

INFLUENCE OF SEROTONIN-RELATED GENES ON BEHAVIOR AND BODY WEIGHT

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Abstract

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Rationale: The neurotransmitter serotonin has been implicated in the regulation of normal behaviors, including food intake, and attributed importance for a variety of common psychiatric conditions, including major depression, suicidal behavior, eating disorders and premenstrual dysphoria. The purpose of these studies was to explore the possible influence of genetic variation in serotonin-related genes on a) body weight, b) binding capacity of the serotonin transporter in the brain of suicide attempters and c) a disorder for which numerous findings suggest serotonin to play a key role, *i.e.*, premenstrual dysphoria. **Observations:** 1) An amino acid substitution (Cys23Ser) in the gene encoding the serotonin receptor 5-HT_{2C} (*HTR2C*) was associated with weight loss in teenage girls. 2) Supporting the above-mentioned finding, the Cys23Ser substitution in the *HTR2C* was associated with low body weight also in a middle-aged female cohort recruited from the general population; in addition, influences on weight of a SNP in the promoter region of *HTR2C*, as well of a polymorphism, 5-HTTLPR, in the gene encoding the serotonin transporter, *SLC6A4*, were found. 3) Both the 5-HTTLPR polymorphism and a variable number of tandem repeats (VNTR) in intron 2 (STin2) of *SLC6A4* were shown to be associated with binding capacity of the serotonin transporter in brains of suicide attempters. 4) Genes coding for the serotonin receptor subunit 5-HT_{3B} and a transcription factor involved in the development and differentiation of serotonergic neurons, *GATA2*, were associated with premenstrual dysphoria. **Conclusions:** Our results add to the growing literature suggesting variations in serotonin-related genes to be of importance for inter-individual differences in behavior.

Key words: serotonin – genes – polymorphism – *HTR2C* – *SLC6A4*– *HTR3B* – *GATA2* – body weight – anorexia nervosa – suicide – premenstrual dysphoria

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This thesis is based on the following papers, which will be referred to in the text by their roman numerals:

- I. Westberg L, **Bah J**, Råstam M, Gillberg C, Wentz E, Melke J, Hellstrand M, Eriksson E. Association between a polymorphism of the 5-HT_{2C} receptor and weight loss in teenage girls. *Neuropsychopharmacology*, Jun;26(6):789-93, 2002
- II. **Bah J**, Westberg L, Baghaei F, Henningsson S, Rosmond R, Melke J, Holm G and Eriksson E. Further exploration of the possible influence of polymorphisms in HTR_{2C} on body weight. *Submitted*, 2008
- III. **Bah J**, Lindström M, Westberg L, Mannerås L, Ryding E, Henningsson S, Melke J, Rosén I, Träskman-Bendz L and Eriksson E. Serotonin transporter gene polymorphisms: Effect on serotonin transporter availability in the brain of suicide attempters. *Psychiatry Research*. Apr 15;162(3):221-9, 2008
- IV. **Bah J**, Suchankova P, Ekman A, Eriksson O, Henningsson S, Holm G, Landén M, Nilsson LG, Nilsson S, Nissbrandt H, Westberg L, Melke J, Eriksson E. A study of 19 serotonin-related genes reveals association between premenstrual dysphoria and genes encoding the GATA2 transcription factor and the 5-HT_{3B} receptor subunit. *Manuscript*, 2008

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LIST OF ABBREVIATIONS

3'-UTR	3'- untranslated region
5'-UTR	5'- untranslated region
5-HIAA	5- hydroxyindole acetic acid
5-HT	5- hydroxytryptamine
5-HTP	5-hydroxytryptophan
5-HTT	Serotonin transporter
5-HTTLPR	Serotonin transporter linked polymorphic region
AADC	Aromatic amino acid decarboxylase
AN	Anorexia nervosa
ATD	Acute tryptophan depletion
BDNF	Brain derived neurotrophic factor
BMI	Body mass index
BN	Bulimia nervosa
CSF	Cerebrospinal fluid
CNS	Central nervous system
DNA	Deoxyribonucleic acid
DSM	Diagnostic and statistic manual of mental disorders
DZ	Dizygotic
FGF	Fibroblast growth factor
GAD	Generalized anxiety disorder
MAO	Monoamine oxidase
MZ	Monozygotic
OCD	Obsessive compulsive disorder
PET	Positron emission tomography
PMD	Premenstrual dysphoria
PMDD	Premenstrual dysphoric disorder
PMS	Premenstrual syndrome
RFLP	Restriction fragment length polymorphism
RNA	Ribonucleic acid
SHH	Sonic hedgehog
SNP	Single nucleotide polymorphism
SPECT	Single photon emission computed tomography
SRI	Serotonin reuptake inhibitor
SSRI	Selective serotonin reuptake inhibitor
STin2	Serotonin transporter intron 2
TCA	Tricyclic antidepressant
TPH	Tryptophan hydroxylase
VMAT	Vesicular monoamine transporter
VNTR	Variable number of tandem repeats

PREFACE

What makes us human?

Humans often take pride in being more refined than other animals. We generally consider ourselves as capable of confining our inner urges, such as anger, impulsivity, appetite, sadness, tiredness and lust, and that this is something one has to do if wanting to be considered as civilized. It is difficult to grasp that all these behaviors, and the control over them, are the result of an exquisitely organized network of small chemical substances in our body and in our brain. Nevertheless, sometimes the machinery fails us, and instead of harmony, balance and control, chaos and destruction take over.

We all differ with respect to our proneness for various emotions, and our ability to control our behavior. Although the reasons for such differences have been the subject of an intensive and long-lasting debate, today few would deny that inter-individual differences in our genes are of significant importance for human diversity in mood and behavior. To pinpoint the precise role of specific genes for various aspects of behavior however has turned out to be extremely difficult.

The objective of this thesis has been to investigate how and if genetic variation in serotonin-related genes is involved in weight regulation and also in the pathophysiology of suicide and premenstrual dysphoria. Three very different conditions, but all connected by a small molecule called serotonin.

INTRODUCTION

Serotonin

Background

In the 1930's, Erspamer and Vialli discovered a small, vasoconstricting substance in entero-chromaffin cells from the gut and named it *enteramine* (Vialli and Erspamer, 1937). Little did they know what role this substance would come to play for our understanding of the human mind and behavior, and for the development of treatments of psychiatric disorders. A decade after their discovery of enteramine, the work of Page, Rapport and Green led to the identification of a substance they

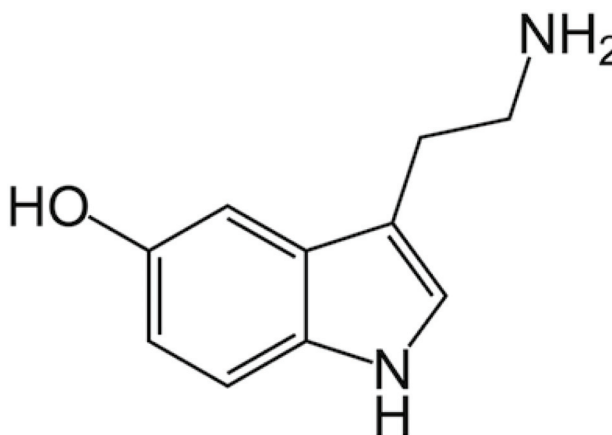


Figure 1. Molecular structure of serotonin

called serotonin, due to the fact that it was found in blood (*serum*) and had vasoconstricting properties (*tonin*) (Rapport *et al.*, 1948). A few years later, the young PhD student Betty Twarog concluded that enteramine and serotonin were in fact the same molecule, and additionally showed that it was present in the human brain (Twarog and Page, 1953). The idea that serotonin possibly could be involved in the regulation of human behavior, as well as in the pathophysiology of psychiatric disorders, initially arose from a study where the psychoactive drug, lysergic acid diethylamide (LSD), was found to exert agonistic effects on 5-HT receptors in peripheral tissue (Gaddum, 1953). Subsequently, reports on the presence of serotonergic nerve terminals in most parts of the central nervous system (CNS) (Amin *et al.*, 1954) as well on the localization of serotonin producing cell bodies to the raphe nuclei followed (Dahlstrom and Fuxe, 1964), and so the story of serotonin as an important brain neurotransmitter begun.

Synthesis and metabolism of serotonin

Serotonin (*5-hydroxytryptamine*, *5-HT*) (Fig 1) is an ancient monoamine, found in almost all organisms from plants to more evolved animals. In the CNS, almost all of the serotonin-producing neurons have their cell bodies located in the raphe nuclei, situated in the midline of the brain stem. They are not many in number, but innervate almost all of the CNS with widespread axons.

Tryptophan is a precursor to serotonin and, as an essential amino acid, assimilated from our diet. Tryptophan is transported from the blood into the brain through a specific amino acid transporter, for which it has to compete with other large, uncharged amino acids. The levels of tryptophan and that of competing amino acids in the blood affect the production rate of serotonin in the brain, and manipulation of this relationship has been demonstrated to affect a variety of behaviors and disorders (see below).

In the neuron, tryptophan is converted by the enzyme *tryptophan hydroxylase* (TPH) into 5-hydroxytryptophan (5-HTP), which in turn is converted into serotonin by the *aromatic aminoacid decarboxylase* (AADC) (Fig 2). In conjunction with the availability of tryptophan, the activity of TPH is believed to be the rate-limiting step in the serotonin synthesis. TPH exists in two isoforms TPH-1 and TPH-2. TPH-2 appears to be responsible for the neuronal serotonin synthesis in the adult organism (Walther *et al.*, 2003) while TPH-1 primarily is expressed in the periphery. However, TPH-1 has been shown to be expressed in the brain during the development, suggesting a role also of this isoform in the CNS (Nakamura and Hasegawa, 2007).

Following synthesis, serotonin is transported into vesicles by the *vesicular monoamine transporter* (VMAT) and stored in the neural endings before being released into the

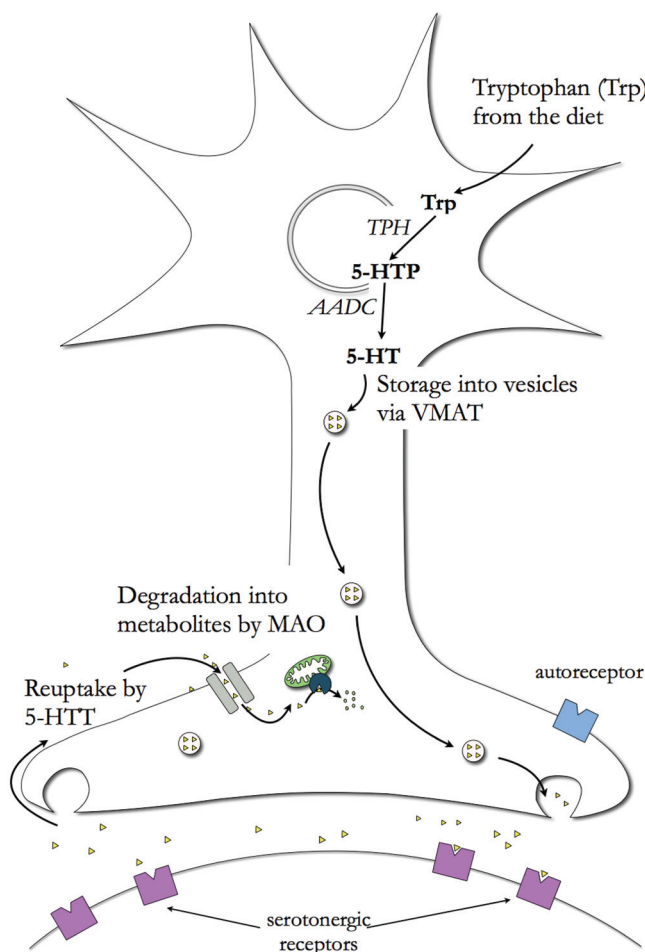


Figure 2. Overview of the serotonergic synthesis, metabolism and synapse

TPH= Tryptophan hydroxylase
 AADC= Aromatic amino acid decarboxylase
 5-HTP=5-hydroxytryptophan,
 5-HT=Serotonin
 VMAT= Vesicular monoamine transporter
 5-HTT= Serotonin transporter
 MAO=Monoamine oxidase
 5-HIAA= 5-hydroxyindole acetic acid

synaptic cleft where it exerts its function by activating designated receptors (see below). The *serotonin transporter* (5-HTT) removes serotonin from the synaptic cleft by transporting it back into the nerve terminal. This transporting process is energy-driven and sodium-dependent.

Serotonin is degraded into its metabolite, *5-hydroxyindole acetic acid* (5-HIAA), by an enzyme called *monoamine oxidase* (MAO). MAO exists in two isoforms – A and B – of which MAO-A is more important for serotonin degradation as compared to MAO-B. Even so, the subtype that is found in the serotonergic neurons is MAO-B (Richards *et al.*, 1992, Thorpe *et al.*, 1987). MAO-B is also expressed in platelets enabling the use of thrombocyte MAO activity as an indirect marker of serotonergic function in relation to different aspects of human behavior (Oreland *et al.*, 2004). 5-HIAA is excreted via the urine and can be measured in both cerebrospinal fluid (CSF) and urine in order to assess central and peripheral serotonergic turnover, respectively. In the pineal gland serotonin acts as a precursor in the two-step synthesis of *melatonin*, a hormone which among other functions is involved in the regulation of the sleep/wake cycle (Hardeland *et al.*, 2006).

Notably, only a small fraction of the total amount of serotonin is present in the CNS (1-2%). The main part of serotonin is stored in enterochromaffin cells in the gastrointestinal tract, and considerable amounts are also found in platelets and mast cells.

Serotonergic receptors

A large number of receptors mediate the effects of the released serotonin. So far as many as 15 subtypes have been characterized (Bockaert *et al.*, 2006). Depending on ligand affinity, molecular structure and intracellular transduction mechanism, the receptor subtypes are divided into seven classes, 5-HT₁- 5-HT₇. All of them, with the exception of the 5-HT₃ receptor, belong to the family of G-protein coupled receptors (Hoyer *et al.*, 2002). The 5-HT₃ receptors are ligand-gated ion channels, and therefore display a very different mode of action compared to the other receptors. Apart from being postsynaptic, the 5-HT_{1A}, 5-HT_{1B} and 5-HT_{1D} receptors also act as *autoreceptors* – 5-HT_{1A} located in the somatodendritic region and 5-HT_{1B/D} in the nerve terminals – exerting inhibitory effects on the serotonergic transmission (Hoyer *et al.*, 2002).

