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The Relation between Serotonergic Biomarkers and Behaviour

– studies on human primates, non-human primates and transgenic mice

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Abstract
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Rationale: The serotonergic system is involved in the modulation of emotion and plays an important role for personality and vulnerability for psychiatric disorders. In the papers included in this thesis, we investigate three biological factors that have been studied in relation to psychiatric symptoms: Platelet monoamine oxidase B (MAO-B) activity, and variations in the MAO-A and the serotonin transporter (5HTT) genes. We also study intensity dependent auditory evoked potentials (IAEP) as an intermediate phenotype for serotonergic capacity. Platelet MAO-B has been shown to be a biological marker for the properties of monoamine systems, with low activity being associated with vulnerability for high scores of sensation seeking, monotony avoidance, and impulsiveness, as well as for susceptibility for alcoholism. Functional polymorphisms in the promoter of the genes encoding MAO-A and the serotonin transporter result in high- or low- activity alleles that have been associated with numerous psychiatric symptoms. One hypothesis for the shaping of personality is that these genotype variants have prenatal effects on the wiring of the brain. Thus, exploring how the development of the brain is affected by different prenatal serotonin levels is relevant in this context.

Observations: (i) Platelet MAOB activity was associated with monoamine metabolites in cerebrospinal fluid from cisterna magna in monkeys, as well as with voluntary alcohol intake, alcohol-induced aggression, and alcohol sensitivity. **(ii)** The long 5HTTLPR allele was associated with increased IAEP. **(iii)** The functional MAOA and 5HTT polymorphisms were associated with symptoms of ADHD-related traits in a population based sample of Swedish adolescents. Associations of these candidate genes with ADHD scores were strenghtened when the platelet MAOB activity was combined with genotype. **(iv)** Our pilot data showed that treatment of pregnant mice with 5HTT blocking antidepressives resulted in more serotonergic cellbodies in lateral wings of dorsal raphe in the offspring, when compared to saline treatment.

Conclusions: Our studies support the notion that platelet MAOB activity and IAEP are endophenotypes for monoaminergic capacity and related behaviours. The functional candidate polymorphisms in MAOA and 5HTT were linked to behaviour, however, the cause-relationship is unclear and the explanation for the associations need to be further investigated, possibly with focus on prenatal effects of the polymorphisms.

Keywords: Serotonin, MAO-A, 5-HTT, MAO-B, alcohol, ADHD

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Att förstå är inte att ursäkta

Illustrations by Emma Källerö

List of Papers

I **Wargelius H**, Fahlke C, Suomi SJ, Oreland L, Higley JD. (2010) Platelet monoamine oxidase activity predicts alcohol sensitivity and voluntary alcohol intake in rhesus monkeys. *Ups J Med Sci*. Feb:115(1):49-55.

II Hensch T, **Wargelius H**, Herold U, Lesch KP, Oreland L, Brocke B. (2006) Further evidence for an association of 5-HTTLPR with intensity dependence of auditory evoked potentials. *Neuropsychopharmacology*. Sep;31(9):2047-54.

III Malmberg K*, **Wargelius H***, Lichtenstein P, Oreland L, Larsson JO. (2008) ADHD and Disruptive Behavior scores - associations with MAO-A and 5-HTT genes and with platelet MAO-B activity in adolescents. *BMC Psychiatry*. Apr 23;8:28. *Shared first authorship.

IV **Wargelius H***, Malmberg K*, Larsson JO, Oreland L. Associations of MAOA-VNTR or 5HTT-LPR alleles with ADHD symptoms are moderated by platelet MAO-B activity. Accepted for publication in *Psychiatric Genetics*. *Shared first authorship.

V **Wargelius H**, Deneris ES, Mackenzie A, Oreland L. Effect of prenatal fluoxetine on serotonergic cell bodies in lateral wings of dorsal raphe nucleus. *Manuscript*.

Additional publications

- Dahlgren A, **Wargelius H**, Berglund K, Fahlke C, Blennow K, Zetterberg H, Oreland L, Berggren U, Balldin J. Do alcohol-dependent individuals with DRD2 A1 allele have an increased risk of relapse? A pilot study. Accepted for publication in *Alcohol and Alcoholism.*

- Nilsson KW, **Wargelius H**, Sjöberg RL, Leppert J, Oreland L. (2008) The MAO-A gene, platelet MAO-B activity and psychosocial environment in adolescent female alcohol-related problem behaviour. *Drug Alcohol Depend*. Jan 11;93(1-2):51-62.

- Hensch T, **Wargelius H**, Herold U, Strobel A, Oreland L, Brocke B. (2008) Electrophysiological and behavioral correlates of polymorphisms in the transcription factor AP-2beta coding gene. *Neurosci Lett.* May 2;436(1):67-71.

- Nilsson KW, Sjöberg RL, **Wargelius H**, Leppert J, Lindström L, Oreland L. (2007) The monoamine oxidase A (MAO-A) gene, family function and maltreatment as predictors of destructive behaviour during male adolescent alcohol consumption. *Addiction.* Mar;102(3):389-98.

- Sjöberg RL, Nilsson KW, **Wargelius H**, Leppert J, Lindström L, Oreland L. (2007) Adolescent girls and criminal activity: role of MAOA-LPR genotype and psychosocial factors. *Am J Med Genet B Neuropsychiatr Genet*. Mar 5;144B(2):159-64.

Contents

Abbreviations

ADHD	Attention deficit hyperactivity disorder
ASP	Antisocial personality disorder
AUD	Alcohol use disorder
CD	Conduct disorder
CNV	Copy number variation
CNS	Central nervous system
CSF	Cerebrospinal fluid
DBD	Disruptive behaviour disorders
DSM-IV	Diagnostic and statistical manual of mental disorders, 4thed.
GABA	Gamma-aminobutyric acid
GWAs	Genome wide association studies
5-HIAA	5-hydroxyindole acetic acid
5-HT	5-hydroxytryptamine (serotonin)
5-HTT	Serotonin transporter
5-HTTLPR	Serotonin transporter gene-linked polymorphic region
HVA	Homovanillic acid
IAEP	Intensity dependent auditory evoked potential
K-SADS-PL	Kiddie Schedule for Affective Disorders and Schizophrenia for School-Age Children-Present and Lifetime Version
LR	Level of response (to alcohol)
MAO-A	Monoamine oxidase A
MAO-B	Monoamine oxidase B
MAOA VNTR	MAO-A variable number of tandem repeats
ODD	Oppositional defiant disorder
PEA	Phenylethylamine
PMD	Premenstrual dysphoria
SNP	Single nucleotide polymorphism
SSRI	Selective serotonin reuptake inhibitor
TCHAD	Twin study of child and adolescent development
TPH	Tryptophanhydroxylase

Preface

It is important to recognize that certain individuals are at greater risk for developing psychiatric disorders than others, and that this vulnerability is a result of environmental influence on, and interactions between, a person's genetic set up.

It is known that there is a genetic component in psychiatric disturbances. This is good news that should receive a hearty welcome because it means that there is a chance that we will be able to understand the mechanisms involved in psychiatric disease, and thus look for cures. Ultimately, the goal of genetic research should be to prevent mental disorder and to refine and perhaps personalize treatment strategies.

In this thesis, I describe certain genetic factors that contribute to the development of alcohol addiction, as well as to the development of various forms of externalizing psychopathology — that is, psychiatric disorders characterized by disinhibited behaviour, such as attention deficit hyperactivity disorder (ADHD) and conduct disorder.

Aims of studies

General aim
To elucidate genetic predispositions for personality traits which increase the vulnerability for psychiatric disorders and deviant behaviour.

Paper I
To study deviant behaviour in a non-smoking animal model (rhesus monkeys), and to relate behaviours and alcohol intake with platelet MAO-B activity.

Paper II
To analyze the effect of 5-HTTLPR on the intensity dependence of auditory evoked potentials in a healthy male student sample that was homogenous for most significant confounding variables.

Paper III
To study serotonergic candidate genes as well as platelet MAO-B activity in relation to ADHD symptoms in a population-based sample of adolescents.

Paper IV
To study the combined effect of platelet MAO-B activity and variations in the 5-HTT or MAO-A genes for expression of ADHD like behaviours.

Paper V
To study aspects of the neuroanatomical effects of high prenatal serotonin levels following maternal intake of a SSRI in mice.

Introduction

The shaping of an individual's personality has been discussed for centuries. Like a pendulum, the trends have shifted between nature and nurture as the determining factors. The swings back and forth have been influenced by political ideologies and scientific hopes as can be followed through history: The newborn as a tabula rasa (in the 1700s); Galton's principles and the rediscovery of Mendel's hereditary laws (1900s); the behaviourism and the back-reaction to the Nazi regime in Germany (1950); twin- and adoption-studies and the discovery of DNA (1960); the socialist-movement and environment focus (1970); development of molecular genetics and initiation of many heredity studies (1980).

Today, these swings seem as old-fashioned ways of looking at the story of how behaviour, emotions and personality traits develop. Heritability is dependent of time point and an environmental context, and the environment operates differently on individuals with different genetic make-ups. This is shown by findings from the field of epigenetics. Hence, we now know that nature and nurture work together. Practically all the major mental disorders, personality dimensions and human behaviours have been found to have a genetic component. Likewise, environmental factors influence almost all behaviours.

To understand personality disorders and other mental disorders, knowledge about the neurobiological basis of personality traits is crucial. In 1967 Dr. Eysenck discussed the connection between low tonic activity of an unspecific activating system in the brains of individuals with high sensation seeking behaviour. Also others discussed extraversion behaviours as a compensation for low activation and under-stimulation of some biological system (Zuckerman and Neeb, 1979; Zuckerman et al., 1984; Hegerl et al., 1995). Most certainly, more than one system is involved in complex behaviours, however, the serotonin system has emerged as one of the key players in mood and personality. Central serotonergic neurotransmission is involved in the pathophysiology of psychiatric disorders such as depression, schizophrenia, generalized anxiety, obsessive-compulsive behaviour, addiction, aggression disturbances, and autism (Canli and Lesch, 2007).

