is that they cannot prove causality, but only significant probabilities of association. Nevertheless, an association suggests the importance of a gene in a biological pathway leading to disease.

Depressive symptoms are of particular importance in late life and the general theory of serotonergic function in psychiatric disorders and behavioral traits seems plausible. It is unlikely that serotonergic function alone is the sole genetic cause for a complex human trait like depressive symptoms, and more likely that serotonin constitutes a part of a pattern of developmental changes and adaptations in concert with other neurotransmitters and other factors. There are several very interesting genes and gene products within the serotonin system that can affect mood in general, several of them being used as target molecules for drug therapies. We have focused on only four serotonergic candidate genes and found a significant association, reiterating the potential involvement of serotonin genes in the etiology of individual differences for depressive symptoms.

Depressive disorders have in many studies and different populations, been positively associated with several components of the serotonergic system. But just like in linkage studies of depressive disorders, the findings have generally been quite inconsistent and difficult to independently replicate. The lack of consistent findings is most likely an effect of specific single genes having more of a major gene effect in certain study settings mixed with heterogeneous effects and spurious findings. Using a broader defined phenotype such as depressive symptoms based on a population sample, rather than diseased burdened families or cases with a narrow clinical definition compared to matched controls, will certainly generate a study setting where the trait is a result of many more genes with smaller effect sizes. One obvious advantage with identifying these “small effect genes” would be the possibility to generalize the risk for the whole population. However, such studies need very large

samples to be able to find the causative genes, since the effect in general is expected to be small. Today a plausible strategy is to collect very large population based samples for molecular studies, much because the technical development within molecular biology has been enormous during the last decade.

A vast number of new tools have been introduced in molecular biology as well as in statistical genetics that will help to disentangle the genes that contribute to susceptibility for these traits. There has also been a massive increase in the number of markers available for association and linkage studies with the advent of the human genome project and its successor projects. In this thesis a sequencing-based system was used for scoring SNPs, an approach that plausibly will minimize the risk for genotyping errors. Parameters controlling the sequencing reaction can be readily optimized; the quality of the reaction depends mainly on the quality of the template. The multi-base reading capacity of Pyrosequencing facilitates optimal positioning of the sequencing primer, and the peaks generated downstream of the SNP may serve as reference signals.

By applying the serotonergic hypothesis to depressive symptoms and by implementing a robust molecular technique in this thesis, an association between depressive symptoms and the –1438G/A promotor polymorphism in the *HTR2A* gene was found in males but not females. This implies that the gene is of importance for men in this population, but with a quite limited effect size. We also know that the heritability is relatively small for men in this population indicating that different genetic factors might be affecting the liability to depressive symptoms differently in men and women. In other words, even if there is a significant association to *HTR2A* in men, this finding does not explain the greater heritability for women than men. On the other hand, this polymorphism may account for a significant portion of the genetic variance for men. We have not evaluated the effect size for this gene in men as compared to women.

When investigating the genetic variation and structure of the MAO locus very few genetic variants were found and a strong LD characterized both *MAOA* and *MAOB*. The MAO locus covering a genomic region of almost 200kb, harbors surprisingly little variation, especially the larger *MAOB* gene that can be referred to as a SNP desert. The reason for this lack of variation is definitely intriguing and one can speculate whether this is due to selection or a founder effect. Selection might be the more likely explanation, due the enzymes implicated effect on human behaviors. The MAO genes are undoubtedly of great importance for the chemical balance in the CNS and genetic changes in these genes could, if they in any way altered the enzymes activity, have substantial consequences on behavior and mental health and would thereby be acting under strong selective pressure. It is also possible that the lack of variation in this region is the result of a limited number of founder individuals for this population. Although such an explanation for Sweden seems unlikely.

The MAO locus does not seem to influence the liability for depressive symptoms in this population. However, there was an interesting additive effect found in females further supporting the possibility of different genetic effects in men and women and implying that the MAO gene possibly could be contributing to a higher heritability in females compared to men. There was also an association between common variants of the *MAOA* locus and trbc-MAO activity. This is perhaps a bit unexpected since the

MAOB gene codes for trombocytic MAO, but it is possible that the *MAOA* haplotypes harbors distant regulatory elements affecting *MAOB*. Sequencing the region between the two genes, approximately 20 kb, would be interesting, since it should generate more exact information on the extent of LD at this locus and also reveal possible regulatory elements in that region.

Taken together, both the finding of a male risk for the *HTR2A* gene and the additive effect found in females for MAO locus haplotypes could possibly contribute to the heritability differences between genders. The study population used in this thesis did not have enough power to disentangle any possible separate genetic effects between males and females, but the cumulative evidence in this thesis certainly point in that direction – that there actually are genes with different effect sizes acting differently on the liability for the trait in men and women.

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APPENDIX

Appendix 1. CES-D Questionnaire.

During the past week:	Rarely or none of the time	A little of the time	A moderate amount of time	Most or all of the time
1. I was bothered by things that don't usually bother me.	☐	☐	☐	☐
2. I did not feel like eating; my appetite was poor.	☐	☐	☐	☐
3. I felt that I could not shake the blues even with help from my family or friends.	☐	☐	☐	☐
4. I felt that I was just as good as other people.	☐	☐	☐	☐
5. I had trouble keeping my mind on what I was doing.	☐	☐	☐	☐
6. I felt depressed.	☐	☐	☐	☐
7. I felt that everything I did was an effort.	☐	☐	☐	☐
8. I felt hopeful about the future.	☐	☐	☐	☐
9. I thought my life had been a failure.	☐	☐	☐	☐
10. I felt fearful.	☐	☐	☐	☐
11. My sleep was restless.	☐	☐	☐	☐
12. I was happy.	☐	☐	☐	☐
13. I talked less than usual.	☐	☐	☐	☐
14. I felt lonely.	☐	☐	☐	☐
15. People were unfriendly.	☐	☐	☐	☐
16. I enjoyed life.	☐	☐	☐	☐
17. I had crying spells.	☐	☐	☐	☐
18. I felt sad.	☐	☐	☐	☐
19. I felt that people disliked me.	☐	☐	☐	☐
20. I could not "get going".	☐	☐	☐	☐

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