Adding to the diversity resulting from the large number of different receptor subtypes, some of the receptors may also undergo posttranslational modifications. For example the 5-HT_{2C} receptor has been shown to be RNA-edited (Burns *et al.*, 1997), and 5-HT_{1B} and 5-HT_{1D} receptors have been demonstrated to be able to dimerize (Xie *et al.*, 1999). The great variety of receptors may be of relevance for the fact that serotonin is involved in so many different aspects of human behavior.

Serotonergic neurogenesis

Serotonin is one of the first neurotransmitters to appear during the development of the CNS. As early as in gestation week 5 or 6, the first serotonergic neurons emerge (Sundstrom *et al.*, 1993). The development of the serotonergic neurons is regulated by an intricate network of transcription factors, as displayed in Figure 3.

The developmental process can be divided into three phases: 1) the specification of serotonergic destiny of the cells, 2) the migration of the cells to the correct location in the CNS, and 3) the expression of proteins specifying the serotonergic phenotype of the cell. Initiated by the secretion of the growth factors sonic hedgehog (SHH), fibroblast growth factor (FGF) 4 and FGF8 from the neural floor plate, a concentration gradient that permits the formation of serotonergic precursor cells in a restricted area is created (Ye *et al.*, 1998). SHH also induces the expression of two homeodomain proteins, NKX2-2 and NKX6-1, which both interact to activate the transcription factors GATA2 and GATA3, which in turn are responsible for the specification and localization of the dorsal and caudal raphe nuclei, respectively (Craven *et al.*, 2004). GATA2 subsequently induces the expression of transcription factor Pet-1 (in humans, FEV) and the homeodomain protein LMX1B which are sufficient for the production of a number of proteins that are responsible for the

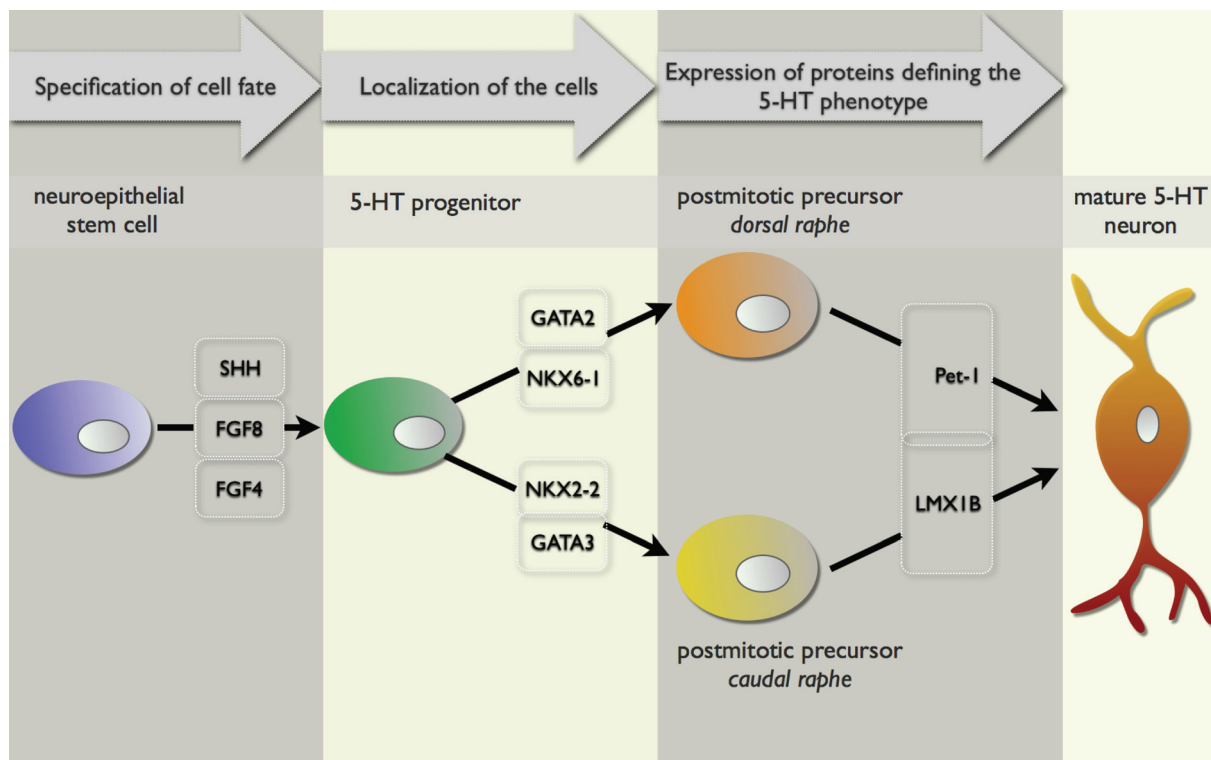


Figure 3. Proposed model of the development and differentiation of the serotonergic neurons and overview of the transcription and growth factors demonstrated to participate in this process. (Alenina *et al.*, 2006, Craven *et al.*, 2004)

synthesis and storage of serotonin, such as TPH, 5-HTT, AADC and VMAT (Cheng *et al.*, 2003, Hendricks *et al.*, 1999). The developmental course of the caudal neurons is thought to be similar to that of the dorsal neurons; however, it is possible that yet unidentified factors are involved in the differentiation process for both the dorsal and caudal raphe neurons (Jensen *et al.*, 2008).

One of the very few behavioral studies performed on knockout mice lacking one of the discussed transcription factors, Pet-1, demonstrates that these mice show a reduction of serotonergic neurons by ~70%, and display increased anxiety-related and aggressive behaviors (Hendricks *et al.*, 2003). It is obviously tempting to suggest that variation in genes coding for these transcription factors may be implicated in the development of psychiatric disorders, as well as personality traits.

Most research concerning the serotonergic neurogenesis by transcription factors has been performed in species other than humans, which of course raises the question whether these findings are applicable to human brain development.

GLOSSARY

Transcription factors	<i>Proteins that facilitate or repress the binding of RNA polymerase to DNA.</i>
Homeodomain	<i>A highly conserved sequence of 60 amino acids, that is found within a large number of transcription factors which can bind to DNA in a sequence-specific manner. Often present in transcription factors that trigger cascades of other genes.</i>
Growth factors	<i>A group of biologically active polypeptides that function as regulatory signals, controlling the growth and differentiation of the target cell.</i>
Neurotrophic factors	<i>A group of proteins that promote axonal growth and proliferation of neurons.</i>

Role of serotonin for the regulation of behavior

Serotonin has been shown to modify normal aspects of behavior, such as food intake, sexual activity and aggression, and seems also to be involved the regulation of respiration, sleep, cardiovascular function, body temperature, pain perception, gastrointestinal function and hormonal release (Lucki, 1998). However, although serotonin influences all these functions, its presence of serotonin does not seem to be a prerequisite for any of them. Animals from which serotonin has been depleted hence survive, and do not display any gross abnormalities (Eriksson and Humble, 1990).

When a new drug, *iproniazid*, was assessed as a putative treatment for tuberculosis in the late 1950s, it was discovered that some of the patients displayed a marked elevation of mood (Loomer *et al.*, 1957). Almost at the same time, another compound, *imipramine*, was also demonstrated to exert an unexpected antidepressant effect (Kuhn, 1958). Whereas iproniazid was subsequently shown to be an MAO inhibitor (MAO-I), imipramine was shown, by Axelrod and co-workers, to be a noradrenaline reuptake inhibitor, and later, by Carlsson and co-workers, to inhibit the reuptake also of serotonin, the later finding paving the way for the subsequent introduction of the selective serotonin reuptake inhibitors (SSRIs) as treatment for depression (Carlsson *et al.*, 1968, Eriksson and Humble, 1990).

The discovery that serotonin depletion by *parachlorophenylalanine* (pCPA), a TPH inhibitor, could reverse the antidepressant effect of reuptake inhibitors or MAO-Is (Shopsin *et al.*, 1975) lent support to the notion that the antidepressant effect is, at least partly, mediated by serotonin. This was also supported by numerous subsequent studies showing that acute tryptophan depletion (ATD) can trigger the onset of depressive symptoms in patients in remission as well as lower mood in healthy subjects with a family history of depression (Bell *et al.*, 2005).

Drugs that modulate the output from serotonergic synapses are effective not only for the treatment of depression, but also for the treatment of several other psychiatric disorders, such as premenstrual dysphoria (PMD), panic disorder, general anxiety disorder (GAD), posttraumatic stress disorder (PTSD), social phobia, obsessive compulsive disorder (OCD) and bulimia nervosa (Eriksson and Humble, 1990). Notably, with respect to some of these conditions, such as PMD (Landen *et al.*, 2006) and panic disorder (Eriksson and Humble, 1990) the effects of SRIs in terms of effect size and response rate are considerably higher than in depression. Further supporting the hypothesis that serotonin influences other aspects of behavior than mood, ATD has been reported to induce an increase in food intake in bulimic patients (Kaye *et al.*, 2000) and an increase in irritability in women with premenstrual dysphoria (Bond *et al.*, 2001, Menkes *et al.*, 1994). With respect to anxiety disorders, ATD alone does not trigger anxiety or anxiety attacks, but enhances the response to an anxiety-provoking challenge, *e.g.*, CO₂ exposure (Anderson and Mortimore, 1999).

Do patients with potentially serotonin-related disorders differ from healthy controls with respect to serotonergic turnover or function, and, if so, how could this be determined? Assessment of 5-HIAA levels in the CSF has been used as one putative marker of serotonergic function, and differences between patients and controls with respect to this measure have been demonstrated for conditions such as suicidality (Nordstrom and Asberg, 1992, Traskman *et al.*, 1981), aggression, impulsive behavior (Placidi *et al.*, 2001, Soderstrom *et al.*, 2001, Stanley *et al.*, 2000) and eating disorders (Carrasco *et al.*, 2000, Kaye *et al.*, 1984). Low MAO activity in platelets, which is another putative marker of abnormal brain serotonergic activity, has been reported in subjects displaying impulsive behavior and sensation seeking (Schalling *et al.*, 1987, von Knorring *et al.*, 1984); likewise, the density of serotonin transporters in platelets, as assessed using [3H]-paroxetine or [3H]-imipramine, has been reported reduced in both depression (Owens and Nemeroff, 1994) and bulimia nervosa (see section 1.2.1). Reduced hormonal responses to serotonergic agonists is another finding reported in certain serotonin-related conditions (Eriksson and Humble, 1990).

All these techniques are however crude or indirect, and none of them yields an estimation of serotonergic activity in specific brain regions of potential importance for the regulation of mood and behavior. The development of advanced imaging techniques, such as *single photon emission computed tomography* (SPECT) and *positron emission tomography* (PET), however have provided means to elucidate possible changes in specific parts of the brain. As yet, radioligands have been developed for the serotonin transporter, the 5-HT_{1A} receptor and the 5-HT_{2A} receptors.

Serotonin in weight regulation and eating disorders

Appetite control and food intake

Appetite, food intake and satiety are regulated by an intricate network, which involves both peripheral and central mechanisms, and in which serotonin plays an important role. Agents enhancing serotonergic transmission induce a decrease in food intake in both animals and humans (Noach, 1994, Sargent *et al.*, 1997), and drugs inhibiting serotonergic transmission may exert the opposite effect (Kluge *et al.*, 2007, Nasrallah, 2008). The influence of serotonin on satiety and food intake is believed to be mediated mainly by two serotonin receptors – 5-HT_{1B} and 5-HT_{2C}. Whereas stimulation of 5-HT_{1B} causes a decrease in meal size and total food intake, activation of 5-HT_{2C} (see below) leads to a decrease in intake speed (Simansky, 1996).

Central appetite regulation is believed to take place *e.g.*, in the hypothalamus and the brainstem (Heisler *et al.*, 2003). The hormone *ghrelin*, produced in the periphery by the stomach as well as centrally in the brain, exerts appetite-increasing effects. Studies have demonstrated that ghrelin inhibit serotonin release (Brunetti *et al.*, 2002) and also that the SSRI, *fluoxetine*, can reverse this effect (Carlini *et al.*, 2007). Other substances

of importance for appetite control are *e.g.*, histamine, noradrenaline, leptin, neuropeptide Y and orexin (Stanley *et al.*, 2005).

Anorexia nervosa and bulimia nervosa

The eating disorders anorexia nervosa (AN) and bulimia nervosa (BN) affect predominately women and are more frequent in western societies, having a prevalence of 0.3% and 1%, respectively, among women in these countries (Hoek, 2006).

The two disorders share several features but also differ on some critical points. Anorexia nervosa is a disorder characterized mainly by an irrational fear of weight gain leading to extreme forms of self-starvation and in some cases also excessive exercising to prevent this. Patients with BN also often display a fixation on weight and a twisted body image; however, in this condition, attempts of strict dieting are interrupted by episodes of binge eating, in most cases followed by vomiting (Fairburn and Harrison, 2003).

Both disorders are often accompanied by symptoms of depression, anxiety, irritability and lability of mood. While anorectic patients, on the one hand, often find pride in successfully maintaining a low weight, and see weight loss as accomplishments, bulimic patients, on the other hand, regard their binge eating sessions as failures, and are ashamed about their vomiting. This makes bulimic patients easier to motivate to receive treatment. However, since patients with BN rarely show clear-cut underweight, this disorder can be more difficult to detect (Fairburn and Harrison, 2003).

Several studies support a role of serotonin in the pathophysiology of both AN and BN. For both disorders there are hence reports of low MAO activity in platelets (Carrasco *et al.*, 2000, Diaz-Marsa *et al.*, 2000, Hallman *et al.*, 1990), decreased thrombocyte [3H]-paroxetine binding (Bruce *et al.*, 2006, Ekman *et al.*, 2006, Ramacciotti *et al.*, 2003, Steiger *et al.*, 2006) and reduced concentration of 5-HIAA in CSF (Jimerson *et al.*, 1992, Kaye *et al.*, 1984). Moreover, ATD induces an increased food intake, a rise in body image concern and an increased desire to overeat and purge in patients with BN (Kaye *et al.*, 2000). SSRIs have been shown to reduce binge eating and purging in BN and to improve relapse rate in weight restored AN patients (Kaye *et al.*, 1998); the latter finding has however been disputed (Holtkamp *et al.*, 2005). In underweight subjects with ongoing AN, one should perhaps not expect SSRIs to be of benefit, since the main effect of serotonin on food intake is to reduce it, and since at least one of the SSRIs, *i.e.*, fluoxetine, often exerts a weight-reducing effect (Dolfing *et al.*, 2005, Norris *et al.*, 2005).