Since brain serotonin capacity at present cannot be measured directly, biochemical markers of the central serotonergic function are valuable. We

have studied two potential markers - platelet MAO-B activity and auditory evoked potentials (AEP) - as well as genotypes of serotonergic candidate genes in the search of a non-invasive way of looking at brain serotonin function.

Behavioural genetics

Behavioural genetic studies have been important in showing that there is a genetic component in almost all behaviours. However, the significance of the genetic component for a certain behaviour can probably vary over time. Genes can actually be expressed differently during an individual's developmental stages and could be involved in different behaviours during these stages (Kendler et al., 2008).

It is now generally agreed that many different genes are involved in personality and behaviour, and that every single gene probably has a very small effect. When researchers talk about a *big effect* of a gene in this field, they typically mean that this gene explains about 2% of the variance. A number of publications report associations between specific genes and psychiatric diagnoses, so called association studies. Most reports published so far suggest that the studied genotypes do not convey much of effect in individuals that grow up in a safe and sound environment. Hence, the environment plays a crucial role for the development of the phenotype, just as gene-environment interactions and genetic heterogeneity, since many different systems are involved in complex behaviours and mental disorders.

Studying associations between genes and behaviour

Association studies can be performed using candidate genes. Candidate genes are chosen by basic known mechanisms in a certain disorder. For example, typical candidates for alcoholism are genes involved in alcohol metabolism. Ideally, a candidate gene should be both biologically relevant and have a functional significance. Thus, polymorphisms in candidate genes typically affect protein amount or protein function. This field has exploded over the last decade, often yielding inconsistent results.

Genome-wide association studies (GWAs) identify chromosome regions that are associated with the investigated behaviour. GWAs are considered to support the idea that a common disease is related to common gene variants. However, the threshold to discover a significant hit is quite high and the method is limited in terms of identifying alleles that are prevalent in the general population. For this reason, large samples are required to detect common genetic variants.

In order to associate a gene variant with a certain behaviour or disease, different kinds of variations in the DNA are used. A single nucleotide polymorphism (SNP) signifies a change of one nucleotide in the genetic code.

Other types of polymorphisms are, for example, insertion or deletion polymorphisms and variable number of tandem repeats (VNTRs). Copy number variations (CNVs) constitute a major type of genetic variation (Sebat et al., 2004; Feuk et al., 2006) and are probably involved in disease (Check, 2005; Nadeau and Lee, 2006). CNVs are deletions or duplications of DNA sequences that consist of more than 1000 nucleotides. Genes with CNVs are of interest for psychiatric disorders where the syndrome consists of extremes of common personality traits.

Environmental factors

The complexity of studies dealing with behaviour and psychiatric disease increases even more because of the role played by environmental factors. Thus, behavioural genetic research also provides evidence for the importance of environmental influences. Individuals with similar genetic backgrounds may be at different risk for a psychiatric disorder depending on life experiences. Hence, it is important to identify the environmental factors that modify the risk for psychopathology.

In this context, 'environmental factors' is a very broad concept including not only parenting and childhood trauma, but also prenatal events and non-genetic biological events such as nutrition. It has been demonstrated that a variation in the serotonin transporter gene increases the risk of developing depressive symptoms, particularly in individuals with a stressful life history (Caspi et al., 2003). Although not undisputed (Munafo et al., 2009; Kaufman et al., 2010). We have, in a series of studies, found that polymorphisms in candidate genes interact with childhood psychosocial environment to explain adolescent deviant behaviour (Nilsson et al., 2007; Sjoberg et al., 2007a; Nilsson et al., 2008). Similar data with regard to gene-environment interactions are retrieved from animal models (Barr et al., 2003).

Genes may be directly influenced by stress, for example, stress hormones regulate the expression of certain genes of importance for brain development and behaviour (Tafet et al., 2001). Another important perspective is that some environmental factors are influenced by genetics, hence, people create their own environment, sometimes protective, sometimes predisposing for hazardous behaviour and psychopathology (Kendler and Baker, 2007). Environmental factors are at play throughout development of the embryo as well as the adult individual. Such epigenetic regulation of gene expression during development and later are of great interest within the field of complex disorders.

Theories of personality traits

Personality traits such as impulsivity and antisocial behaviour are linked to an increased risk of high alcohol consumption. These traits are also common in ADHD, and children with ADHD often tend to get into alcohol problems.

Personality refers to the temper or persistence of behaviour or to the emotional expression that accompanies it. According to a pioneer in personality psychology, Gordon Allport, there are 17 953 words describing human traits in the Webster's New International Dictionary, 1925. These words were used by Allport and others to create models of personality that are used both in research and in clinic within the fields of psychology, psychiatry and medicine. The heritability of many personality traits has been estimated to be between 40-60% (Loehlin, 1982; Jang et al., 1996). In 1983, Oreland and Shaskan proposed that serotonin is related to impulsivity (Oreland and Shaskan., 1983). Cloninger et al. further argued that various temperament traits are associated with specific neurochemical substrates that have a genetic background (Cloninger, 1987). Cloninger's personality model is based on four dimensions of temperament and three character traits (Cloninger et al., 1993). Another frequently used model is the Big Five Factor Model where five dimensions are used to describe human personality. Both the Big Five and Cloninger's model are believed to have a biological base. Previously professor Zuckerman had introduced the concept of sensation seeking (Zuckerman et al., 1964; Zuckerman, 1990) which is a trait that highly correlates with Cloninger's novelty seeking and with impulsiveness, making it an interesting trait for biological studies of vulnerability for risk behaviours and psychiatric disorders. Karolinska Scales of Personality (KSP) was developed by professor Schalling at Stockholm University and has mainly been used in psychological and psychiatric research. KSP is a personality test that has been developed from existing theories described above.

In summary, there are several different models to describe personality and certain personality traits seem to have a biological base and are associated with vulnerability for psychiatric disorders.

Classification of psychiatric symptoms

It is difficult to create universal criteria for psychological disease that apply to all people. Psychiatric disorders are diagnosed with a clinical interview using a diagnostic tool such as the Diagnostic and Statistical Manual of mental disorder Text Revised, 4th edition (DSM IV TR) (American Psychiatric Association, 2000). The DSM is a categorical classification system, which is carefully validated, however, whether the diagnostic criteria also mirror the genetic or biological aetiology is not clear. For the forthcoming edition of

DSM, the possibility of a dimensional classification of some diagnoses is discussed (www.dsm5.org).

Dimensional or categorical diagnoses

To what extent are for example alcohol addiction and ADHD distinct illnesses or extremes of a spectrum? Dimensions of temperament account for much of the liability to psychiatric disorders. There is a range of different risk dimensions that are not synonymous with disorder (Figure 1). The effect of risk genes that affect a certain psychiatric disturbance may also have an effect on the behavioural variation in the normal population with regard to closely related behaviours. Furthermore, the same trait can be a risk factor for one form of psychopathology but a protective factor for another. Most probably, genes that contribute to e.g. clinically diagnosed alcoholism also contribute to problematic alcohol abuse at a subclinical level (and to the variation in drug use in the normal population). A significant portion of the genetic influence on alcohol addiction is through a general predisposition toward externalizing disorders (Kendler et al., 2003). Dysfunction of the serotonergic system also characterizes subclinical symptoms that are shared by patients with different psychiatric disorders.

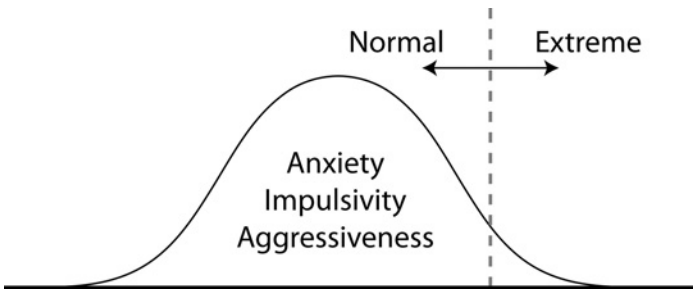


Figure 1. The dimensional way of looking at personality traits or behaviours. Continuously distributed dimensions of a trait in the population. Where should the line for a diagnosis be drawn and what about individuals just below the threshold?

With dimensional scales no information is lost, it is more individual and more detailed descriptions are obtained. Categories on the other hand are easy to use and well established, however there is heterogeneity within a disorder and inadequate biologic base.

Furthermore, there are individuals that do not fulfill the criterias for full syndrome diagnoses but who still have high scores on important items, this is sometimes referred to as subthreshold diagnosis.

Comorbidity

Coexistence of several psychiatric diagnoses is rather a rule than an exception. For example, alcohol addiction typically overlaps with other kinds of addictions, with externalizing behaviour, anxiety and depression and with antisocial behaviour. In our material of teenaged twins we have found that individuals with high scores of ADHD related symptoms also express anxiety and high alcohol consumption (Malmberg et al., submitted). Such comorbidity could for instance be explained by that one condition causes the other or that the risk for one problem increases if you have the other problem, or perhaps the same biological system affects both conditions.

Although we talk about comorbidity, it is possible that it is not the question of several independent diseases, but rather alternative expressions of one underlying behavioural disturbance. This would mean that a certain set of genes contribute to multiple behavioural problems while other genes would be more specific for a certain problem (Figure 2) (the generalist gene philosophy, see (Butcher et al., 2006)). Different genes are involved in different routes to what is diagnosed as the same disorder. Thus, there are indications that certain genes are more important in specific subtypes of psychiatric disorders, e.g. with regard to the difference between Cloninger's type I and type II alcoholism. For instance, genetic variation linked to the serotonergic system seems to be especially important in alcoholism with early onset, type II, the subtype that also include occurrence of antisocial behaviour and poor impulse control.