Twin studies report that both AN and BN are hereditary, the heritability assessments varying from 60 to 80% in anorexia, and from 30 to 80% in BN (Bulik *et al.*, 2000, Fairburn and Harrison, 2003). Because of the involvement of serotonin in appetite regulation as well as in other traits commonly occurring in patients with eating disorders, such as anxiety, depression and poor impulse control, association studies in

this field have to a great extent been focused on serotonin-related genes, *e.g.*, *HTR2A* (Campbell *et al.*, 1998, Collier *et al.*, 1997, Gorwood *et al.*, 2002, Nacmias *et al.*, 1999, Sorbi *et al.*, 1998, Ziegler *et al.*, 1999), *HTR1D* (Bergen *et al.*, 2003, Brown *et al.*, 2007), *TPH1* (Monteleone *et al.*, 2007) and *SLC6A4* (Di Bella *et al.*, 2000, Frieling *et al.*, 2006, Fumeron *et al.*, 2001, Lauzurica *et al.*, 2003, Matsushita *et al.*, 2004, Sundaramurthy *et al.*, 2000). The group of genes that have been assessed with respect to eating behavior and eating disorders however also include, *e.g.*, those encoding dopamine receptors (Bergen *et al.*, 2005, Nisoli *et al.*, 2007) and ghrelin (Dardennes *et al.*, 2007). Apart from rather consistent findings regarding an association between the gene encoding the *brain derived neurotrophic factor* (BDNF) and both AN and BN (Gratacos *et al.*, 2007, Ribases *et al.*, 2004), results from association studies in this field however have been inconclusive (Rankinen and Bouchard, 2006, Steiger and Bruce, 2007).

Drug-induced weight gain

Weight gain is a commonly occurring adverse reaction to treatment with atypical antipsychotics, especially *clozapine* and *olanzapine*. Furthermore certain antidepressants, such as TCAs and the tetracyclic agent *mirtazapine*, are known to induce weight gain (Vanina *et al.*, 2002). These effects have been attributed the antagonistic actions of the drugs predominantly on 5-HT_{2C} and/or histamine receptors. Since the weight gain can be substantial, *i.e.*, up to 30% for the atypical antipsychotics, it is a common cause of discontinuation and low compliance (Nasrallah, 2003). Mirtazapine has actually been suggested as a treatment in AN, with a number of smaller studies have reporting beneficial effects of this treatment (Hrdlicka *et al.*, 2008, Jaafar *et al.*, 2007).

Different genes have been assessed in the search for genetic factors influencing the level of weight gain elicited by treatment with atypical antipsychotic drugs, including, *e.g.*, the adrenoreceptor alpha1A gene, the leptin receptor gene, and the histamine 1 receptor gene (Ellingrod *et al.*, 2007, Hong *et al.*, 2002, Lane *et al.*, 2006, Park *et al.*, 2006, Saiz *et al.*, 2008b, Wang *et al.*, 2005a, Wang *et al.*, 2005b, Zhang *et al.*, 2007). The most consistent finding in this area so far, however, is the association between drug-induced weight gain and a promoter polymorphism (rs3813929) in the 5-HT_{2C} receptor gene, *HTR2C* (De Luca *et al.*, 2007, Reynolds, 2007, Reynolds *et al.*, 2002).

Serotonin in suicidal behavior

Suicidal behavior can be defined as self-directed injurious acts with at least some intent to end one's life, ranging from the most severe form, the completed suicide, to low-lethality attempts. Since the vast majority of reported suicides and suicide attempts are performed by individuals with a psychiatric diagnosis, suicidal behavior may often be regarded as a complication of a psychiatric illness. Most suicides and suicide attempts occur in patients with mood disorders (60%), but patients with schizophrenia, alcoholism, anxiety and substance abuse are also at higher risk (Mann,

2003). However, obviously not all individuals with severe psychiatric illness attempt to commit suicide, indicating that psychiatric illness alone is usually not a sufficient risk factor for suicidal behavior.

The finding that suicide attempters not only display reduced levels of 5-HIAA in CSF, but that low levels are also predictors of increased future suicide risk, suggests that serotonin is involved in the predisposition for suicidal behavior (Asberg *et al.*, 1986, Traskman *et al.*, 1981); it should however be mentioned that some studies have failed to confirm an association between low CSF 5-HIAA and suicide (Engstrom *et al.*, 1999). The hypothesis that a dysfunction in serotonergic transmission is of importance for suicidal behavior is further supported by studies showing fewer presynaptic serotonin transporters, and up-regulation of serotonergic receptors, *e.g.*, the 5-HT_{2A} receptor, in certain regions of the brain of suicide victims as assessed post mortem (Turecki *et al.*, 1999). In addition, tentatively serotonin-related personality traits such as impulsivity and aggression, correlate strongly with proneness for suicide (Baud, 2005).

There is a considerable genetic component in the susceptibility for suicidal behavior, as illustrated by the fact that individuals with a family history of suicide are at ~4 times higher risk for engaging in suicidal behavior as compared with those without such a family history (Brent and Mann, 2005). This notion is also supported by data from twin and adoption studies (Roy *et al.*, 1997), a recent meta-analysis estimating the heritability for suicidality to 30-55% (Voracek and Loibl, 2007). Genetic studies on possible candidate genes in this field have been most encouraging for the serotonin-related ones; association with suicide or suicide-related personality traits have thus been found for *SLC6A4*, *HTR2A* (Anguelova *et al.*, 2003), *TPH1* (Bellivier *et al.*, 2004) and *TPH2* (Zhou *et al.*, 2005). The literature is not unanimous with respect to any of these findings, but some results appear more promising, *e.g.*, those regarding *TPH1* and *SLC6A4*, than others, *e.g.*, those regarding *HTR1A* (Bondy *et al.*, 2006).

Serotonin in premenstrual dysphoria

Premenstrual syndrome (PMS), and more severe forms of this condition, such as premenstrual dysphoria (PMD) and premenstrual dysphoric disorder (PMDD), are characterized by the emergence of increased irritability, depressive symptoms, mood lability and tension during the luteal phase of the menstrual cycle. Most women experience some degree of symptoms during this phase, and in ~2-8% the symptoms are severe enough to affect their work capacity and social functioning (Wittchen *et al.*, 2002).

The pathogenesis is as yet not understood, but changes in the levels of progesterone and estrogen around ovulation in the luteal phase of the menstrual cycle (Fig 4) appear to trigger the symptoms (Backstrom *et al.*, 2003).

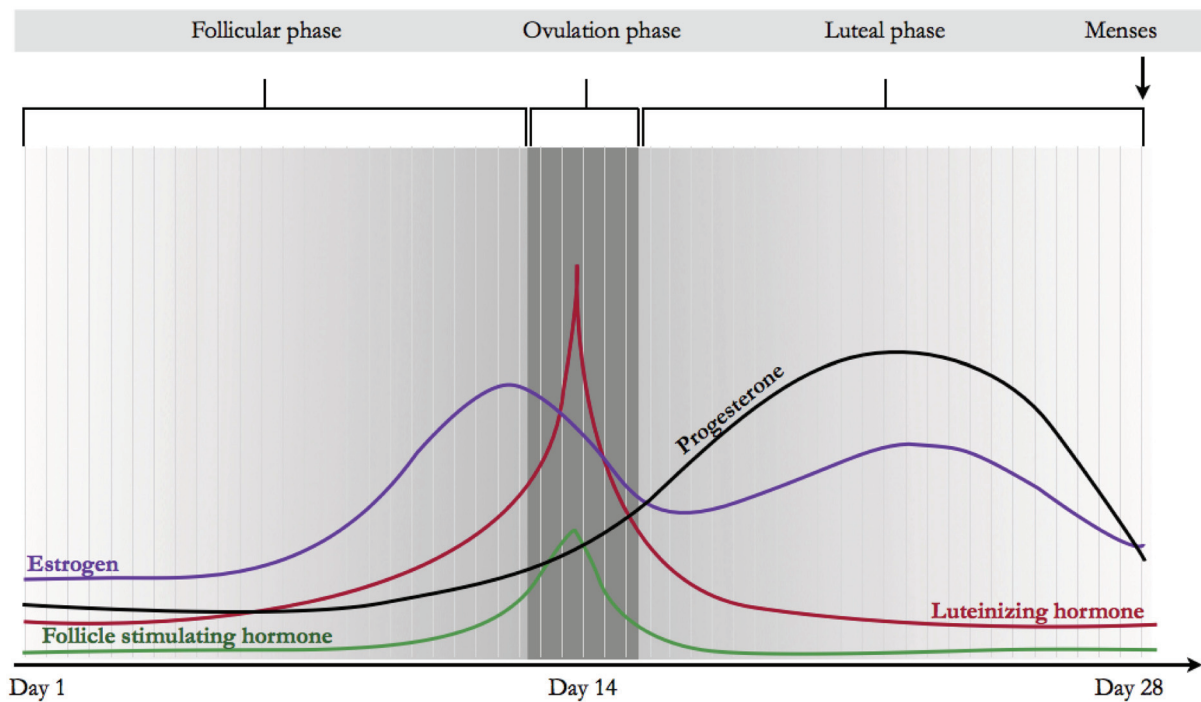


Figure 4. Hormonal fluctuations over the menstrual cycle.
PMD symptoms appear during the luteal phase and usually disappear after menses

There are many reasons to believe that serotonin is involved in PMD. First, animal studies indicate that a major role of serotonin is to dampen sex steroid-driven behavior, including the most characteristic feature of PMD, *i.e.*, anger (Ho *et al.*, 2001). Second, the SSRIs reduce premenstrual irritability with a larger effect size and response rate than they display in any other SSRI indication, and this effect correlates better in time with the influence of these compounds on serotonergic levels in the synapse than do the antidepressant and anti-anxiety effects (*i.e.*, the effect is more rapid in onset)(Landen *et al.*, 2006). Third, also other compounds facilitating serotonergic neurotransmission, such as the serotonin precursor tryptophan (Steinberg *et al.*, 1999), the serotonin-releasing agents mCPP (Su *et al.*, 1997) and fenfluramine (Brzezinski *et al.*, 1990), and the 5HT1A agonist buspirone (Landen *et al.*, 2001), reduce premenstrual irritability. Fourth, PMD symptoms are aggravated by ATD (Menkes *et al.*, 1994). And fifth, women with PMD have been reported to differ from controls with respect to a number of serotonin-related indices, including 5-HTT binding in platelets (Melke *et al.*, 2003a), platelet MAO activity (Ashby *et al.*, 1988, Hallman *et al.*, 1987, Rapkin *et al.*, 1988) and prolactin response to serotonin receptor agonists (Bancroft *et al.*, 1991, Fitzgerald *et al.*, 1997, Rasgon *et al.*, 2000, Yatham *et al.*, 1989). Moreover, differences between healthy controls and PMD patients were recently reported with respect to the influence of the menstrual cycle on 5-HT1A

receptor binding (Jovanovic *et al.*, 2006) as well as with respect to brain uptake of a serotonin precursor (Eriksson *et al.*, 2006).

It is well established that estrogen and progesterone receptors co-localize with serotonergic neurons, and that estrogen and progesterone exert profound effects on serotonergic turnover and receptor density (Amin *et al.*, 2005, Bethea *et al.*, 2002). It is thus possible that the influence of sex steroids on behavior in part is mediated by serotonin, and, for that reason, can be effectively counteracted by SRIs. These interactions between sex steroids and serotonin are likely to take place, *e.g.*, in the amygdala and the hypothalamus.

Twin studies suggest that the heritability of PMD is ~40% (Kendler *et al.*, 1992, Treloar *et al.*, 2002). So far only a small group of genes have been assessed in relation to this disorder, *i.e.*, genes encoding the estrogen receptor alpha and beta (*ESR1*, *ESR2*), *COMT* (Huo *et al.*, 2007), the 5-HT_{1A} receptor (Dhingra *et al.*, 2007), 5-HTT (Magnay *et al.*, 2006, Melke *et al.*, 2003a), TPH-1, MAO-A (Magnay *et al.*, 2006) and AP2-beta (Damberg *et al.*, 2005). All these studies have been small in terms of number of included subjects.