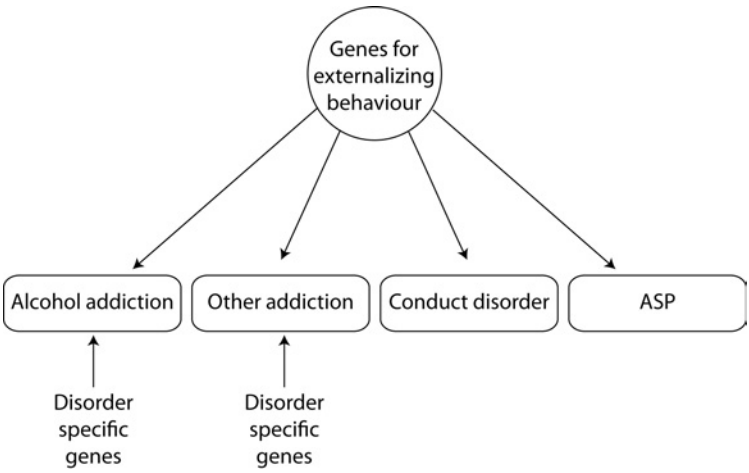


Figure 2. Liability probably requires a combination of susceptibility genes. One set of genes could be involved in more general liability whereas other genes could be involved in specification of how this liability is manifested. Figure modified from (Kendler et al., 2003). ASP: antisocial personality disorder.

ADHD and DBD

ADHD affects 3-5% of school aged children (Larsson et al., 2004). The aetiology of ADHD is not fully understood and as with other psychiatric disorders there is a discussion going on as to whether the categorical diagnostic tools used for diagnosis manage to catch ADHD correctly.

A strong genetic component with heritability of 70-80% has been reported for ADHD (Biederman, 2005; Freitag et al., 2010). However, no consistent linkage regions have been identified with GWAs. ADHD is a disorder with two separate underlying symptom dimensions; a hyperactive-impulsive dimension including excessive activity and impulsive responding and an inattentive dimension including difficulties in sustained attention, distractibility, disorganization and lack of task persistence.

Included in disruptive behaviour disorder (DBD) diagnosis are oppositional defiant disorder (ODD) and conduct disorder (CD) that are characterized by a sustained pattern of chronic argumentativeness and anger associated with compromised social relations with parents and peers and a variety of persistent antisocial behaviours including acts of aggression, destruction of property, deceitfulness, theft and violation of commonly adhered to social problems (DSM IV). ODD is a significant precursor of adolescent CD in children with ADHD (Figure 3) (Loeber et al., 2000). Individuals with ADHD are at heightened risk for poor educational attainment, lower income, underemployment, legal difficulties, and impaired social relationships. Children with ADHD and comorbid CD have an increased risk for criminality and drug abuse in adolescence and adulthood (Biederman, 2005).

Several studies suggest that many of the co-morbid conditions associated with ADHD are also substantially heritable. Furthermore, ADHD symptoms are now recognized as persisting into adulthood (Jacob et al., 2007).

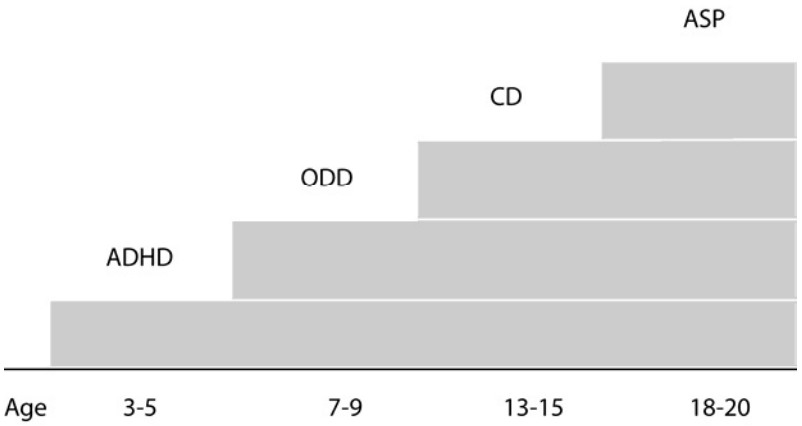


Figure 3. Risk profile for ADHD diagnosis. ADHD in bottom is associated with high risk of later ODD, CD and even antisocial personality disorder (ASP).

Alcohol abuse

Alcohol is the world's third largest risk factor for disease burden (WHO, 2011). In Sweden, about 450 000 persons have severe problems with alcohol (addiction or abuse). Alcohol abuse in itself is problematic, however, the burden on family members and society goes beyond the addiction, creating problems like e.g. childhood trauma and poor psychosocial environment in the family. Furthermore, and perhaps less acknowledged is the great disease burden represented by neuropsychiatric disorders related to alcohol problems (Figure 4).

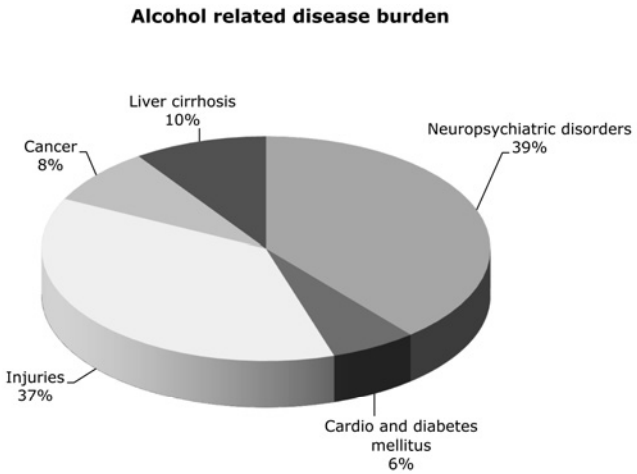


Figure 4. Figure represents DALY data from WHO 2011. DALY: disability-adjusted life year, which is a measure of overall disease burden.

For most people alcohol in itself is a very small risk for alcohol use disorder (AUD). Hence, other factors are important or even necessary for normal alcohol use to develop into alcoholism. Such factors are the social environment (e.g. access) and genetic predisposition. Thus, the heritability estimates for alcohol addiction is 50-60% (Goldman et al., 2005). Addiction spans several disciplines including, psychology, psychiatry, sociology, behavioural neuroscience, genetics, molecular biology and pharmacology. Hence, AUD is a heterogeneous disorder and attempts have been done to classify alcoholics into different groups and subgroups. Personality traits are suggested to contribute to alcohol addiction and Cloninger's type I and type II system is frequently used in biological research. Type I alcoholics have a late debut and typically score high for harm avoidance and reward dependence on personality scales. Type II alcoholism, or early-onset alcoholism, is characterised by high scores of sensation seeking or impulsiveness (Von Knorring et al., 1985) and are frequently associated with social problems when drunk. Furthermore, it has been stated that that the type II alcoholic has a heavier genetic load (Cloninger et al., 1981). Pattern of alcohol use similar to the

type I/type II concept has also been found in monkeys (Barr and Goldman, 2006; Wargelius et al., 2010).

It should be mentioned that other classifications of alcohol abuse also exists (Leggio et al., 2009; Pombo and Lesch, 2009). However, the classifications are to a large degree overlapping.

Alcohol induced aggression

Alcohol is often involved in violent crimes (Arseneault et al., 2000). It has been noted that alcohol potentiates aggressive behaviour in some (Buchsbaum et al., 1976; Ito et al., 1996; Giancola et al., 2002) but not in all individuals (Miczek et al., 1994; Nilsson et al., 2007). Similar results have come from animal studies on rodents and non-human primates (Miczek et al., 1992; Higley, 2001). Some authors have concluded that alcohol intake could cause aggression potentially through reduced inhibition (Bushman and Cooper, 1990). Hence, there is research indicating a stronger relationship between alcohol intake and aggression in subjects with low serotonin levels (Virkkunen et al., 1995; Dougherty et al., 1999). Impaired serotonin functioning has also been shown to increase the risk for alcohol-induced aggression in non-human primates (Higley, 2001).

Endophenotypes

An endophenotype is a measurable behaviour (other than diagnosis) that reflects part of the pathophysiology of a certain condition. It is often a less complex behaviour or a biologic measure that is hypothesised to be "closer to the genes". Consider how many complex processes and events there are between the expression of e.g. the MAO-A gene and the development of a deviant behaviour or a certain personality trait. A way to get closer to the biological function of the gene could be by the use of a measurable variable which can be associated with both diagnoses and genetic data, i.e. an intermediate phenotype bridging between genetic data and clinical data, that is, phenotypes that are more easy to quantify and that possibly are closer to the biological function. In psychiatry, an endophenotype may be any of the measurable variables obtained by biochemical, endocrinologic, neuroanatomical, neurophysiological, neuropsychological, or cognitive investigations. Four relatively independent, genetically influenced intermediate phenotypes that affect the risk for alcoholism, include alcohol-metabolizing enzymes, disinhibition/impulsiveness, several psychiatric disorders, and the response to alcohol.

Suitable endophenotypes should be hereditary and common in high-risk individuals and also stable over time. Gottesman and Gould listed four criterias, which should be fulfilled for endophenotypes in psychiatric genetics (Gottesman and Gould, 2003): According to them an endophenotype is

1. Associated with illness in the population
2. Heritable
3. State-independent
4. Co-segregates with illness within families

It must be said that there are difficulties to meet all these criterias, however, examples of intermediate phenotypes relevant in the context of this thesis are: Biochemical markers such as platelet MAO-B activity; low level of response to a drug; electrophysiological markers such as amplitude of event-related potentials; personality traits or behaviours (certainly more far away from the genetic level than physiological measurements, but perhaps closer than diagnoses) (Figure 5.). Moreover, behavioural phenotyping could be used for animal studies where it is impossible to talk about full syndrome diagnoses.

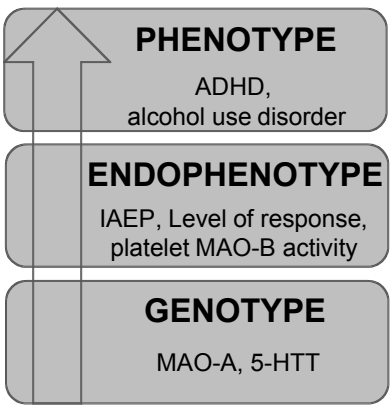


Figure 5. Schematic illustration of the relationship between genotype, endophenotype, and phenotype.

Level of response to alcohol

Level of response (LR) to alcohol, i.e. physical responses like body sway or stumble, as well as subjective feelings like dizziness and high, show inter-individual differences between subjects. Low LR to alcohol is an intermediate phenotype believed to be a marker for susceptibility to alcohol abuse (Schuckit, 1994; Morean and Corbin, 2010). The need for higher doses of alcohol to get the desired effect is hypothesised to lead to higher alcohol intake (Barr et al., 2003; Boyce-Rustay et al., 2006; Chappell and Weiner, 2008), which in turn contributes to subsequent acquired tolerance. In line with this hypothesis are reports of that LR has been shown to predict drinking frequency and quantity as well as alcohol abuse (Heath et al., 1999; Schuckit et al., 2006).