GLOSSARY

PMS	<i>Recurring symptoms emerging in the luteal phase of the menstrual cycle and disappearing in the follicular phase. This term can be used regardless of the type and severity of the symptoms.</i>
Severe PMS	<i>Recurring symptoms emerging in the luteal phase of the menstrual cycle and disappearing in the follicular phase. The symptoms, that could be of any type, are severe enough to significantly reduce quality of life and normal functioning</i>
PMDD	<i>Recurring symptoms emerging in the luteal phase of the menstrual cycle and disappearing in the follicular phase. . The symptoms, of which at least one must be a mood symptom, are severe enough to significantly reduce quality of life and normal functioning. Detailed criteria, that are provided in the Diagnostic and Statistic Manual of Mental Disorders (fourth edition) (DSM-IV) (1994), should be met for this diagnosis to be made.</i>
PMD	<i>Severe mood and behavioral symptoms emerging in the luteal phase of the menstrual cycle and disappearing in the follicular phase.</i>

Molecular genetics

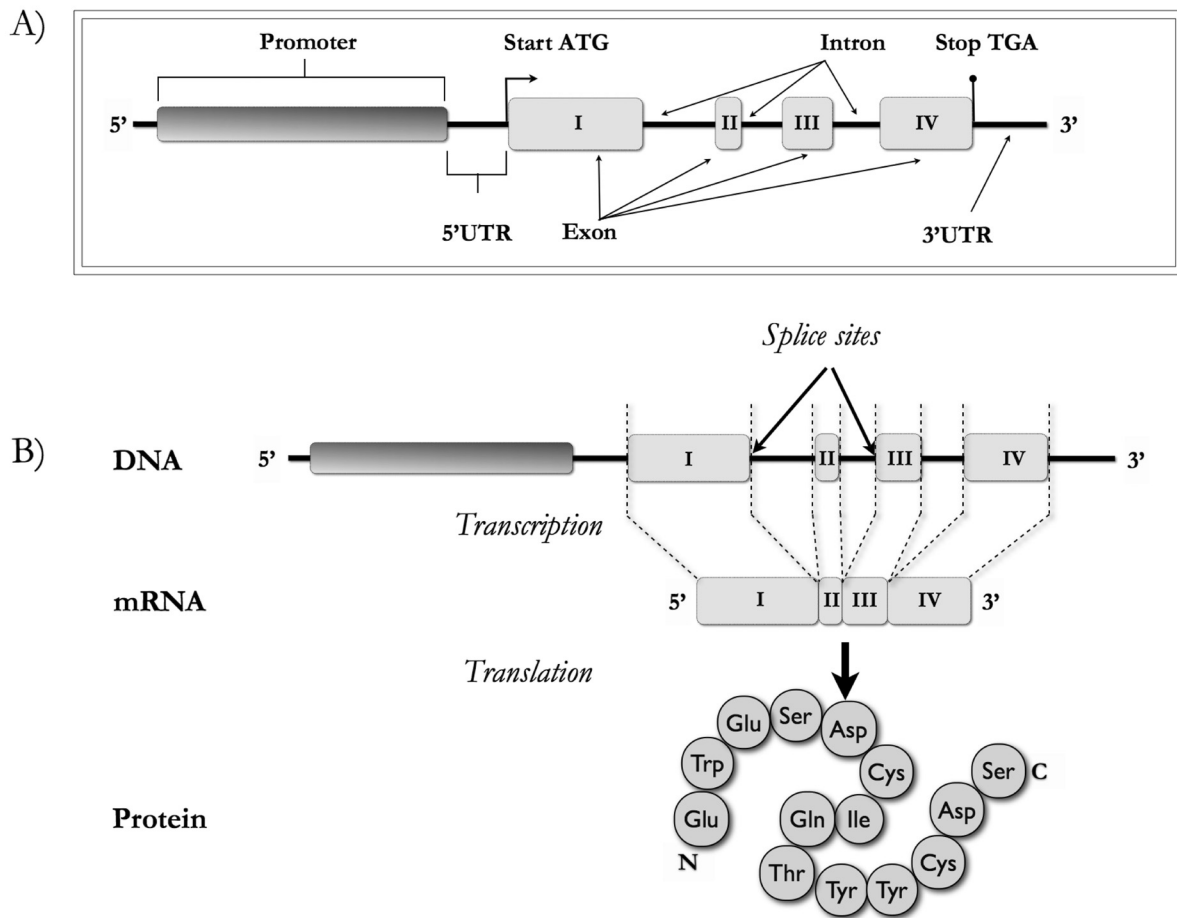


Figure 5. A) Schematic picture of a gene. B) From DNA to protein – overview of the transcription/ translation process.

The genetic code

The human genome is composed of a class of molecules called *deoxyribonucleic acid* (DNA). DNA is composed of 4 different *nucleotides* or *bases* – adenine (A), cytosine (C), guanine (G) and thymine (T) – assembled as strands in a specific order that constitutes the *genetic code*. In 1953 Watson and Crick proposed a model of the organization of the DNA molecule, the *double helix* (Watson and Crick, 1953), according to which the DNA strings are coupled in a specific way: A with T and C with G. This structural arrangement allows the DNA to replicate itself by using one string as template, which occurs when a cell divides. The DNA molecules are intricately folded and packed into structures called *chromosomes*, of which all the cells in our body contain 23 pairs.

The basis for the genetic code is the sequence of three bases called *codons*. One codon corresponds to one specific amino acid; however, since there are 20 amino acids and 64 possible codons, different codons can match the same amino acid. A *gene* is made

up by sequences of codons organized as coding (exons) and non-coding (introns) regions (Fig 5). The first estimates proposed that there are 35000 genes in the human genome (Ewing and Green, 2000), but this number has been reevaluated, and a recent paper suggests that the actual number is considerably smaller, *i.e.*, around 20500 (Clamp *et al.*, 2007).

In the *transcription* process (Fig 5), the sequence of bases on one of the two DNA strands is copied into single stranded RNA by a protein called *RNA polymerase*. The intronic, non-coding sequences are subsequently removed by a process called *splicing*. The remaining sequence is called *messenger RNA* (mRNA), and serves as a template in the final step, the *translation*, where the mRNA is decoded into the specific order in which the amino acids are attached when forming a *protein* (Jorde *et al.*, 2006).

Gene regulation

It is essential that the correct proteins are expressed in the accurate tissue and at the right time. Consequently, gene expression is controlled through several processes. One is at the transcriptional level, where the binding of the RNA polymerase is dependent on a group of proteins called *transcription factors*. There are different classes of transcription factors, the *general* transcription factors that interact directly with the RNA polymerase, initiating the transcription process, and the *specific* transcription factors that bind to specific sequences in the DNA, which can be far from the transcription initiation site, and by doing so, repress or activate the transcription of a gene (Jorde *et al.*, 2006).

There are also other means of regulating the expression of a gene, including imprinting (Bartolomei and Tilghman, 1997) and post-translational processes such as phosphorylation (Reinders and Sickmann, 2007). Further, other mechanisms, *e.g.*, alternative splicing of the mRNA (Kim *et al.*, 2008) and RNA editing (Amariglio and Rechavi, 2007) may alter protein structure and function.

Genetic variation

All genetic variation stems from *mutations* that can be defined as changes in DNA sequence. When a genetic variant occurs more frequently than 1% of a population it is called a *polymorphism*. There are several different types of polymorphisms, *e.g.*, single nucleotide polymorphisms (SNPs), insertions and deletions (I/D), repeat polymorphisms and copy number variants (CNVs). Depending on where in the genome these polymorphisms are located, they can influence the gene product in different ways.

The most common form of polymorphism is the *SNP*, which consists of one base that is exchanged for another (Brookes, 1999). It has been suggested that the human genome contains one SNP every 100-300 base pair (bp) (Kruglyak and Nickerson, 2001), and so far over 11 million SNPs are available in public databases (Madsen *et al.*,

2007), e.g., dbSNP (<http://www.ncbi.nlm.nih.gov/SNP/>) (Sherry *et al.*, 2001) and UCSC (<http://genome.ucsc.edu/>) (Kent *et al.*, 2002). *Insertions* or *deletions*, which may vary in length from just one bp up to sequences of several kbp, are added or removed from the DNA sequence. *Repeat polymorphisms* consist of fragments of DNA sequence that are repeated variable number of times. They vary from small di- or trinucleotide repeats (microsatellites) (Jeffreys *et al.*, 1985) to sequences involving up to over 100 bases (variable number of tandem repeats; VNTRs) (Nakamura *et al.*, 1987). Recently, larger deletions and duplications (>1 kbp in size), have also been shown to be an important source of genetic variation; this phenomenon is called *copy-number variants* (CNVs) or *copy-number polymorphisms* (CNPs) and is probably more commonly occurring than previously believed (Iafate *et al.*, 2004, Sebat *et al.*, 2004). CNVs may have dramatic effects on the phenotype but are difficult to detect with conventional genotyping methods.

It is the placement and nature of the polymorphism that determines whether it will be *functional* or not. The most obvious effects are exerted by polymorphisms in coding regions, where SNPs can cause amino acid substitutions or stop codons, and by insertions and deletions, which can change the reading frame for the polymerase. However, also polymorphisms in non-coding regions, such as the 5'- and 3'untranslated regions (UTRs), the promoter and the introns can be of functional importance since they might affect transcription rates by creating or interrupting binding sites for transcription factors or microRNAs (miRNA) (Bentwich, 2005, Sauna *et al.*, 2007) and thereby influence the expression rate of the protein. Some polymorphisms do not affect the final product at all, e.g., *silent* SNPs in the exons.

Linkage disequilibrium

A specific combination of markers on one chromosome is termed *haplotype* (Jorde *et al.*, 2006). The shorter the distance between two markers, the higher the probability that they will be inherited together, which leads to a non-random association between these alleles. Such association is termed linkage disequilibrium (LD). The concept of LD can be used in candidate gene studies where some combinations of alleles in a region are more frequent in a certain cohort than would be expected by chance (*haplotype block*), and where knowledge regarding one of these hence may provide indirect information regarding other loci on the same chromosome.

Genetic studies

The intricate interplay between genetic variation and environment is the basis for the evolution. The fact that people are different in many aspects – physically, *e.g.*, with respect to eye and skin color, and psychologically, *e.g.*, with respect to intelligence and personality traits – is partly explained by genetic variation. The importance of inter-individual differences becomes most apparent when it comes to disease. Why are some persons afflicted and others not? Some disorders like *e.g.*, Huntington’s disease (Gusella *et al.*, 1983), are *monogenic*, *i.e.*, they are due to a mutation in one single gene. For more *complex* illnesses, such as most psychiatric disorders, the relation between inherited and acquired pathology is not clear-cut, which makes the underlying causes more difficult to establish. It is believed that such disorders are *polygenic*, and that the genetic variation in this context generates vulnerability for the disease, rather than being the direct cause.

Two different strategies have been used to identify the specific genes involved in different traits or disorders: *linkage analyses* and *association studies* (Glazier *et al.*, 2002). A *linkage analysis* aims at finding the region that is inherited together with the trait or disorder at interest. By using a large set of genetic markers, evenly distributed throughout the genome, and by studying which of these markers that are inherited together with the disorder in families, it is possible to identify chromosomal regions where the involved gene/genes are located. This strategy may be performed without any knowledge of underlying pathophysiological mechanisms. *Association studies*, on the other hand, are based on an *a priori* hypothesis regarding a possible etiological role for a certain gene.

GLOSSARY

Allele	<i>One of the variants of a gene on one chromosome. An individual carries two alleles for every given loci (with the exception that men carry only one X chromosome)</i>
Genotype	<i>The genetic constitution of an organism (always comprising two alleles at any given loci, with the exception that men carry only one X chromosome).</i>
Homozygous	<i>Carrying two identical alleles at any given loci.</i>
Heterozygous	<i>Carrying two different alleles at any given loci.</i>
Haplotype	<i>Set of alleles on one chromosome.</i>
Phenotype	<i>The traits of an individual, <i>e.g.</i>, hair color or body weight. The phenotype may or may not be influenced by genes.</i>
Endophenotype	<i>A measurable component, other than a diagnosis, that may reflect parts of the pathophysiology of a certain condition. Often a less complex behavior, or some underlying biological function, <i>e.g.</i>, brain activity as assessed by means of neuroimaging.</i>

Instead of families, a classical case-control design is often used, where affected individuals and unrelated controls are compared with respect to the allele- and genotype frequencies of one or several polymorphisms in the gene in question. A significant difference between these groups implies that the investigated gene is involved in the disease or trait.

In recent years, a third strategy has been increasingly used, *i.e.*, the *whole-genome association analysis*. This approach is similar to the conventional association study apart from the fact that cases and controls are compared with respect to the whole genome rather than with respect to only a few polymorphisms (Fan *et al.*, 2006). The advantage of this technique is, of course, the vast amount of information that is obtained, and the fact that entirely unexpected findings can be made. The problem, on the other hand, is the need to correct all associations found for the fact that multiple comparisons have been undertaken, *i.e.*, to separate genuine findings from those that have appeared by chance.

Lately, the importance of including the environmental aspect in the analysis of genetic susceptibility has become evident, as demonstrated, *e.g.*, by Caspi and co-workers, when studying the 5-HTTLPR polymorphism in *SLC6A4*, in combination with stressful life events with respect to possible influence on risk for depression (Caspi *et al.*, 2003). Depression was significantly associated neither with genotype nor with environmental factors when assessed *per se*, but the combination of a susceptibility allele and stressful life events was found to predict the disorder. This finding has been replicated in a number of studies (Kaufman *et al.*, 2004, Nilsson *et al.*, 2007, Sjöberg *et al.*, 2006) but is not undisputed (Chipman *et al.*, 2007).

Problems and possible solutions in genetic studies

Substantial problems with non-replication of highly significant findings in association studies on complex disorders (Hirschhorn *et al.*, 2002) have raised the question how to most efficiently assess potential candidate genes. The inheritance patterns of, *e.g.*, most psychiatric disorders, in conjunction with the outcome of twin studies, suggest that multiple genes and environmental factors are involved in the etiology of these conditions. Moreover, as judged by the association studies undertaken so far, including whole-genome studies, it is likely that the effects exerted by individual genes are small. To further complicate the association studies, the affected cohorts are often heterogeneous, the diagnostic criteria used in psychiatric research may be misleading, and our knowledge of the underlying mechanisms, that is to guide us in our selection of candidate genes, is as yet limited.

Phenotype definitions: More and more effort has been devoted to finding the optimal phenotypes to study in the context of genetic variation. For the majority of psychiatric disorders, we still know very little about which genes that are involved; also, we do not know the size of the effect that the as yet unidentified genes might exert. Associations

between less complex traits that may be correlated to the investigated disorder, so-called *endophenotypes*, and genetic variation, however may contribute to a greater understanding of the disorder in question. In addition, aspects such as early onset of the investigated disorder and treatment responsiveness may be used to obtain more uniform study samples. Those that share one or several properties like these may thus have a higher probability of also sharing the same genetic influences. However, it is of course a possibility that several different combinations of genes and environmental factors could produce the same phenotype.

Gene – Environment interactions: As mentioned previously, the interaction between genetic variance and environmental factors has proven to be of great importance, and this should be considered when studying conditions with an expected high occurrence of environmental influence, *e.g.*, eating disorders and obesity. Many negative genetic findings might be reinvigorated when assessed in conjunction with environmental factors. One challenge is however, to establish how to define these external, environmental determinants in such analyses. Exactly what is, for example, a stressful life event suitable to investigate in this context? What is to be considered as traumatic enough to be regarded as important, and how will these different factors relate to each other?