Children of alcoholics are typically characterised by low LR to alcohol (Schuckit and Smith, 2000) which suggests that the LR is genetically influenced. The heritability of LR has in twin studies been estimated to 40-60% (Heath et al., 1999). Genes with functions within different aspects of the LR to alcohol are probably involved. For example, polymorphisms that alter the activity of the alcohol metabolizing enzymes can increase the LR and thereby decrease the risk of heavy drinking. Relating to alcohol's effect in the body, variations in GABAergic receptor genes (binding sites for alcohol) could result in less sensitivity to the pharmacological effects of alcohol (Hanchar et al., 2005). Furthermore, alcohol's effect on the monoamine system may be moderated by polymorphisms in e.g. serotonergic genes and may thereby affect LR. Thus, levels of 5-HIAA as well as serotonin transporter availability have been associated with LR in monkeys (Heinz et al., 1998), and a polymorphism in the serotonin transporter has been related to low LR as measured by high inherited tolerance in human subjects (Turker et al., 1998).

Evoked potentials

Many brain diseases give rise to alterations in the brain that can be detected by electroencephalography (EEG). EEG measures electric activity from the brain via electrodes placed at specific positions on the scalp. The temporal resolution of the EEG is precise since changes in the activity can be detected instantly, whereas, spatial resolution is poor.

Individual characteristics of cortical sensory processing is stimulus intensity dependent and can be measured by intensity dependent auditory-evoked potentials (IAEP). IAEP show how much the amplitudes of the auditory-evoked potentials rise in response to tones of increasing intensities (Figure 6).

Evoked potentials as markers of biological functions have become a promising endophenotype as knowledge about anatomical function increases. An auditory stimulus causes the release of cortical neurotransmitters (such as GABA and glutamate) and induces intracortical currents that underlie the electric activity that is measured by the electrodes on the scalp (Mitzdorf, 1994). Intensity dependence of the cortical response measures the more general reactivity of cortical neurons. This reactivity is influenced by neuromodulators like serotonin. Differences in the auditory evoked potentials may reflect individual differences in serotonergic innervation to the primary auditory cortices. Late auditory-evoked potentials have been associated with individual differences in monoaminergic neurotransmission and associated personality traits. Individuals with large IAEP have higher scores on sensation seeking and impulsivity traits (Zuckerman, 1990). Thus, IAEP has been suggested to be an indicator of central serotonergic neurotransmission (Hegerl and Juckel, 1993). Because the highest serotonergic innervation

is found in the primary auditory cortices as compared to the lower levels of serotonin in secondary sensory areas (Azmitia and Gannon, 1986; Lewis et al., 1986), the response of especially the primary auditory cortices should be influenced by the serotonergic system. The intensity dependence can be best seen in the negative component N1 and the positive component P2, which appears after about 100 ms and 170 ms after the tone, respectively.

The N1/P2 component is mainly generated in the primary auditory cortex and has been put forward as a way of capturing central serotonergic transmission (Hegerl and Juckel, 1993). A strong increase in amplitudes of the auditory N1/P1 component is thought to reflect low serotonergic activity, which, has been shown in animal studies where reducing serotonergic activity increases N1/P2 IAEP in the primary auditory cortex (Juckel et al., 1997; Juckel et al., 1999). Furthermore, several studies have related IAEP to serotonergic modulated disorders or personality traits (Hegerl et al., 2001) and two studies investigated IAEP in relation to genetic variation of the serotonergic system (Gallinat et al., 2003; Strobel et al., 2003). Additionally, some studies suggest that it is possible to influence IAEP with serotonergic medications in healthy subjects (Roon et al., 1999; Nathan et al., 2006; Simmons et al., 2011). The IAEP is reduced by chronic administration of serotonin enhancing drugs (Hegerl and Juckel, 1993) which suggests that IAEP is a marker for long-term or chronic rather than acute changes in the serotonin system. In addition, patients with low serotonergic activity, as measured by intensity dependence, showed a better response to drugs that increase serotonergic neurotransmission than did patients with normal or high serotonergic activity (O'neill et al., 2008).

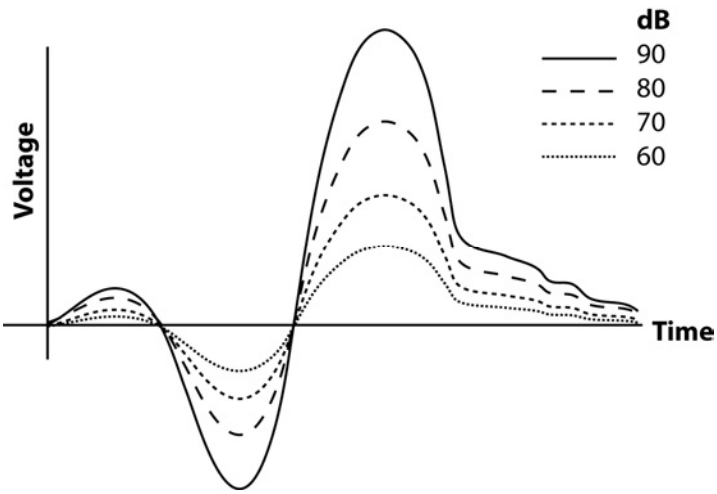


Figure 6. Intensity dependent auditory evoked potentials (IAEP) of the N1/P2 component for tones of intensities between 60 dB and 90 dB.

A problem with the evoked potential is that the electrodes on the scalp report potentials that are composed by several components. Many subcomponents generated by different cortical structures, such as the activity of primary and secondary auditory cortices, are detected within one scalp potential. Using different dipoles as sources for the measurements, as in paper II, has shown to be a successful approach to separate the overlapping subcomponents of the auditory evoked potentials and to relate them to the cortical structures that generate them.

In summary, IAEP fulfills some of the demands of a serotonin endophenotype but there are several questions that remain to be resolved. Data on associations of IAEP with serotonin-related polymorphisms or behavioural traits are sparse. Furthermore, confounding factors are not always being sufficiently controlled for which, probably, explains some of the conflicting results.

Platelet MAO-B activity

MAO-B metabolizes dopamine both in the brain and in the periphery and has been shown to be stable over time and with a heritability factor of 0.75 (Pedersen et al., 1993). Platelet MAO-B activity has in a large number of studies been associated with temperament, however, a functional link between peripheral MAO-B activity and central activity has not been established, why platelet activity must be regarded as a marker and not as a cause. This, however, does of course not rule out that there really is a biologically functional explanation for the reported associations with higher cognitive functions.

A U-shaped relationship with high or low platelet MAO-B activity being associated with psychopathology has been suggested (Af Klinteberg et al., 1987). However, it is low MAO-B activity that has been linked to sensation seeking, novelty seeking and impulsiveness (Oreland, 2004), and it was early shown that low platelet MAO-B activity is associated with type II alcoholism in males (Von Knorring et al., 1985). Although a recent study found no associations between psychopathology and platelet MAO-B activity (Gustavson et al., 2010), we have shown that females with low platelet MAO-B activity had an increased risk of alcohol-related problem behaviour in an unfavourable environment (Nilsson et al., 2008).

Besides an association to behaviours known to rely on a serotonergic component, platelet MAO-B activity has also been linked to serotonin metabolite levels in cerebrospinal fluid which points to connections with the serotonergic system (Oreland, 2004) but there are also reports on correlations with dopamine metabolites (Oreland et al., 1981; Wargelius et al., 2010).

Oreland and Hallman have proposed four theories to explain the association between peripheral MAO activity and behaviour (Oreland and Hallman, 1995). Two of these theories are relevant in the context of the papers presented here: Either is platelet MAO-B activity influencing the concentration of endogenous trace amines that are of importance for behaviour, or, MAO-B activity in platelets shares genetic control with e.g. monoamine transmitter genes.

Smoking

The MAO-B enzyme is irreversibly inhibited by compounds in cigarette smoke, however after quitting smoking MAO-B activity is restored probably by *de novo* synthesis (Fowler et al., 2000). In a recent study it is suggested that smoking regulates MAO protein concentrations by de-methylation of the MAO-B promoter (Launay et al., 2009). Alcoholics are often smokers, hence, in light of these findings, studies showing low platelet MAO-B activity in alcoholics can be questioned. However, the association between platelet MAO-B activity and personality/psychopathology has been shown to remain even when controlling for smoking (Oreland et al., 2002; Ruchkin et al., 2005; Wargelius et al., 2010).

The Serotonin System

Serotonin (5-hydroxytryptamine, 5-HT) was first described as a vasoconstrictor compound in serum (Rapport et al., 1948) and was later identified as a neurotransmitter (Gaddum, 1953; Twarog and Page, 1953; Amin et al., 1954). In the CNS, serotonergic neurons are located in the raphe nuclei of the brain stem and innervate almost the whole brain. Thus, virtually every cell in the brain is in close proximity to a 5-HT fibre.

Serotonin is an important mediator of mood, fear, impulsivity, aggression, sleep, and appetitive behaviours, including alcohol consumption (Lucki, 1998). Many compounds that target the serotonin system are used as treatment for psychiatric disorders. For instance, selective serotonin reuptake inhibitors (SSRIs) are effective for premenstrual disorder (PMD), suicidal behaviour, anxiety, major depression, obsessive-compulsive disorder (OCD) and anorexia (Vaswani et al., 2003). Furthermore, a variety of psychotomimetic drugs are acting by interfering with serotonergic mechanisms (e.g. LSD and ecstasy) (Aghajanian et al., 1970).

Serotonergic neurogenesis and development

While serotonergic neurons appear early in development, the network of connections is established throughout the whole gestation period and is completed after birth (Sundstrom et al., 1993). The transcription factor Pet-1 is necessary for the development of the majority of serotonergic neurons.