Gene – Gene interactions: The putative importance of gene–gene interaction has since long been acknowledged. However, it has been problematic to reach a conclusion of how to approach this issue, *e.g.*, from a statistical point of view. Lately technical achievements, *e.g.*, the development of high-throughput genotyping platforms, such as *Illumina*, *Affymetrix* and *Sequenom*, have enabled the detection of multiple SNPs at the same time in large study samples, hence making it possible to assess gene–gene interaction at a large scale. To assess information regarding many polymorphisms in one study, and then to use this information for assessment of gene–gene interaction, is however not uncomplicated, since the power to find associations decreases the more components one adds into the equation. Gene–gene interaction assessment is however probably more in accordance with how genes actually exert their influence on the phenotype, and, lately, new reports have emerged presenting interesting results primarily from assessments of the possible interaction between two genes (De Luca *et al.*, 2005, Schmidt *et al.*, 2007, Smolka *et al.*, 2007, Vandenberg *et al.*, 2007). Development of more appropriate statistical models will hopefully open up the possibility to add more genes to the analysis, and hence to improve the potential of finding out how susceptibility genes interact to generate normal and abnormal human behaviors.

Epigenetics: Modifications that affect gene expression, but not DNA sequence, *e.g.*, imprinting, are called epigenetic variation, and have lately drawn much attention. For example, the fact that monozygotic twins differ more in their phenotypes than would be expected from their genetic construct is believed to be due partly to epigenetic alterations (Fraga *et al.*, 2005). The notion that environment can influence the

phenotype partly by turning on or off the expression of genes, however, does not necessarily warrant a re-evaluation of our view on the relative role of the genome *versus* that of the environment for various disorders, unless it can be shown that such epigenetic changes can be transferred to the next generation. Epigenetics do however add an interesting point of view, both by providing a biological mechanism by which the environment may affect us, and by re-viving the intriguing issue of to what extent acquired qualities can be transferred to the next generation.

Statistical analysis: One of the major obstacles in genetic research may be the missing of potential associations due to the harsh criteria for significance that are often applied when conducting multiple testing. Strict statistical models reduce the risk of unwanted false positive findings, but are not well adjusted to the biology of genetic variance, and its influence in complex traits, where many components with low independent effect are to be examined. However, new statistical models to be applied in genetic research are being developed (Montana, 2006).

Serotonin-related genes in behavior and psychiatric disorders

This section will review psychiatric genetic research in regard to serotonin transmission in general, but will focus on the genes involved in the main findings of the subsequent papers, *i.e.*, *HTR2C*, *SLC6A4*, *HTR3B* and *GATA2*.

A variety of studies on transgenic mice lacking different serotonin-related proteins (5-HT1A, 5-HT1B, 5-HT2C, MAO-A/B, SERT) implicate these genes in several functions and behaviors such as anxiety, impulsivity and aggression (Gross *et al.*, 2002, Lesch and Mossner, 2006, Shih and Chen, 1999, Zhuang *et al.*, 1999). These findings support conclusions drawn from previous preclinical and clinical studies using various pharmacological tools to modulate the serotonergic activity (see above). The large body of evidence implicating serotonin in the pathophysiology of psychiatric disorders have encouraged the exploration of genetic variation in serotonin-related genes and the possible influence such variation may have on the susceptibility for psychiatric disorders.

As expected, given that serotonin has been in focus for psychiatric research for over 50 years, the literature on this subject is immense. Numerous significant associations between genetic variations and psychiatric disorders have been reported, but for most of these the gathered results are inconclusive (Veenstra-VanderWeele *et al.*, 2000).

One of the more thoroughly examined genes is the one encoding the serotonin transporter, *SLC6A4*. Findings regarding polymorphisms in this gene will be discussed below. Other well-investigated genes, being runners-up on the hot-list, are the *HTR1A* (Albert and LEMONDE, 2004) and *HTR2A* (Norton and Owen, 2005) genes, which are decent. The bias of attention on these particular genes may in part have something to do with the fact of the receptor proteins they encode have been intensely studied thanks to the development of compounds displaying affinity for them; in addition, for these receptors there are ligands available enabling brain imaging studies.

The hypothesis that associations between gene variants and various traits are due to an influence exerted during brain development, and not in the adult organism, has lately been increasingly acknowledged. Despite this, surprisingly little research has been done with respect to the transcription factors involved in the regulation of the early neurodevelopment (Damberg *et al.*, 2001). As we propose in this thesis this group of genes deserves more attention than they have been given so far.

HTR2C

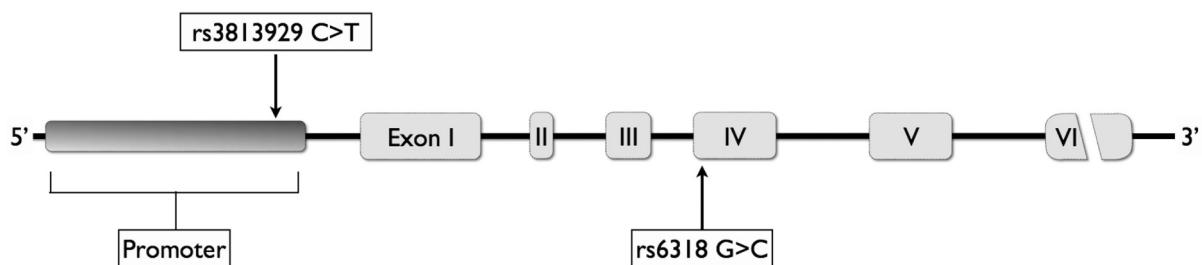


Figure 6. Schematic view of HTR2C (NC_000868.2)
Polymorphisms studied in this thesis are marked with arrows.

The 5-HT_{2C} receptor is a G-coupled receptor. When activated, *e.g.*, by serotonin or by the 5-HT_{2C} agonist LSD, it stimulates phosphatidylinositol (PI) turnover.

This receptor was first discovered in the choroid plexus where it is expressed at high concentration (Pazos *et al.*, 1984). It has since been described in several brain regions such as the cerebral cortex, hippocampus, amygdala and the hypothalamus (Pasqualetti *et al.*, 1999). Initially it was believed to belong to the same family as the 5-HT₁ receptors and hence called 5-HT_{1C}. However, cloning of the cDNA revealed it to be more related to 5-HT_{2A}, and consequently the name was changed to 5-HT_{2C} (Julius *et al.*, 1988, Salzman *et al.*, 1991). So far this receptor has not been found outside the CNS (Hoyer *et al.*, 2002). Atypical antipsychotics, such as clozapine, olanzapine and risperidone, display high affinity for several 5-HT receptors, including the 5-HT_{2C} subtype on which they act as antagonists (Canton *et al.*, 1990, Jenck *et al.*, 1993, Roth *et al.*, 1992).

The *HTR2C* gene has been mapped to human chromosome Xq24. *HTR2C* knockout mice display adult obesity, high frequency of epileptic seizures (Tecott *et al.*, 1995), compulsive behaviors (Chou-Green *et al.*, 2003) and, according to a recent report, reduced anxiety-like behavior (Heisler *et al.*, 2007). Two polymorphisms in this gene have been extensively studied: a Cys23Ser substitution in exon 4 (rs6318) (Lappalainen *et al.*, 1995) and a -759C>T SNP in the promoter region (rs3813929) (Yuan *et al.*, 2000). Suggesting a functional importance of the Cys23Ser polymorphism, it has been shown to be associated with clozapine response (Sodhi *et al.*, 1995), affective disorders (Lerer *et al.*, 2001, Massat *et al.*, 2007), psychotic and depressive symptoms in Alzheimer's disease (Holmes *et al.*, 2003b, Holmes *et al.*, 1998), higher risk for extrapyramidal side effects in drug-treated schizophrenic patients (Gunes *et al.*, 2007, Segman *et al.*, 1997) and differences in regional cerebral blood flow (Kuhn *et al.*, 2004). Results from *in vitro* studies exploring the potential influence of this SNP on receptor function or expression have, however, been conflicting (Fentress *et al.*, 2005, Lappalainen *et al.*, 1995, Okada *et al.*, 2004).

With respect to the -759C>T SNP, one of the more consistent findings is a possible association with antipsychotic-induced weight gain (Reynolds *et al.*, 2006). Furthermore, it has also been associated with ADHD (Li *et al.*, 2006). As in the case for Cys23Ser, studies on the effect of -759C>T on 5-HT_{2C} function are, however, few and inconsistent (Buckland *et al.*, 2005, Hill and Reynolds, 2007). In addition to genetic variation, *HTR2C* is also the subject of RNA-editing in the region encoding the 2:nd intracellular loop of the receptor, *i.e.*, the part of the receptor with which the G-protein interacts (Burns *et al.*, 1997). Editing of 5-HT_{2C} changes the properties of the receptor and has been suggested to be of importance in schizophrenia, depression, and suicide (Gardiner and Du, 2006).

SLC6A4

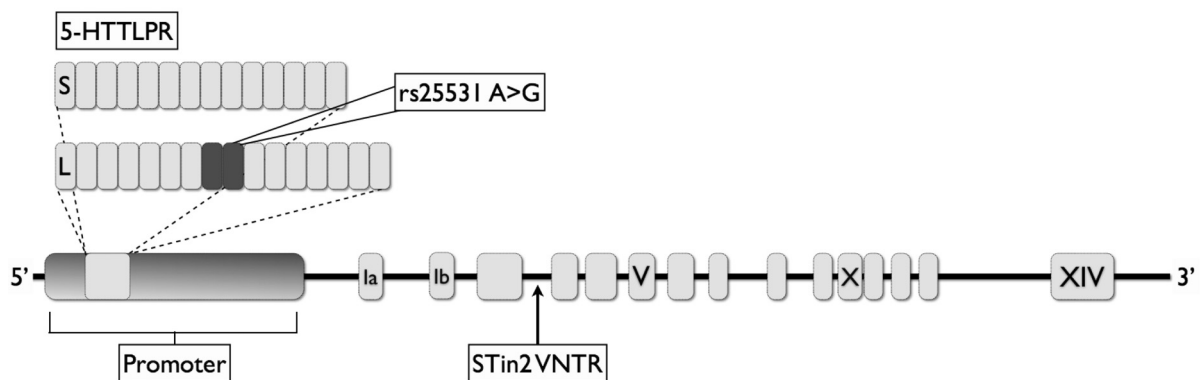


Figure 7. Schematic overview of the serotonin transporter gene, *SLC6A4* (NM_001045)

Given the important role the serotonin transporter plays in the synapse, and also during brain development (Ansgorge *et al.*, 2004), genetic variation in the gene encoding this protein would be expected to have a broader influence on serotonergic function than genetic variation in the gene for a specific receptor. It is hence not surprising that *SLC6A4* is one of the most thoroughly examined genes in serotonergic transmission. Needless to say, encoding the target protein for many of the commonly used antidepressant drugs also makes it a candidate of interest when searching for genes with possible influence on the different disorders responding to these drugs, as well as in studies assessing possible genetic predictors of treatment response.

SLC6A4 is located on chromosome 17q12 (Fig 7). Knockout mice lacking 5-HTT demonstrate an increase in anxiety-related and depressive-like behaviors, and a decrease in aggression (Holmes *et al.*, 2003a). Several polymorphisms have been detected in *SLC6A4*. One of the most scrutinized is a 44 base pair insertion/deletion, which is situated in the promoter region (the serotonin transporter gene-linked

polymorphic region; 5-HTTLPR) (Lesch *et al.*, 1996), resulting in a short (S) and a long (L) variant of the transporter (Fig 7). The S-allele of 5-HTTLPR has been shown to result in a 50% decrease in transcriptional activity *in vitro* (Lesch *et al.*, 1996); data on this issue are however not unanimous (Alenina *et al.*, 2006). The most replicated finding regarding the 5-HTTLPR has been the association between the S-allele on the one hand, and anxiety-related traits and neuroticism on the other, as first reported by Lesch and co-workers (Lesch *et al.*, 1996, Melke *et al.*, 2001, Schinka *et al.*, 2004, Sen *et al.*, 2004). Furthermore, 5-HTTLPR has been associated to major depression (Anguelova *et al.*, 2003, Caspi *et al.*, 2003, Gutierrez *et al.*, 1998), amygdala activation in response to emotional stimuli (Bertolino *et al.*, 2005, Dannlowski *et al.*, 2007, Furmark *et al.*, 2004, Hariri *et al.*, 2006, Heinz *et al.*, 2007, Munafo *et al.*, 2007, Smolka *et al.*, 2007), SSRI response (Smits *et al.*, 2004), drive for thinness (Akkermann *et al.*, 2008) and obesity (Sookoian *et al.*, 2007).

Recently an A>G SNP (rs25531) was discovered in the long variant of the 5-HTTLPR region (Hu *et al.*, 2006, Kraft *et al.*, 2005) (Fig 7). The resulting alleles are L_G, L_A and S_A, where L_G and S_A are low transcriptional alleles and L_A exerts a higher transcriptional activity (Hu *et al.*, 2006). This SNP has been associated with obsessive compulsive disorder (Hu *et al.*, 2006, Wendland *et al.*, 2008), increased amygdala response in response to emotional stimuli (Dannlowski *et al.*, 2007) and melancholic depression in women (Baune *et al.*, 2007, Dannlowski *et al.*, 2007, Kraft *et al.*, 2005, Wendland *et al.*, 2007, Wendland *et al.*, 2006). It is possible that presence of this SNP could explain some of the conflicting results in previous 5-HTTLPR association studies in the past but this remains to be clarified. It should be noted that this polymorphism has not been investigated in this thesis.

Another polymorphism of interest in *SLC6A4* is a variable number of tandem repeats (VNTR) polymorphism situated in the second intron of the gene (STin2) (Battersby *et al.*, 1996). The three most common alleles of the STin2 polymorphism are designated 9, 10 and 12, based on the relative number of VNTR elements. There is evidence that also the STin2-polymorphism may act as a transcriptional regulator, the 12-repeat allele displaying a higher transcriptional activity than the 10-repeat allele (Fischerstrand *et al.*, 1999, MacKenzie and Quinn, 1999). With respect to possible relationship to psychiatric morbidity, the STin2 polymorphism is less explored than 5-HTTLPR, but it has been reported to be associated with anxiety-related personality traits (Melke *et al.*, 2001), anxiety disorders (Ohara *et al.*, 1999), bipolar disorder (Kunugi *et al.*, 1997), schizophrenia (Fan and Sklar, 2005), OCD (Baca-Garcia *et al.*, 2007, Saiz *et al.*, 2008a) and affective disorders (Battersby *et al.*, 1996, Domotor *et al.*, 2007, Lopez de Lara *et al.*, 2006).

GATA2

GATA-binding protein 2 (GATA2) is a 486 amino acid protein with its gene, *GATA2*, located on chromosome 3q21 (Fig 8). It belongs to a family of zinc-finger

proteins, named from GATA1 to GATA6, and binds to specific GATA-containing regions in the DNA. GATA2 is a transcription factor of importance in hematopoiesis, but recent studies have implicated it also in neural development, more specifically in the differentiation of serotonergic neurons. Animal studies have shown that lack of this factor leads to a total loss of rostral serotonergic innervation (Craven *et al.*, 2004). The function and importance of GATA2 in serotonergic development makes it an interesting candidate gene to study in relation to the etiology of serotonin-related disorders and traits.

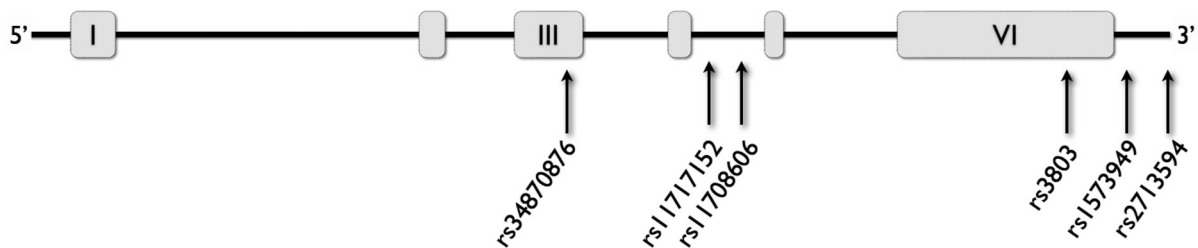


Figure 8. Schematic overview of the GATA2 gene (NM_032638)
Polymorphisms studied in this thesis are marked with arrows.

So far only one association study regarding *GATA2* has been published; in this report, this gene is suggested to be associated with coronary artery disease (Connelly *et al.*, 2006), which is of particular interest given the well established co-morbidity between serotonin-related disorders, such as depression and anxiety disorders on the one hand, and cardiovascular disorders on the other (Joynt *et al.*, 2003).

HTR3A & *HTR3B*

The 5-HT₃ receptor is the only ligand-gated ion-channel among the serotonin receptors. It consists of different combinations of five subunits named 5-HT_{3A}-3E, of which only A-C are expressed in the brain (Niesler *et al.*, 2003). Most extensively studied are the 5-HT_{3A} and 5-HT_{3B} subunits for which the encoding genes are located in close proximity on chromosome 11q23 (Fig 9). 5-HT₃ receptors are expressed at high concentration in regions of the brain stem of importance for the regulation of the vomiting reflex, and drugs with an antagonistic effect at 5-HT₃ are antiemetic (Barnes and Sharp, 1999). 5-HT_{3A} null mutant mice display reduced anxiety-like behaviors indicating a role for 5-HT₃ also in regulation of anxiety (Kelley *et al.*, 2003).

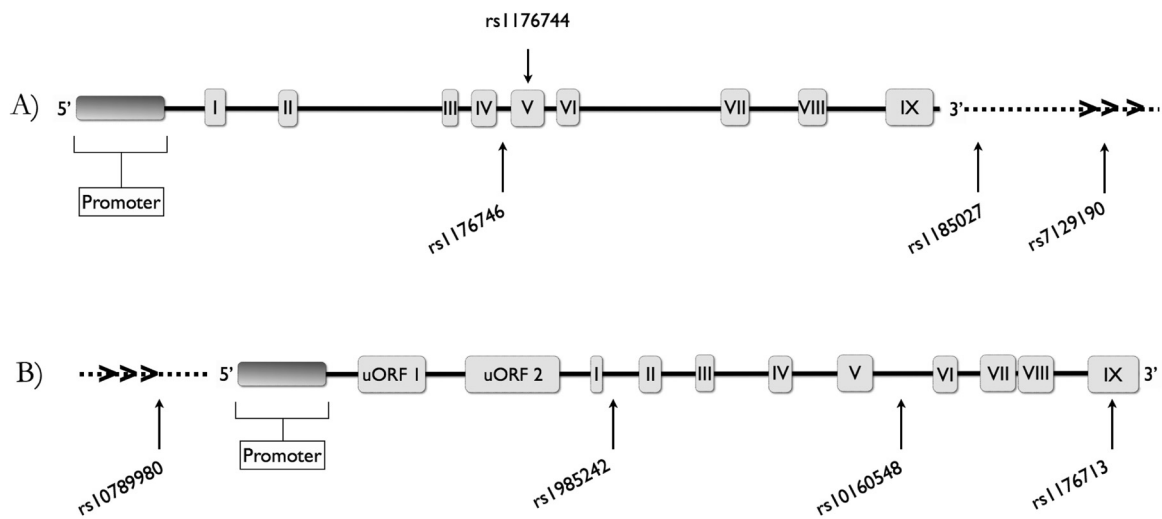


Figure 9. Overview of the HTR3A (NM_000869) (B) and HTR3B (NM_006028) (A) genes. Polymorphisms assessed in subsequent papers are marked with arrows. uORF= upstream open reading frame

The observation by Melke and co-workers that variation in *HTR3A* is associated with anxiety-related personality traits in women (Melke *et al.*, 2003b) has subsequently gained direct indirect support from a study assessing amygdala activation (Iidaka *et al.*, 2005). Polymorphisms in the *HTR3B* gene, on the other hand, have been associated to major depression (Krzywkowski *et al.*, 2008, Yamada *et al.*, 2006) and bipolar disorder (Frank *et al.*, 2004).

AIMS

In the following papers the aim has been:

- I. ... to assess the possible association between a SNP in the gene coding for the serotonin receptor 5-HT_{2C}, *HTR2C* on the one hand, and weight loss and anorexia nervosa on the other.
- II. ... to attempt to replicate previous findings of an association between two SNPs in *HTR2C* and body weight in a sample from the general population as well as to assess the possible role of the serotonin transporter gene in this context.
- III. ... to evaluate the possible effect of two polymorphisms in the gene coding for the serotonin transporter on binding capacity of the serotonin transporter in the brain of suicide attempters and matched controls.
- IV. ... to study the possible association between genetic variation in 20 serotonin-related genes and premenstrual dysphoria.

RESULTS AND DISCUSSION

HTR2C and weight (Paper I and II)

Serotonin and serotonin-stimulating compounds have been found to exert an appetite-suppressive effect in both animal studies and in studies on humans (Blundell, 1992, Wurtman *et al.*, 1993). Since 5-HT_{2C} agonists - such as m-chlorophenylpiperazine (mCPP) and 1-(2,5-dimethoxy-4-iodophenyl)-2-ami-nopropane (DOI) - cause decreased food intake, the 5-HT_{2C} receptor has been proposed to be the mediator of the appetite-reducing effects of serotonin (Simansky, 1996). Supporting this theory, mice lacking this receptor have been reported to display obesity as adults as a consequence of hyperphagia (Tecott *et al.*, 1995). Moreover, it has been shown that dieting may cause an increase the responsiveness of 5-HT_{2C} receptors (Cowen *et al.*, 1996).

Eating disorders, as well as weight regulation, are to a large extent heritable (Bulik *et al.*, 2003, Bulik and Tozzi, 2004, Loos and Bouchard, 2003). Given the alleged influence of the 5-HT_{2C} receptor on food intake, the *HTR2C* gene is an obvious candidate gene in this context. The aim of the first paper was hence to elucidate if a potentially functional SNP in *HTR2C*, rs6318, is associated with weight loss in teenage girls (with or without AN).

To this end, the rs6318, which causes a cysteine to serine substitution at amino acid position 23 (Cys23Ser), was assessed in three groups of teenage girls; one group with marked weight loss and diagnosed with AN, one with marked weight loss but not

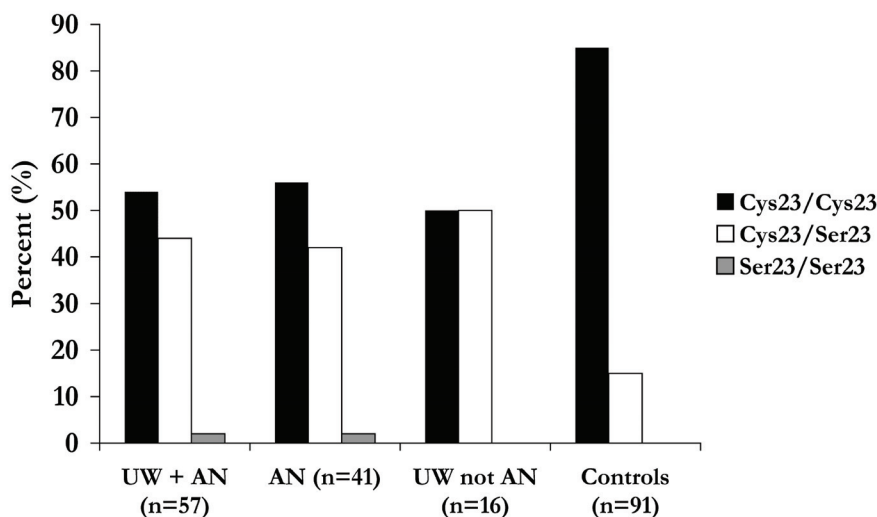


Figure 11. Genotype distribution of Cys23Ser in underweight girls without AN (UW), girls with AN and healthy controls.

fulfilling diagnosis criteria for AN and one group of healthy, age-matched controls. The less common Ser23-allele was shown to be more frequent in girls with marked weight loss, independently of diagnosis.

As the sample studied in this paper was small, the results should be interpreted with caution, but do suggest the Cys23Ser SNP in *HTR2C* to be associated with weight loss *per se* regardless of whether the subjects meet AN diagnostic criteria or not. This study was the first to demonstrate an association between Cys23Ser and weight, and has since gained support from a study where the Ser23-allele was associated with low BMI in subjects with AN (Hu *et al.*, 2003), and by a study on women with seasonal affective disorder in which carriers of the Ser23-allele displayed lower BMI than Cys23 carriers (Praschak-Rieder *et al.*, 2005).

In an attempt to further test our hypothesis of an involvement of *HTR2C* Cys23Ser in the regulation of weight, we assessed this SNP in a cohort from the general population, comprising both men and women in which BMI had been assessed (Paper II). In addition, a SNP in the promoter region of *HTR2C*, rs3813929 (-759C>T), that has previously been associated with antipsychotic-induced weight gain, was investigated (Reynolds *et al.*, 2002).

In accordance with the first study, the Ser23-allele was found to be more common in underweight subjects compared to normal-weight and overweight subjects. When analyzing men and women separately, this association was significant in the female group only. This could suggest that men and women differ regarding the expression or function of appetite-regulating 5-HT_{2C} receptors. However, the lack of significant associations in the male group in this study could also be the result of the anthropometric characteristics of this cohort, very few subjects being underweight and a substantial number being overweight.

The less common T-allele of -759C>T occurred at a higher frequency in non-overweight compared to overweight subjects. This result is in line with several studies where the T-allele has been shown to exert a protective effect against anti-psychotic-induced weight gain (Reynolds *et al.*, 2006). Our data suggest that the T-allele might be protective against weight gain in general rather than merely against drug-induced obesity.

No complete LD was found between Cys23Ser and -759C>T, which is in line with other studies (De Luca *et al.*, 2007, McCarthy *et al.*, 2005). In addition to the single marker analysis, analysis of possible synergistic effects of the two SNPs were performed; as would be expected, the combination of the Ser23-allele and the -759T-allele was more frequently occurring in non-overweight subjects. Partly in accordance with this, a recent study demonstrated a haplotype comprising the Ser23 allele and the -759C-allele to have a small but significant protective effect against antipsychotic induced weight gain (De Luca *et al.*, 2007). The functionality of the polymorphisms

described in these papers, Cys23Ser and -759C>T, remains a matter of controversy (Buckland *et al.*, 2005, Fentress *et al.*, 2005, Kuhn *et al.*, 2004, Okada *et al.*, 2004).

The data presented in this paper are in perfect agreement with the observation that *HTR2C* null mutant mice display obesity, as well as with the reports of the appetite-suppressing effects of 5-HT_{2C} receptor agonists. Moreover these associations have been replicated in a number of independent studies, which further support the notion that genetic variation in *HTR2C* exerts a small but significant influence on the regulation of food intake and body weight.

Genetic variation of *SLC6A4* and weight (Paper II)

Paper II also assessed the possible association of the 5-HTTLPR repeat in the gene coding for the serotonin transporter (*SLC6A4*) with body weight. Although this polymorphism has been extensively studied with respect to possible association with other aspects of human behaviors, including susceptibility for a number of psychiatric disorders (see below), its possible relation to body weight has not been studied to the same extent (Sookoian *et al.*, 2007).

Our data showed that those being homozygous for the S allele more often were underweight, and that this difference was most prominent in men. In accordance with these results, previous studies have found an association of the S-allele with AN (Fumeron *et al.*, 2001, Matsushita *et al.*, 2004). The literature is however not unanimous: whereas our data demonstrate that the 5-HTTLPR SS-genotype is significantly associated to underweight, *e.g.*, Sookoian and co-workers reported an association of the S allele with obesity (Sookoian *et al.*, 2007), and the same allele has also been associated with treatment-induced weight gain (Bozina *et al.*, 2007). In addition, there are also negative studies (Hinney *et al.*, 1997, Mergen *et al.*, 2007).

Potential interaction between 5-HTTLPR and the two *HTR2C* SNPs were assessed as well. There was a significant interaction between the 5-HTTLPR and -759C>T genotypes, suggesting that homozygosity with respect to the L-allele of the 5-HTTLPR is a prerequisite for the T-allele of the -759C>T to exert a protective effect on overweight.

Serotonin transporter knockout mice display increased 5-HT_{2C} binding but down-regulated receptor signaling in response to DOI (Li *et al.*, 2003, Qu *et al.*, 2005). These data suggest that there might be a relationship between 5-HTT function during development and 5-HT_{2C} responsiveness in the adult animal. While each of the two genes may exert certain individual effects on appetite and body weight, by means of interaction they may exert influences on the studied phenotype that are somewhat different from those they exert *per se*.