Thus, Pet-1 is required for expression of tryptophan hydroxylases (TPHs) and for expression of the serotonin transporter (Hendricks et al., 1999). Mice with deletion of Pet-1 show a 70% reduction of 5-HT neurons (Hendricks et al., 2003). The 5-HT neurons that seem to be independent of Pet-1 were recently proposed to be a unique subpopulation with selective anatomical targets (Kiyasova et al., 2011).

During development, 5-HT has neurotrophic effects (reviewed in (Gaspar et al., 2003)). Thus, prenatal serotonin levels are of importance for the development of the central serotonergic system, which is supported by molecular genetic, pharmacological and brain imaging studies (Hariri et al., 2002; Gaspar et al., 2003; Ansorge et al., 2004). Furthermore, serotonin has been shown to be important for CNS morphogenesis in rodents (Cote et al., 2007).

Serotonergic neurotransmission

Serotonin is synthesised from tryptophan in a two-step process where tryptophan hydroxylase (TPH) is the rate-limiting enzyme. Serotonin is stored in vesicles until release into the synaptic cleft where it binds to receptors and subsequently is re-uptaken into the cell via the serotonin transporter (5-HTT). Serotonin is degraded into 5-hydroxyindole acetic acid (5-HIAA) by monoamine oxidase A (MAO-A). The metabolite 5-HIAA can be detected in the urine and in cerebrospinal fluid (CSF) and has been put forward as a marker for serotonin turnover (Asberg et al., 1986; Placidi et al., 2001).

No other neurotransmitter system has more receptors than the serotonin system. At the moment at least 14 different receptors have been identified (Hannon and Hoyer, 2008). Some of these receptors are located also presynaptically on the soma, on dendrites or on nerve terminals where they are involved in the autoregulation of serotonergic neurotransmission.

SSRI

Selective serotonin reuptake inhibitors (SSRIs) block serotonin transporter function which results in elevated extracellular 5-HT levels. However, acute SSRI manipulations have been shown to have different effects on 5-HT levels in different areas of the brain (Beyer and Cremers, 2008). Treatment with SSRI show instant reduction of irritability in subjects with premenstrual dysphoria (Landen et al., 2009). In contrast, when used for depression and anxiety disorder, SSRI typically show effect after about 4 weeks, suggesting that secondary effects such as structural plasticity are also necessary (Zhou et al., 2006; Pittenger and Duman, 2008). Moreover, studies on rodents show that the effects of SSRIs seem to have paradoxical behavioural outcomes depending on if it is administered during early development or in adult life. For example, in adults, SSRIs have anxiolytic effects while, during development, SSRI causes anxiety-like behaviours in rodents (Homberg et al., 2010).

Polymorphisms in serotonin-related genes

Genetic variations in serotonergic genes could add to the risk of developing mood- and personality-related disorders (Lesch and Mossner, 1998; Nilsson et al., 2007; Sjoberg et al., 2007b). Two proteins of major importance for the levels of serotonin in the brain are MAO-A and 5-HTT. The genes encoding these proteins have functional polymorphisms that affect their transcription.

5-HTT LPR

The serotonin transporter (5-HTT) is responsible for the presynaptic reuptake of serotonin and thereby terminates and modulates serotonergic neurotransmission, which gives the transporter a pivotal role in serotonergic function. A nowadays very extensively studied polymorphism has been identified within the human 5-HTT gene (SLC6A4): The serotonin transporter gene-linked polymorphic region (5-HTT LPR) that is a functional 44bp insertion or deletion in the transcriptional control region (Figure 7). The short variant has been shown to have lower transcriptional efficiency than the long variant (Heils et al., 1995; Lesch et al., 1996; Mortensen et al., 1999). The short allele has repeatedly been associated with harm avoidance and anxiety-related personality traits (Ebstein et al., 2000; Canli and Lesch, 2007), affective disorders (Lotrich and Pollock, 2004), alcohol dependence (Feinn et al., 2005). The short 5-HTTLPR allele has also been related to lower serotonin metabolites (5-HIAA) in cerebrospinal fluid (CSF) (Williams et al., 2003) and to stronger amygdala response in brain-imaging studies (Hariri et al., 2002; Furmark et al., 2004; Pezawas et al., 2005).

The 5-HTTLPR is a highly polymorphic region (Nakamura et al., 2000) and has been proposed to be functionally triallelic (Hu et al., 2006). No CNVs have been found in the 5-HTT gene (Alaerts et al., 2009).

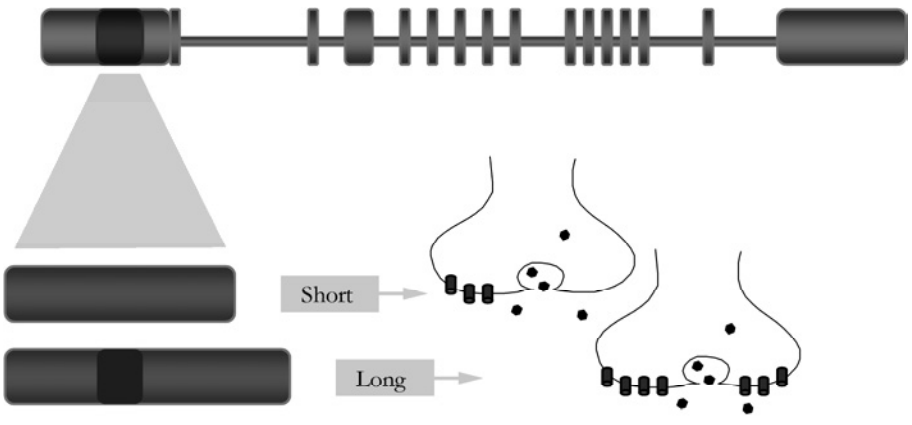


Figure 7. The 5-HTT LPR. The 44bp inssertion/deletion in the promoter region affects transcription of the gene.

MAO-A VNTR

The MAO-A enzyme metabolizes serotonin, dopamine and norepinephrine (Shih and Thompson, 1999). Deletion of MAO-A activity in males resulted in remarkable aggressiveness in mice (Cases et al., 1995) as well as in a Dutch family (Brunner et al., 1993). The MAO-A gene is located on the X-chromosome and displays a variable number of tandem repeats (VNTR) in the promoter region. The VNTR polymorphism consists of a 30 bp repeated sequence present in 3, 3.5, 4 or 5 copies. The most common variants are the 3-repeat and the 4-repeat alleles, with population frequencies of about 36% and 62%, respectively. The gene variants with more than 4 copies of the repeat sequence are transcribed 2-10 times more efficiently than the shorter alleles (Sabol et al., 1998; Deckert et al., 1999). The short, low activity, variant has been associated with several behavioural deviances and psychiatric disorders, e.g. aggression (Manuck et al., 2000), antisocial alcoholism (Samochowiec et al., 1999), drug abuse (Huang et al., 2004), attention deficit hyperactivity disorder (Lawson et al., 2003b), especially in combination with an unfavourable environment (Caspi et al., 2002; Foley et al., 2004; Nilsson et al., 2007). However, we and others have found that also long alleles are associated with deviant behaviour (Manor et al., 2002; Nilsson et al., 2008).

The effect of altered serotonin levels during early development

It has been shown that alterations in serotonin homeostasis during early neuronal development modulate the fine wiring of brain connections including neuronal growth, synaptogenesis and apoptosis (Gaspar et al., 2003).

Hypothetically, individuals with a low functioning 5-HTT or MAO-A gene could have a different serotonergic milieu during development than individuals with high functioning gene variants (Ansorge et al., 2004). This would result in different effects on the development of the brain and possibly on behaviour and personality later on. Hence, it is not clear whether the response to an actual behaviour is regulated by acute effects of neurotransmission, or if developmental effects of e.g. the 5-HTTLPR affects neurogenesis early in life. Thus, serotonergic induction of brain-derived neurotrophic factor (BDNF) promotes neuroplasticity and BDNF has been shown to be of importance for the antidepressant effect of SSRIs (Alboni et al., 2010; Hodes et al., 2010). Furthermore, administration of SSRI during early brain maturation resulted in abnormal emotional behaviour in adult mice (Ansorge et al., 2004).

In paper V, we aimed at mimicking a situation of less effective MAO-A or 5-HTT during development. Since the MAO-A and 5-HTT polymorphisms (resulting in changed 5-HT levels) do not exist in rodents, we treated pregnant mouse dams with SSRI and investigated the offspring.

Animal models used in this thesis

There is no perfect animal model for psychiatric disorders. Nevertheless, animal models are used to study psychological problems. These studies often focus on some aspect of the disease that seems to be comparable in humans and animals. The use of animal models is critical for understanding the biological mechanisms involved. However, whether the aetiology or neuropathology that result in the human and animal symptoms are similar, is not known. Hence, one should be cautious with translations of animal behavioural studies.

Non-human primates

In paper I, we have used the rhesus monkey as an animal model for alcohol abuse and associated behaviours. Much like humans, non-human primates have complex behaviours and complex social structures. Because most psychopathology involves social functioning, rhesus monkeys are useful in studying such behavioural traits. Furthermore, non-human primates share neuroanatomical properties and are genetically related to humans.

Rhesus monkeys also share numerous features with humans regarding alcohol abuse and associated behaviours (Higley and Bennett, 1999; Barr and Goldman, 2006). For example, rhesus monkeys with excessive alcohol consumption have an impaired social competence, exhibit severe aggression and have reduced CNS serotonin function as measured by low levels of 5-hydroxyindoleacetic acid (5-HIAA) in CSF (Higley et al., 1996; Barr et al., 2004). An advantage to study non-human primates is that alcohol exposure is controlled. Thus, it is possible to study alcohol-naïve animals, while humans that have already been exposed to alcohol will have neurochemical and behavioural differences that are caused by the alcohol use.

Furthermore, it reduces complexity to study personality traits in a non-smoking model since smoking is a confounding factor that has been shown to inhibit MAO-B activity.

Finally, environmental background is very complex and different for all individuals. With an animal model it is possible to control more of the environmental input.