Our results should be considered as preliminary until replicated, preferably in larger samples. Nevertheless, in the light of our present data, and in line with previous

reports implicating it in AN, BN and binge eating disorder (BED) (Di Bella *et al.*, 2000, Monteleone *et al.*, 2006), the 5-HTTLPR emerges as a possible participant in the complex machinery regulating body weight.

Polymorphisms in *SLC6A4* and serotonin transporter availability in the brain (Paper III)

Since the brain is a highly inaccessible and complex organ, the examination of it in living subjects has since long been a major challenge for scientists. The development of advanced brain imaging techniques, such as SPECT and PET, however, has expanded the possibilities to investigate how receptors and other proteins in the brain are associated with behavior and influenced by drugs.

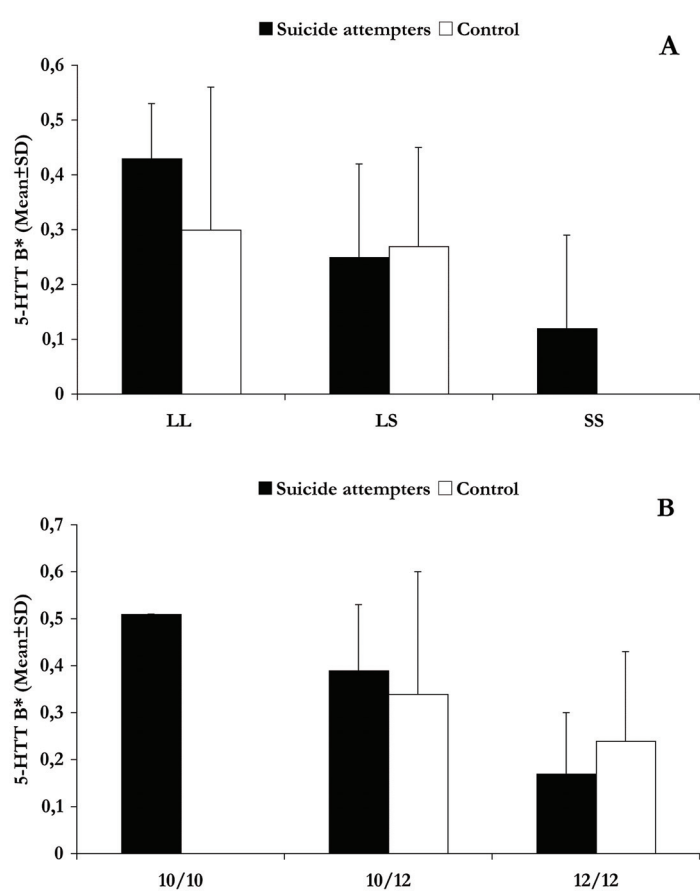


Figure 12. Binding capacity and genotype effect of 5-HTTLPR (Linear regression, $p=0.03$, $r^2=0.44$) (A) and STin2 ($p=0.03$, $r^2=0.44$) (B) in suicide attempters and controls.

Genetic variation in *SLC6A4* has been studied in relation to numerous psychiatric disorders, and replicated associations with, *e.g.*, anxiety-related personality traits, suicidality and depression, implicate this polymorphism in these conditions (Melke, 2003). Both the 5-HTTLPR S-allele and the 10-repeat allele of STin2 have been shown to cause a decreased transcription rate of 5-HTT in vitro. For the interpretation of studies suggesting an association between these polymorphisms and various traits, it is important to establish if they influence the expression of 5-HTT also in the human brain, and if such an influence may differ between patients and controls. When the study presented in Paper III was planned and performed, no studies on the possible influences of polymorphisms in *SLC6A4* on the density of 5-HTT, as assessed in vivo in humans, had been published.

The aims of this project (Paper III) hence were 1) to evaluate whether two repeat polymorphisms in *SLC6A4*, 5-HTTLPR and STin2, displayed any effect on binding

capacity of the serotonin transporter in the brain, and 2) whether such a potential effect might differ between healthy controls and suicide attempters. To this end, a group of suicide attempters (n=9) and age-matched controls (n=9) were assessed with SPECT ^{123}I - β -CIT. None of the participants had received any type of psychoactive drugs within 6 months before the examination.

Both the S-allele of 5-HTTLPR and the STin2 12-allele were shown to be associated with a lower binding potential in a dose-dependant manner. Our data are in line with the *in vitro* studies showing that the S allele of the 5-HTTLPR causes a decrease in transcription rate (Lesch *et al.*, 1996). On the other hand, the finding that the 12-repeat allele is related to *low* 5-HTT availability is not in line with previous *in vitro* data (Fiskerstrand *et al.*, 1999, MacKenzie and Quinn, 1999). However, the 12-repeat allele, like the S-allele of the 5HTTLPR polymorphism, has previously been associated with anxiety-related personality traits (Melke *et al.*, 2001), suggesting that these two alleles to some extent may exert similar effects on serotonergic transmission.

Recent studies that have assessed the possible influence of 5-HTTLPR on central 5-HTT availability have not been consistent, and the data are not easily interpreted. The majority of previous studies do not find any significant effects of genotype on binding capacity (Jacobsen *et al.*, 2000, Parsey *et al.*, 2006, Shioe *et al.*, 2003, Willeit *et al.*, 2001). One study, by Reimold and co-workers, reports higher 5-HTT availability in the brain of LL-carriers, which is in line with our results (Reimold *et al.*, 2007); however the subjects in their study were healthy, in contrast to ours, where the association was significant only in suicide attempters. In a study on abstinent alcoholics and healthy controls, Heinz and co-workers found different effect of genotype in different cohorts, as did we. However, while the LL genotype was associated with enhanced availability in controls, it was associated with reduced binding capacity in alcoholics (Heinz *et al.*, 2000). One study, finally, suggests that the heterozygotes have the lowest transcription rate (van Dyck *et al.*, 2004).

If there indeed is a difference between suicide attempters and healthy controls with respect to the influence of genotype on binding capacity of 5-HTT, one explanation could be that while in healthy subjects there are mechanisms that compensate for the influence of the 5-HTTLPR S-allele and the STin2 12-repeat allele on the density of serotonin transporters (or on the density of serotonergic neurons harboring these proteins), suicide attempters may have defects in this compensatory machinery. This would also partly explain the lack of associations between 5-HTT availability and genotype in studies performed in healthy controls only.

In conclusion, our data suggest that the 5-HTTLPR and STin2 polymorphisms influence central 5-HTT availability, and that this influence is stronger in suicide attempters than in healthy controls. The major limitation of this study is the small number of subjects included; for this reason, the results should be interpreted with caution and regarded as preliminary.

Serotonin-related genes in premenstrual dysphoria (Paper IV)

As discussed above, there are strong reasons to believe that serotonin is involved in the pathophysiology of PMD. In spite of the fact that the heritability of PMD has been estimated to ~40%, association studies in this area are as yet sparse. The aim of this study was to assess genetic variation in a group of serotonin-related genes comprising genes encoding various serotonin receptors, the serotonin transporter, tryptophan hydroxylase 1 and 2, the vesicular monoamine transporter 2, a growth factor of possible importance for the maintenance and plasticity of serotonergic neuron, *i.e.*, brain-derived nerve growth factor (*BDNF*), the *BDNF* receptor *TrkB* (*NTRK2*), and various transcription and growth factors of importance for the development of brain serotonergic neurons, *i.e.*, *ASCL1*, *FEV*, *FGF4*, *GATA2*, *GATA3*, *NKX2-2*, *NKX6-1*, and sonic hedgehog (*SHH*).

When 293 women with PMD were compared to 825 controls recruited by means of the population register, two SNPs were found to display an association with PMD for which the *p*-value was below 0.01 before correction for multiple comparisons: rs2713594 in the *GATA2* gene and rs1176746 in the *HTR3B* gene. After correction for multiple comparisons using permutation, the association remained significant for rs1176746 but not for rs2713594. When subsequently an independent group of cases (*n*=57), with self-reported PMD, was compared with the controls, both SNPs however again displayed a significant difference between groups. When all patients were compared to the controls, *p*-values for both rs1176746 in the *HTR3B* and rs2713594 in the *GATA2* remained significant after correction for multiple comparisons.

To further assess the possible association between PMD and *HTR3B* and *GATA2*, respectively, we performed sliding window analyses, comparing, in cases and controls, the frequencies of haplotypes generated with a specified number (=window size) of contiguous markers. For the *GATA2* and the *HTR3A/B*, a window size of three markers was used. These analyses revealed associations between PMD on the one hand, and haplotypes around exon 5 and 6 of the *HTR3B*, and in the 3' end of *GATA2*, on the other.

The association between *GATA2* and PMD is intriguing, since it lends support for the notion that genes encoding transcription factors of importance for the early development of serotonergic neurons are reasonable – but as yet under-studied – candidates in association studies on serotonin-related psychiatric disorders ((Damberg *et al.*, 2005). And the association between PMD and a SNP in the *5HT3B* gene, finally, fits nicely with recent studies suggesting this receptor subtype to be involved in the regulation of anger and aggression (McKenzie-Quirk *et al.*, 2005).

In a recent study, Dhingra and co-workers (Dhingra *et al.*, 2007) presented data suggesting a possible association between PMD and a SNP in the *HTR1A* gene that has previously been shown to affect the transcription rate of the 5-HT_{1A} receptor

(Lemonde *et al.*, 2003), *i.e.*, the -1019C>G (Dhingra *et al.*, 2007). Even though our sample was significantly larger, hence providing more power to detect a difference in allele distribution, this finding was not replicated in our study. Several other potential candidates, including *SLC6A4*, also were not associated with PMD.

To conclude, this study, that is the largest association study on PMD undertaken so far, supports the involvement of three serotonin-related genes in this condition: *GATA2* and *HTR3B*.

CONCLUDING REMARKS

The data presented in this thesis support the influence of genetic variance in serotonin-related genes on weight regulation, on binding capacity of 5-HTT in the brain of suicide attempters and on risk for PMD.

The mystery of how and why psychiatric disorders affect some individuals, but not others, has occupied scientists for a long time. Serotonin modulates normal emotions and behaviors, such as aggression, appetite, sexuality, mood and fear, which are all of fundamental importance for the evolution and survival of mankind.

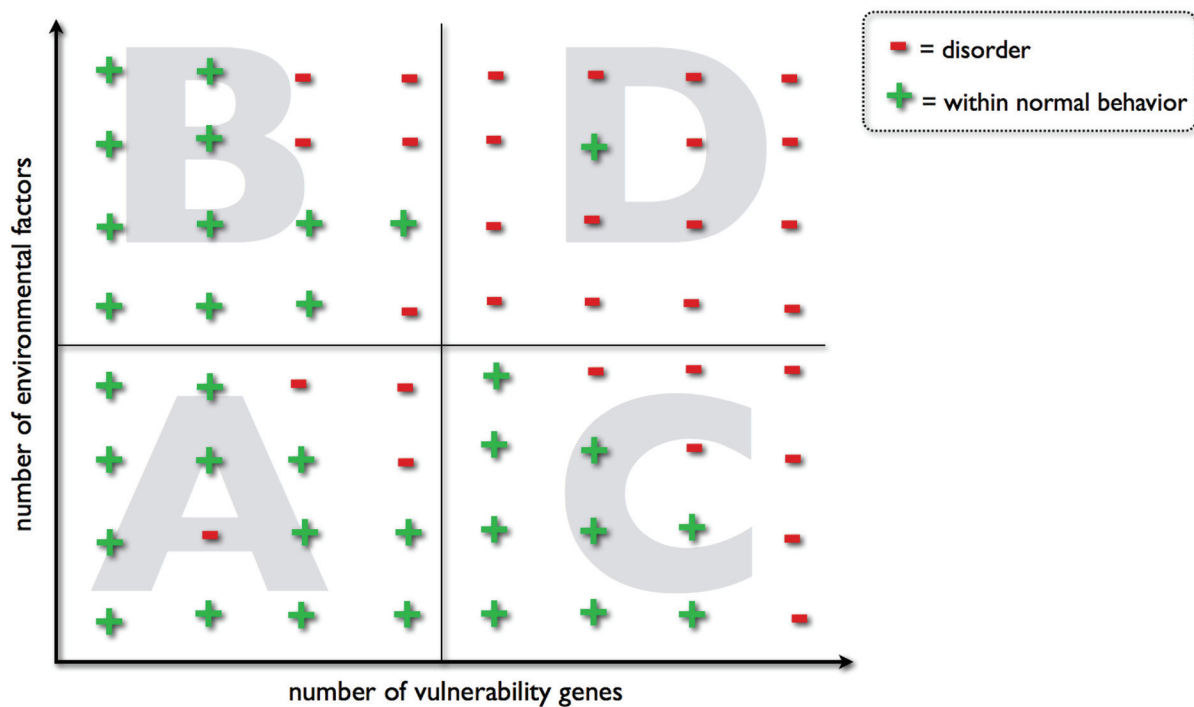


Figure 13. Hypothetical graph over how genes and environment may affect behavior and psychiatric morbidity. In the part of the graph with modest presence of vulnerability genes and environmental factors (A), most persons are healthy, but some may display subtle traits that are the result of the presence of some of the relevant risk factors. The minus in this part of the graph is supposed to symbolize the fact that morbidity also depends on which risk factors one carries or is exposed to, some having stronger effects than others, and that hence few but important risk factors may, though rarely, lead to illness. For some individuals the environmental factors may be more important (B), and for others the genetic factors (C), but usually illness is caused by a combination of genes and environmental risk factors (D).

One hypothesis is that serotonin-related psychiatric disorders should be considered as extreme variants of normal and important emotions and behaviors, but that vulnerability genes in combination with environmental factors push some beyond the line of what can be considered normal and functional. In other words, in those who are affected by disease, many of the risk factors have aggregated, whereas in

unaffected subjects risk alleles and environmental factors may just contribute to the inter-individual variation in personality traits and behavior (Fig 13).

In the work of Caspi and co-workers (see Introduction), a straightforward form of relationship between gene and environment is demonstrated. Carriers of only the risk allele but without stressful life events, do not develop depression significantly more often than would be expected, and neither do those with no genetic risk but with stressful life events in their past. However, the combination of the genetic risk, in this case the S-allele of 5-HTTLPR, and traumatic experiences in the youth, significantly increases the risk of becoming depressed (Caspi *et al.*, 2003).