Transgenic mice

Since neuronal wiring or cell body number during development cannot be studied in the human brain, we use a mouse model. Rodents are widely used in research and especially mice have a given role in genetics because the possibility to manipulate them genetically. In paper V, we have used a mouse that has a fluorescent tag introduced into an enhancer region of the *Pet-1* gene (Scott et al., 2005). Pet-1 is a transcription factor that co-localizes with TPH positive neurons and the transcription of which is restricted to serotonergic neurons in the brain preceding the appearance of serotonin by half a day (Hendricks et al., 1999). Hence, with this mouse it is possible to see whole serotonergic neurons before other serotonin-specific traits appear.

Material and methodological considerations

Preparation of plasma and DNA

With regard to paper I, blood was drawn from *vena femoralis* under ketamine anesthesia (15 mg/kg, intra muscular) into tubes with ethylenediaminetetraacetic acid (EDTA). Platelets were counted within 24 hours. Plasma was stored at -80°C and transported to Sweden on dry ice. For paper II, whole blood was transported in EDTA tubes from Dresden University of Technology in Germany and DNA was prepared at BMC in Uppsala using the Qiamp® DNA extraction kit (Qiagen GmdH, Hilden, Germany) blood kit. Blood samples from the TCHAD-study (paper III and IV) were sent in EDTA tubes from Karolinska institutet and were always analyzed within 24 hours. If samples were delayed they were retaken. For genotyping of mice, DNA was prepared from the tail tip.

For estimation of platelet MAO-B activity (paper I, III, IV), platelet-rich plasma was prepared by low speed centrifugation. 0.33µl capillary pipetted platelet rich plasma was dissolved in 10 ml isoton solution and platelet concentration was estimated electronically with a Thromobocunter-C® (coulter Electronics Ltd, UK) or with a Z1™ Coulter Counter® (Beckman coulter Inc. USA) for paper I.

Genotyping and sequencing

Polymerase chain reaction (PCR) was used for genotyping and the different alleles were detected by gel electrophoresis. PCR reactions were performed on a GeneAmp 9700\. For MAO-A, PCR products were ran on 3% agarose gels for 3h, for 5-HTT: 2% 2h, for Pet-1: 1.5% 45min. Ethidium bromide (paper II) or SYBR safe (paper III-VI) was used to detect DNA.

For genotyping of MAO-A VNTR, a 50 µl reaction mixture was used containing 75 ng genomic DNA, PCR buffer (200 mM Tris-HCl, pH 8.4, 500 mM KCl), 1 mM MgCl2, 400 µM dNTP (100 µM each of dATP, dCTP, dTTP, dGTP), 4 pmol of each primer (table 1.), 0.03 % W-1 buffer and 1.5 units Taq-DNA polymerase (Invitrogen™). PCR reactions were performed at the following profile: 95°C for 60 seconds, 35 x (95°C for 60 seconds, 63.5°C for 60 seconds, and 72°C for 90) and finally 72°C for 5 minutes.

For genotyping of 5-HTTLPR, a 40 µl reaction mixture was used containing 75 ng genomic DNA, PCR buffer (200 mM Tris-HCl, pH 8.4, 500 mM KCl), 1 mM $MgCl_2$, 400 µM dNTP, 4 pmol of each primer, DMSO and 1.5

units Taq-DNA polymerase (Roche Diagnostics). PCR reactions were performed at the following profile: 94°C for 60 seconds, 35 x (94°C for 45 seconds, 63°C for 60 seconds, and 72°C for 90).

For detailed PCR protocol used for YFP, see the paper IV.

Table 1. Primer sequences used for the PCRs.

Gene	Primer sequences	PCR product, bp
***h*5-HTT LPR**	F:5'-CAACCTCCCAGCAACTCCCTGTA-3'	458/414
	R:5'-GAGGGACTGAGCTGGACAACCAC-3'	
***h*MAO-A VNTR**	F:5'-ACA GCC TGA CCG TG GAGA AG-3'	294/309/324
	R:5'-GAA CGG ACG CTC CAT TCG GA-3'	339/354/384
YFP	F:5'-GAA CTC CAG CAG GAC CAT GT-3'	219
	R:5'-TAT ATC ATG GCC GAC AAG CA-3'	

In order to confirm the region amplified with PCR, sequence analysis was performed using a BidDye™ Terminator Cycle Sequencing Ready Reaction kit (ABI PRISM™, Perkin elmer, Foster City, CA, USA) with AmpliTaq®DNA polymerase on a ABI PRISM™ 310 Genetic Analyzer, that we had at the department at this time (paper II-IV). Sequenced products were used as references when calling genotypes from the gel electrophoresis (Figure 8).

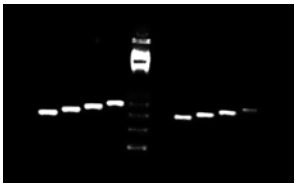


Figure 8. Genotyping performed as in the old days. Gel electrophoresis showing the four most common MAO-A VNTR genotypes.

MAO-B activity measurement

For MAO-B activity measurement, platelet rich plasma (PRP) was prepared from fresh blood by low-speed centrifugation. Platelet concentrations of the plasma samples were estimated electronically and the plasma was stored at -80°C. Catalytic activity of platelet MAO-B was analyzed with C^{14}-labelled β-phenylethylamine (PEA) as substrate. Before analysis, the samples of platelet rich plasma were thawed and sonicated at 0°C. C^{14}β-PEA (0.1 mM, 0.5 μCi/ml) in 0.1 M sodium phosphate buffer was added to the PRP and the reaction mixture was incubated at 37°C for 4 minutes. The reaction was terminated by the addition of 1 M HCL. Thereafter, the radioactive aldehyde product formed was extracted by toluene: ethylacetate (1:1). The samples

were then centrifuged at room temperature. The aldehyde product was transferred into vials with scintillation fluid and the amount of radioactive aldehyde product subsequently quantified by scintillation analysis. Enzyme activity is expressed as nmol of substrate oxidized per 10^{10} platelets per minute. All samples were analyzed blindly and in duplicate.

Preparation of mouse brains

Mice pups were taken at postnatal day zero (P0) and the brains were dissected and fixed in formaldehyde for 4 hours. After infusion in 20% sucrose for 24 hours, the brains were frozen in tissue tec and stored at -80°C. Brains were coronally sectioned and mounted with Fluoroshield containing DAPI (4,6-Diamidino-2-phenylindole dihydrochloride, Sigma-Aldrich®). The slides were analyzed using a confocal microscope system. Z-stack pictures were taken on the lateral wings dorsal raphe nuclei (lwDR) and YFP fluorescent serotonergic cell bodies of the nuclei were counted with the ImageJ 1.43u software (NIH, USA).

Animals and treatment paradigms

Rhesus macaques

Rhesus macaques (*macaca mulatta*) originally from Morgan Island outside the east coast of USA were used in this work. The monkeys were in the seventies moved to Poolesville, Maryland where they are housed to mimic the free-living conditions as much as possible. Hence, monkeys are group-housed and have access to outdoor playfields.

In paper I, a total of 78 rhesus monkeys were used (31 males, 47 females). However, in total 200 animals were assessed during my time in Poolesville. The monkeys weighed between five and ten kilogram depending on age and sex (mean: 5.8±1.2). The monkeys used in this study were young adults, 3.5-5 years old.

All handling of the monkeys was approved by the Institutional Animal Care and Use Committee of the National Institute on Alcohol Abuse and Alcoholism (NIAAA) and National Institute of Child Health and Human Development, National Institute of Health (NIH), USA. Ethics number LCSDH-13 and LCSDH-09.

Alcohol was administered intravenously into *vena saphena magna* and animals were placed in a cage (about 6x6m) that was prepared with a thick layer of straw in order to protect the animals from potential stumble injuries. Animal intoxication was rated during a 30 minutes period. Three observers

subjectively rated the monkey's general degree of intoxication on a scale 1-5. Specific behaviours indicating of pharmacological effect (e.g. sway, fall, unsucessful jumps) were also recorded. Thereafter incidents of provoked aggressive behaviour were recorded for five minutes for each monkey. Procedures known to elicit aggression in macaques were used (Barr et al., 2004): An investigator that was unknown to the monkeys entered the room and maintained eye contact with the monkey. For the last 2.5 minutes the investigator also imitated a macaque open-mouth threat once every 30 seconds. The human intruder wore big capture gloves as protection. Frequencies of aggressive behaviours were recorded by the observers with special behaviour scoring computers.

Approximately two months after the intoxication procedure, animals went through a standardised alcohol-training period of two weeks whereafter the oral alcohol consumption experiment started. Animals had free access to an aspartame-sweetened 8.4% ethanol solution, an aspartame vehicle, and home-cage water during two weeks.

Monkeys were anesthetized with ketamine (15mg/kg, intramuscular) before any invasive intervention. This was forced to be a very hasty procedure in order to have time to put down all animals before the first ones woke up again. Animals were anesthetized for about 20 minutes during which time blood was drawn and the animals were weighed.

CSF sampling from *cisterna magna* was only performed by specially trained personnel who were certified for the procedure. Animals were administered pain-killers in connection with this procedure.

ePet-EYFP mice

Mice were kept at the Biomedical Center (BMC) in Uppsala and were housed according to the Uppsala ethics committé agreements. ePet-EYFP mice were shipped from Prof. Deneris, USA and mated with wild type C57Bl/6 female mice. Females were housed two and two. For mating, two females and one mating male were put in a new cage and stayed together throughout the experiment. Offspring were weaned at day 21 and same-sexed litters were kept together 2-5 per cage. Enrichment in the cages was two wooden houses, a tunnel and tissue paper. Food and water were constantly available. Female mice had one litter before the experiment started. Treatment was performed with saline (0.9%) or fluoxetine (10mg/kg) sub cutaneously every day during pregnancy. Injection site was varied between four sites. For detailed treatment procedure see paper V.

Clinical series and assessments

EEG study group

In paper II, the subjects were healthy male students aged 19-27 years (mean = 23.3; SD=1.9). Inclusion criteria were selected in order to minmize confounding factors that are related to serotonergic neurotransmission or auditory-evoked potentials. Thus, the individuals were non-smoking Caucasian without any psychiatric or neurological disorder. Individuals using centrally acting medications or drugs were also excluded. Ninety-one subjects met the inclusion criteria and participated in the study.

EEG recordings were performed in an acoustically and electrically shielded room at Industrial Acoustics Company (IAC), Niederkrüchten, Germany.

EEG methodology is described in more detail in paper II.

TCHAD study group

The subjects used in paper III and IV were recruited from the population-based twin study of child and adolescent development (the TCHAD-study) (Lichtenstein et al., 2007) and included twins living in Stockholm and born between May 1985 and December 1986. The sample collection procedure is described in figure 9. Complete data was obtained from 247 individuals, genetic associations were carried out on 197 individuals. Age ranged from 14.6 to 16.7 years (mean 16 years). The children and parents were invited to an assessment including a structured clinical interview using a test that assesses the symptoms according to DSM-IV: The Kiddie Schedule for Affective Disorders and Schizophrenia for School-Aged Children-Present and Lifetime Version (K-SADS-PL). In the interview procedure, the interview was first performed with the child alone and thereafter with at least one parent. The clinical interviewer (MD Kerstin Malmberg) then summarized the information from the parent (about the child) with the information from the child and classified the symptoms as not present, possible, or certain. Symptoms for the following diagnoses were scored: ADHD inattentive type, ADHD hyperactive type, ADHD combined type, ODD, CD and disruptive behaviour (presence of ODD or CD). For a more detailed description see paper III.

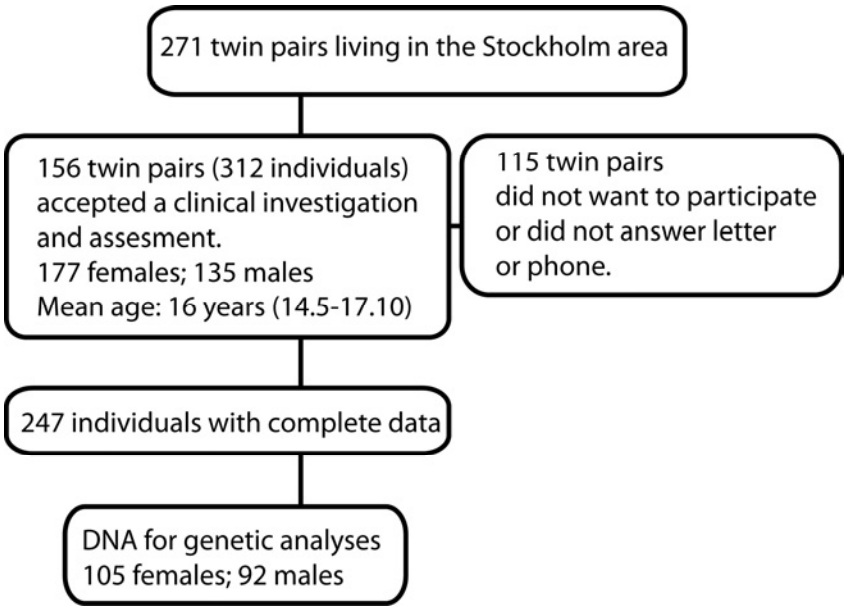


Figure 9. Sample selection procedure for The twin study of child and adolescent development (TCHAD) (paper III-IV).

Results and Discussion

IAEP and platelet MAO-B activity as indicators of central 5HT neurotransmission and 5HT-influenced behaviours (paper I, III, IV)

With regard to platelet MAO-B activity, we could, in paper I, replicate results of low levels of monoamine metabolites in CSF of individuals with low platelet MAO-B activity (Oreland et al., 1981; Von Knorring et al., 1986; Fahlke et al., 2002). CSF metabolites are often used as a measure of neurotransmitter turnover although the relation to levels in the brain can be questioned. However, in paper I CSF was drawn locally from cisterna magna, which should increase the predictive value. Hence, we hypothesise that the link between peripheral MAO-B activity and central serotonin metabolites (5-HIAA) and dopamine metabolite (HVA) are in favour of a relation between platelet MAO-B activity and central monoamine system activity. We could also replicate the findings from 2002 (Fahlke et al., 2002) with regard to low platelet MAO-B activity in monkeys that voluntarily consumed higher amounts of alcohol.

Furthermore, we could link low platelet MAO-B activity to low level of response (LR) to a given amount of alcohol. Low level of response to alcohol has been suggested to increase the risk of alcohol use disorders and to be an intermediate phenotype for alcohol problems (Schuckit et al., 2004). The non-human primate animal model is especially well suited to study LR since we can be sure that the monkeys were alcohol-naïve when exposed to alcohol. Low platelet MAO-B activity was also associated with alcohol-elicited provoked aggression. This could be interpreted as individual differences in behavioural disinhibition of alcohol, which is generally considered to be a serotonin-related mechanism. It is also in line with data of stronger reactions in terms of evoked potentials, in individuals with low serotonergic tone (Hegerl and Juckel, 1993) as well as our notion of an inverse relationship between platelet MAO-B activity and intensity dependence of auditory evoked potentials (IAEP) (Hensch, Wargelius et al., unpublished data).

Finally, in a clinical sample, low platelet MAO-B activity was associated with high scores of oppositional defiant disorder (ODD) and disruptive behaviour disorder (DBD) in girls but not in boys (paper III). In other studies, the number of girls has been very low or only boys have been studied. Nevertheless, a trend towards an association of low platelet MAO-B activity and ADHD in girls has been reported (Coccini et al., 2009). In contrast to the

relatively limited results of MAO-B activity in paper III, taking also the genotype of variations in the serotonin transporter (5-HTTLPR) or in the (MAOA-VNTR) genes into account, considerably changed the picture. Thus, when individuals with either high or low platelet MAO-B activity where further divided into subgroups with regard to 5-HTTLPR genotype, boys with a short 5-HTTLPR variant in combination with high MAO-B activity in platelets scored high on symptoms of ADHD combined type, ODD, CD and DBD. Furthermore, when the same analysis was performed with regard to the MAOA-VNTR polymorphism, a similar pattern evolved. The association of high MAO-B activity could be explained by a U-shaped relationship between MAO-B activity and behaviour, with extreme levels (high or low) being associated with different dimensions of deviant behaviour (Af Klinteberg et al., 1987). An hypothesis of a functional mechanism might relate to reports of low levels of the MAO-B enzyme substrates phenylethylamine or dopamine in the CNS as a result of high CNS MAO-B activity in ADHD (Baker et al., 1991).

With regard to the endophenotype, IAEP, we report an association between the long variant of 5-HTTLPR and high IAEP in healthy males. The study sample was carefully chosen in order to avoid known confounding factors such as sex, smoking, psychiatric disorder and medications. There were only two studies on this topic at the time of our study, however contradictory, and we could confirm a relation between 5-HTTLPR and IAEP, which is in line with the result of Strobel and colleges (Strobel et al., 2003) but opposed to the result of Gallinat and colleges (Gallinat et al., 2003). In a recent, fourth, study, no influence of 5-HTTLPR on IAEP was found. The study sample in that study, however, was sex-mixed, ethnically mixed and included only 14 individuals (Simmons et al., 2011). Thus, the different findings may be explained by differences in methodology and study samples.

In summary, the studies in this thesis support both platelet MAO-B activity and IAEP as intermediate phenotypes for central monoamine system activity and associated behaviours. However, the functional link remains to be explained. Moreover, complexity is added in that it may be relevant to take other genes into account when using markers such as platelet MAO-B activity.

5HT-related candidate genes and the role of 5HT during early development (paper II-V)

The short MAOA-VNTR allele was associated with disruptive behaviour in boys. This is in line with data on conduct disorder in boys (Lawson et al., 2003a) and with a recent study where individuals with the short MAOA-

VNTR and a history of childhood sexual abuse reported more symptoms of conduct disorder (Derringer et al., 2010).

In combination with platelet MAO-B activity, the short MAOA-VNTR allele was associated with symptoms of ADHD combined type in boys. This study seems to be the first of its kind and suggests that it may be important to consider combinations of genes and biological markers.

With regard to 5-HTTLPR, the long allele was associated with stronger IAEP in males as described above. The heterozygous 5-HTTLPR genotype was associated with conduct disorder in both boys and girls. Such molecular heterosis, i.e. that the heterozygous genotype differ, has been reported with regard to 5-HTT availability (Van Dyck et al., 2005) and serotonin transporter binding in cocaine users (Little et al., 1998). Heterosis has also been observed in some previous studies (Comings and Macmurray, 2000) as well as in a recent study of impulsiveness (delay aversion) in children with ADHD (Sonuga-Barke et al., 2011). In combination with platelet MAO-B activity, presence of the short 5-HTTLPR allele was associated with symptoms of ADHD combined type, ODD, conduct disorder and DBD in boys. Two previous studies have investigated the 5-HTTLPR in combination with platelet MAO-B activity, however, other phenotypes were tested in those studies. Thus, in the study by Akkermann and colleagues, girls with long 5-HTTLPR alleles and high platelet MAO-B activity had higher scores of drive for thinness. In the study by Paaver and colleagues, individuals with a short 5-HTTLPR allele and low platelet MAO-B activity scored higher on impulsiveness (Paaver et al., 2007; Akkermann et al., 2008). However, it is difficult to compare those studies with each other and with our study because different populations were used and different phenotypes were measured.

Our preliminary data about the role of 5-HT during development, showed that there were more serotonergic cell bodies in the dorsal raphe of offspring from SSRI treated mouse dams as compared to offspring from saline treated dams. Offspring from non-treated dams showed the highest number of cell bodies but due to the small group size (n=2) it was not possible to rely on data from this group. It could be speculated that the stress caused by the injections affect development of serotonergic cell bodies and that these effects were in part counteracted by fluoxetine. In line with our results are data from rodents that have been directly exposed to SSRI. Thus, Zhou and colleagues report of increased density of 5-HT axons in the frontal cortex (Zhou

et al., 2006). However, our results on cell body number in the offspring of SSRI treated mice need to be validated with larger groups.

In summary, genotype data alone was not conclusive but pointed towards an involvement of the serotonergic system in ADHD. Combination of genotype with the monoaminergic marker platelet MAO-B activity suggest that effects of the MAO-A VNTR or 5-HTTLPR are moderated by platelet MAO-B activity. Validation of the data is needed in clinical samples of ADHD.

At present, prediction of genotype effects on protein expression and behavioural phenotype is not easily made. Complexity is added in terms of e.g. developmental events, environmental factors acting on gene expression as well as interactions between genes. Thus, the actual function of the candidate polymorphisms in vivo is not clear, and the final result with regard to the size of CNS monoamine systems and wiring patterns that is seen in individuals with a certain genotype is not known. The effects of subscription of antidepressants to pregnant women may need to be evaluated in more detail with regard to molecular mechanisms in the foetus. Thus, increased prenatal levels of 5-HT as a result of SSRI treatment may affect the development of the early brain.

Summary of papers

Paper I

In this study, platelet MAO-B activity was analysed in 78 rhesus macaques (*macaca mulatta*), and its relation to voluntary alcohol intake and behaviours after intravenous alcohol administration was observed.

Monkeys with low platelet MAO-B activity had low levels of 5-hydroxyindole acetic acid (5-HIAA) in cerebrospinal fluid ($t=-2.39$; $p<0.05$) and showed excessive aggression after alcohol administration ($t=4.55$; $p<0.001$). A novel finding was that animals with low platelet MAO-B activity showed less intoxication following alcohol administration ($t=-2.10$; $p<.0.05$). The monkeys also voluntarily consumed more alcohol ($t=2.15$; $p<0.05$).

We here replicate results from studies on both humans and non-human primates, showing the utility of platelet MAO-B as a marker for risk behaviours and alcohol abuse. Furthermore, we link platelet MAO activity to alcohol sensitivity.

Paper II

Intensity dependence of auditory-evoked potentials (IAEP) has been suggested as an indicator of central serotonergic neurotransmission. In two studies (Gallinat et al., 2003; Strobel et al., 2003) a possible association of IAEP with 5-HTTLPR, has been investigated. However with conflicting results that requires further evaluation.

In this paper we investigated the effect of 5-HTTLPR on IAEP in a healthy male student sample (N=91; age=23 years, SD=1.9) that was homogenous for most significant confounding variables.

A stronger IAEP was shown in l/l individuals, irrespective of the method of IAEP parameterization ($F_{1,88}$=9.663; p=0.003). This also held at re-test after 3 weeks in a subsample (N=18).

Given the successful replication of the paper by Strobel et al, several possible reasons for conflicting results with regard to the paper by Gallinat et al are discussed. It is argued that the most significant difference between Gallinat et al on the one hand, and Strobel et al and this study on the other, is that different intensity ranges were used which impact IAEP.

Paper III

The heritability estimate of attention deficit hyperactivity disorder (ADHD) is 75%. In the present report we studied serotonergic candidate genes in relation to ADHD symptoms in a population-based sample. Platelet MAO-B activity was also measured in the subjects. The study group consisted of 156 adolescent twin pairs, i.e. 312 individuals. ADHD symptoms were scored with a structured clinical interview of both the twin and a parent using Kiddie Schedule for Affective Disorders and Schizophrenia for School-Age Children-Present and Lifetime Version (KSADS-PL).

There was an association in girls between low platelet MAO-B activity and symptoms of Oppositional Defiant Disorder (ODD). In both girls and boys, there was an association between the heterozygous long/short 5-HTTLPR genotype and symptoms of conduct disorder. In boys, hemizygosity for the short MAOA-VNTR allele was associated with disruptive behaviour.

In summary, the results of the present study were not conclusive but pointed towards an involvement of the serotonergic system in ADHD.

Paper IV

In paper four, we have studied ADHD symptoms with regard to the combination of platelet MAO-B activity and MAOA-VNTR or 5-HTTLPR genotype. Hence this study is a re-examination of the material in paper III.

When combining the genetic factors a much more stringent picture evolved. Presence of short 5-HTTLPR or short MAOA-VNTR in combination

with high levels of platelet MAO-B enzyme activity was associated with higher scores of ADHD like problems ($p<0.001$; $p<0.01$).

This re-examination of ADHD scores in a non-clinical sample suggests that effects of the MAOA-VNTR or 5-HTTLPR are moderated by platelet MAO-B activity.

Paper V

The ultimate aim of the present paper was to explore some of the effects of maternal intake of a selective serotonin reuptake inhibitor (SSRI), as well as increasing the knowledge about the biomedical background of personality and psychiatric disorders. The acute effect of SSRI results in increased levels of serotonin in the brain. Here, we have studied if the number of serotonergic cell bodies in the lateral wings of dorsal raphe nucleus is altered by high prenatal levels of serotonin in the offspring of SSRI treated mothers.

Pregnant female mice were divided into three groups. One group was treated with SSRI during the whole period of gestation, while the second group was treated with saline as a control. The third group remained un-treated. Brains from mice pups were taken at postnatal day zero.

Preliminary data show that there were more serotonergic cell bodies in offspring from SSRI treated dams as compared to offspring from saline treated dams ($p<0.05$; $t=-2.13$). Offspring from non-treated dams had the highest number of cell bodies but due to the small group size ($n=2$) it is not yet possible to rely on data from this group. It could be speculated that the stress caused by the injections affect development of serotonergic cell bodies and that these effects are in part counteracted by fluoxetine.

General summary of papers

- The studies in this thesis support both platelet MAO-B activity and IAEP as intermediate phenotypes for central monoamine system and associated behaviours. Platelet MAO-B activity is for the first time linked to low level of response to alcohol, a promising finding that should be replicated.
- We further suggest that the MAO-A VNTR and 5-HTTLPR polymorphisms are involved in the aetiology of ADHD related behaviours, especially if combined with platelet MAO-B activity.
- Finally, preliminary data suggests that administration of SSRI early in development could affect serotonergic cell number.

Reflections and Perspectives

The association studies that this thesis is based on were initiated in 2004. At that time behavioural genetics had moved from traditional linkage studies of single-gene disorders towards finding genes for complex traits. Avshalom Caspi's study initiated a golden age for candidate gene studies and threw light on the possibility to incorporate environmental factors into the models. Since then, this field has evolved quickly and although the candidate gene approach is still valid, the new era of huge genome wide association studies is now exploding. Although we have just started to see the results of these efforts, it would not be surprising if also this approach will be proven to be a disappointment. Thus, we can already see a movement towards genome wide *sequencing* of families.

In this perspective, the great hopes that accompanied the candidate gene studies may seem dashed. However, although it is unlikely that anything new will come from candidate gene studies, they have brought to us the understanding of psychiatric disorders as being polygenic.

Furthermore, it has come clear that prediction of genotype effects on behavioural phenotype is not easily made. Environmental factors acting on gene expression as well as interactions between genes need to be considered. In addition, environmental factors may have different effects depending of when in a person's life they occur and depending on the person's genetic constitution.

The focus of this thesis is on serotonin-related genes and biomarkers, however many other players are also involved in personality and psychiatric disorders. For example, there are other enzymes that break down monoamines, and there are enzymes that are important in the synthesis of neurotransmitters or in the synthesis of receptors and transporters. These proteins all together are important in the regulation of neurotransmitter amount and availability in the synapse or in the vesicles.

Furthermore, critics could be raised to the expression studies that are done *in vitro*. Cell culture is a totally different system than the human body. In addition, many neurotransmitter systems have dual or mixed effects on the behavioural response. This may include having opposite effects in different areas of the brain or exerting opposing effects depending on which neurotransmitter receptor-subtype that is activated. Finally, neurotransmitter systems do not act in isolation.

It seems obvious that the field of psychiatric genetics will become increasingly more important with advances with regard to approaches and methodology. Though, so far, studies often show inconsistencies and failures in replication. However, it is probably unrealistic to expect an unambiguous answer and outcome when dealing with complex systems like cognition and higher brain functions, behaviour and personality. There are multiple sources to why different studies get divergent results. The complexity of what is being studied is of course a major cause. Furthermore, there are sampling issues. Thus, every study includes different populations, and small differences in these can have crucial effects. Ethnicity, age, living area, interviewer and low power (due to e.g. low prevalence of adversity/maltreatment) are all examples of where measurements can be at variance. It is in fact quite amazing that we get replications at all within this field.

For the nearest future it is also necessary to take copy number variations (CNVs) into account. Thus, CNVs have for instance recently been found in ADHD and autism (Lesch et al., 2011). Hence, as genetic technology advances, the field of psychiatric genetics changes track or adds on complexity. Epigenetics – the constant dialogue between environment and genetics - is also an interesting issue that probably will add new dimensions to future genetic studies.

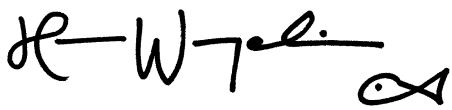
I am convinced that the serotonergic system is strongly involved in psychopathology and that there are certain personality traits that carry higher risk. But how do we best study this? Association studies have played their most important role. It will become interesting to look closer on the characterization of the risk behaviours that have been associated with specific genes. Since the signal in the genetic studies is weak, there is a possibility that we look at the wrong things. Perhaps phenotyping should move from the categories of DSM towards a more dimensional approach without focusing on disease-non disease. Thus: ***We can have the best genetic tools in the world, but without getting the phenotype right, it is to no help.***

With regard to genetics, we do have evidence that genes *are* involved and to some extent also evidence of *which* systems that seem to be important. However, a statistical interaction between a genotype and an environmental risk factor requires further research to reveal the biological machinery involved in the interaction. Hence, the next step will be to look at *how* this genetic component acts. My hope is that statistical associations involving serotonergic key players, as reported here, will represent a fruitful step for developmental neuroscience research into the underlying causal mechanisms involved in the aetiology of psychopathology. Such knowledge could make it possible to base diagnostic criteria on aetiology rather than symptoms. Furthermore, it will add to our understanding of the molecular mechanisms for

how psychopharmacological drugs work and may allow for tailor made medicine. Thus, it is today almost impossible to predict which patient that will respond to a certain treatment.

Finally, it is not to forget that there are positive sides of traits in e.g. ADHD, such as high creativity and energy, as well as curiosity for finding new solutions.
And, the complexity of psychiatric disorders implies that genes are not destiny. However, genes do provide the recipe that the environment works with.

Svårigheten ligger kanske främst i att fånga det man vill fånga, att mäta rätt saker, att veta vad som är rätt saker att mäta och att veta att det är dem man mäter.



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