When discussing *how* risk alleles exert their influence on psychiatric morbidity, it must always be taken into consideration that this influence may be exerted during development rather than in the adult organism. Studies of mice subjected to SSRIs during a specific period during postnatal development (Borue *et al.*, 2007) hence lend indirect support for the possibility that associations between 5-HTTLPR and various phenotypes may be exerted during development rather than being the result of modified serotonin reuptake in the adult brain. Consequently, genes encoding transcription factors of importance for the early development of the brain may deserve larger attention in psychiatric association studies than they have received so far.

Future perspectives

In the case of the serotonin-related psychiatric conditions, the outcome of genetic research has so far been somewhat disappointing. Numerous studies have been performed, but still very few findings have proven to be solid and possible to replicate. The capacity and power of genetic analysis to identify vulnerability genes in serotonin-related disorders thus seems to have been overstated in the early years of association studies. It has taken nature millions of years to form the intricate creatures of human beings – is it realistic to believe that it would be possible to crack that code in just ten or twenty?

On the other hand, the potential for revolutionary discoveries in this area is obvious. Uncovering which genes that are involved in, *e.g.*, depression and anxiety disorders, will thus probably provide means for improved treatment as well as a better understanding of these individuals. Finding more efficient and accurate methods to pinpoint genetic and environmental influence in these conditions is thus of immense importance.

So which way is the one to go in future studies? The strategy of whole genome association studies has its advantages, but is marred by the risk of missing true associations with small effect size due to the correction for multiple testing that will be required. The strategy of including very large study samples is also attractive, since this enhances the power of detecting small effects; when very large samples are to be

recruited, on the other hand, the reliability of the diagnoses is often jeopardized. The use of endophenotypes to obtain homogenous study cohorts is also a possible way to go, although the relation between a putative endophenotype and the clinical manifestations of a disorder is not always obvious. The road to choose is probably to combine all these strategies, and to add a bit of good sense.

Notwithstanding the difficulties, and the large number of observations that have proven impossible to replicate, there are however some findings in this field that seem reliable, including the importance of the *SLC6A4* and *MAOA* genes for certain traits. Given the obvious importance of the 5-HTTLPR polymorphism in *SLC6A4*, we believe that our observations that this polymorphism influences transporter density in the brains of suicide attempters, as well as body weight in healthy volunteers, are of interest. Our observation of an association between *HTR2C* and body weight is intriguing, given that it fits so excellently with data from other fields of research suggesting this receptor to regulate food intake, and also given that it has proven possible to replicate. And our observations that polymorphisms in *GATA2* and *HTR3B* are associated with PMD are also of putative importance, since we were able to find the same aberration in two different cohorts of patients, and since the theory that serotonin is involved in PMD gains support from a large number of other findings.

SUMMARY

- ◆ We suggest that two putatively functional polymorphisms in *HTR2C* influence body weight both in subjects with eating disorders and in the general population; in addition, we found support for an influence of the 5-HTTLPR polymorphism in *SLC6A4* on body weight in the latter cohort. We believe that the 5-HT_{2C} receptor is now established as a factor of importance for weight regulation. The finding of an association between 5-HTTLPR and BMI, on the other hand, warrants replication.
- ◆ Furthermore, we propose two polymorphisms in *SLC6A4* to have an influence on serotonin transporter binding capacity in the brain of suicide attempters. Since the previous literature on the possible influence of this polymorphism on transporter density in non-suicidal subjects is inconsistent, and since the sample size in our study was small, these data are, however, to be interpreted with caution until replicated.
- ◆ We finally suggest, on the basis of the largest association study conducted in PMD so far, that genetic variation in *HTR3B* and *GATA2* are involved in the etiology of this condition. Studies aiming to replicate this finding, and to explore the possible importance of these genes for other conditions characterized by anger or irritability, are warranted.

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APPENDIX: MATERIAL AND METHODS

Samples

Ethics

The studies included in this thesis were approved by the Ethics committees at the University of Gothenburg, the Lund Medical Faculty, the Umeå University and the Uppsala University. All patients and controls provided written informed consent to participate.

Selection of study cohorts

The basis for genetic studies with high quality is a well-defined study sample. The following investigations have been performed on rigorously diagnosed patients groups including AN (+ matched controls), suicide attempters (+ matched controls) and premenstrual syndrome.

In addition to our patient groups, samples of subjects recruited from the general population have been used as controls in an association study on premenstrual syndrome (control group I and II) (paper IV) and in the investigation of weight (control group I)(paper II).

Paper I: Anorexia nervosa

The original purpose of collecting this cohort was to establish the rate of AN in 16-year-old girls in Göteborg, and to compare AN girls with age-, sex-, and school-matched controls with respect to family background, personality traits, early development, co-morbidity, and certain biological markers. To be included for further study, the girls had to meet one of several criteria raising suspicion of possible AN; for details, see previous publications (Rastam et al. 1989; Rastam et al 1992). By means of this procedure, blood samples were obtained from 58 girls displaying weight reduction not explained by somatic illness, defined as a real or projected weight loss of at least 10%. The girls were subjected to a structured interview comprising a checklist of the DSMIII-R criteria for AN (Rastam et al. 1989; Rastam 1992). A majority (78%) but not all of the subjects met the criteria of AN, including a real or projected weight loss of 15%. For the genetic investigation (presented in paper I), two groups of weight loss girls were hence included: those meeting the DSM-III-R criteria of AN (n=41), and those reporting real or projected weight loss of 10% or more without meeting AN criteria (WL group) (n=17), respectively.

As controls (n=93) to the AN and WL groups, the corresponding number of girls of the same age and in the same school—in most cases born within a month of the index child—were identified by school nurses; furthermore, to enlarge the control group, a number of additional normal-weight 16-year-old girls also identified by school nurses were recruited. To be included as a control subject, the girl had to display a completely normal weight curve and report no signs of abnormal eating behavior at interview.

Paper II and IV: General population

The Gothenburg control cohort

Both the male and the female cohorts were originally recruited for a study on body anthropometry, the results from which have been reported elsewhere. (Rosmond and Bjorntorp, 1998, Rosmond *et al.*, 1998) The primary cohort from which the female group was recruited comprised all women born on uneven days in the year of 1956 and living in Göteborg, Sweden (n=1137). Of these subjects, 80 % reported self-measured body weight, height, and circumferences over the waist and hips. From this group, 450 women in total with low, medium and high self-assessed waist-hip-ratio (WHR), respectively, were selected; of these, 270 (60%) volunteered to provide blood samples for genotyping. In the population that was genotyped, WHR, as assessed at the clinic, was almost normally distributed. Body weight was measured to the nearest 0.1 kg, with the women in underwear, height was measured to the nearest 0.01 m, and BMI was calculated. At the time of investigation, all women were 42 years old. The male subjects (n = 240) also were selected from a larger cohort, comprising all men born during the first 6 months of 1944, and living in Göteborg, Sweden (n=1302), using a similar strategy as for the women, and they were also assessed in the same way with respect to BMI; for details on this population, see Rosmond et al (1998) (Rosmond *et al.*, 1998). At the time of investigation, the men were 51 years old.

The Betula control cohort

Another control population was drafted from the population of the Betula project – a large, population-based cohort recruited from the Northern Sweden that is described in detail elsewhere (Nilsson *et al.*, 1997). The subgroup from this cohort used as controls in paper IV comprised 187, women and 197 men.

Paper III Suicide attempt

Patients (male, n=9) were recruited after being admitted at the hospital because of a suicide attempt. Exclusion criteria were use of any antidepressant or antipsychotic drug at any time during the 6 months preceding the suicide attempt.

All the patients were diagnosed according to the DSM IV and rated according to the Suicidal Intent Scale (Beck *et al.*, 1974) by an independent psychiatrist; those who rated 18 or more were included in the study. A more detailed description of each patient is given in paper III. For each case, a male control subject matched for age and season of the year was recruited, mainly from the hospital staff. The subjects have been described previously by Lindstrom et al (2004) (Lindstrom *et al.*, 2004). The study was approved by the Lund Medical Faculty Ethics Committee.

Paper IV: Premenstrual dysphoria

Included in this study were 293 women with severe PMD that had previously been recruited for randomized drug trials. When included, the women were $37,2 \pm 6,1$ years of age, had regular menstrual cycles (22-35 days), and were living in Göteborg (91.8%) or Uppsala (8.2%), Sweden. They all met DSM-IV criteria A-C for PMDD and displayed a clear-cut increase in at least one of the symptoms irritability or depressed mood in the luteal phase as assessed by means of daily visual analogue scale (VAS) rating during two consecutive menstrual cycles, hence also meeting the American College of Obstetricians and Gynecologists (ACOG) criteria for PMS (ACOG, 2003). For further information regarding inclusion criteria, see previous reports (Eriksson *et al.*, 2008, Landen *et al.*, 2001, Landen *et al.*, 2006). As a second independent PMD cohort served 57 women belonging to the cohort recruited from the general population that was assessed in paper II (see above), and who had confirmed suffering from severe PMD by means of a questionnaire.

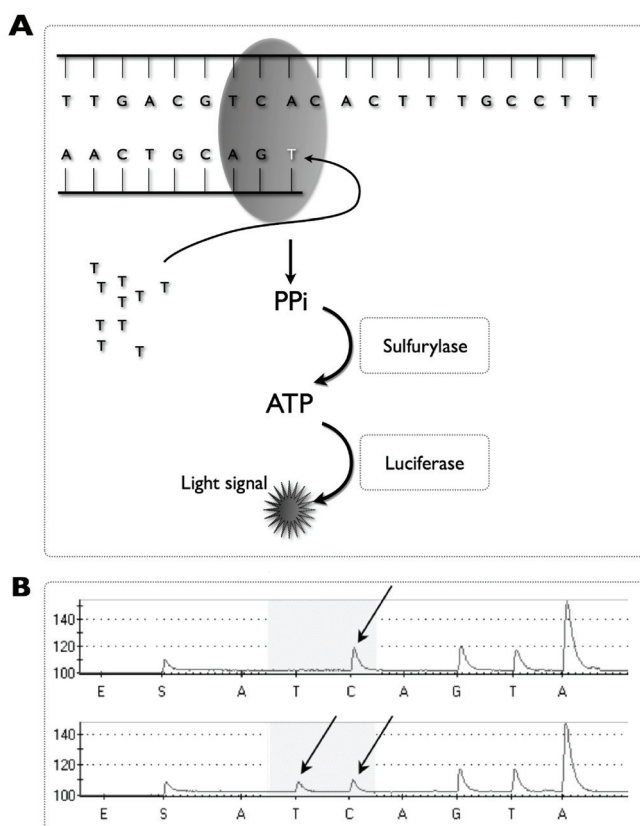


Figure 14. A) Schematic view of the Pyrosequencing process. B) The resulting pyrograms produced, exemplified by a homozygote (one arrow) and a heterozygote (two arrows), and used for genotype analysis

Pyrosequencing®

Pyrosequencing (Nordfors *et al.*, 2002) is based on the same principles as sequencing, but instead of dyes to distinguish the nucleic order, the polymerase transcription process itself is utilized. To

Genotyping

In all studies venous blood was collected from each subject, and genomic DNA was isolated using the QIAamp DNA blood Mini Kit (Qiagen). PCR products for all genotyping analysis were performed on GeneAmp PCR system 9700 (Applied Biosystems). Software Oligo6.7 and Pyrosequencing assay design software version 1.0 were used to design PCR and Pyrosequencing primers. All primers used in the analyses are described in detail in the individual papers (I-IV).

Agarose gel detection

Genotyping the polymorphisms in *HTR2C* and *SLC6A4* were performed by PCR amplification of the polymorphism-containing DNA regions. Amplified products were separated on agarose gels supplemented with ethidium bromide and visualized by ultraviolet transillumination. The PCR fragments for *HTR2C* were first digested with, *HinfI*, a restriction enzyme. The *SLC6A4* polymorphism could be detected without restriction enzyme cleavage. Agarose gel detection was used in papers I, III and IV.

initiate incorporation of nucleotides the DNA polymerase uses a short sequencing primer, which hybridizes close to the polymorphism in question. The nucleotides are added in a preprogrammed order, and when a nucleotide is incorporated, the release of pyrophosphate (PPi) starts an enzymatic reaction generating visible light. Hence if a nucleotide is added, but not incorporated, no light will be produced. The light produced in the luciferase-catalyzed reaction is detected by a charge coupled device (CCD) camera and seen as a peak in a pyrogram, each light signal being proportional to the number of nucleotides incorporated (Fig 14). Pyrosequencing analysis was used in papers II and IV. Assay Design Software, Biotage Version 1.0.6

Sequenom®

Sequenom is a high-throughput SNP analysis tool, based on multiplex PCRs with subsequent single base primer extension, followed by an analysis with MALDI TOF MS (van den Boom and Ehrich, 2007). One extension primer per SNP (up to 36-plex) is added to the PCR products together with the nucleotides. The extension primers anneal to their specific PCR product, the base before the SNP and the following extension is depending on the allele. The extension product will be elongated either with one or two bases and their different masses are separated in the mass spectrometer, rendering a spectrogram with the genotypes of all SNPs in the plex (Fig 11). This genotyping approach was used in Paper IV. TyperAnalyzerFS © software Version 1.0.1.46 was used to assess the results.

Imaging

Single photon emission computed tomography (SPECT)

SPECT is an imaging technology utilizing gamma rays to get a 3 D view of the organ in question *e.g.*, the brain. A radioactive ligand is injected in the subject and when the isotope starts to decay, gamma rays are emitted and can be captured by a gamma camera.

The radioligand 2-beta-carbomethoxy-3-beta-(4-iodophenyl)-tropane labeled with ¹²³I-iodine (¹²³I-β-CIT) is a potent ligand for both 5-HTT and the dopamine transporter (DAT). In Paper III ¹²³I-β-CIT was used to assess 5-HTT binding capacity. The methodology is described in detail by Ryding et al, 2004 (Ryding *et al.*, 2004).

Statistical analyses

The following statistical methods were used in the present papers:

Chi-square analysis (Paper I, II, IV)

Fisher's exact test (Paper II and III)

Linear regression (Paper II, III and IV)

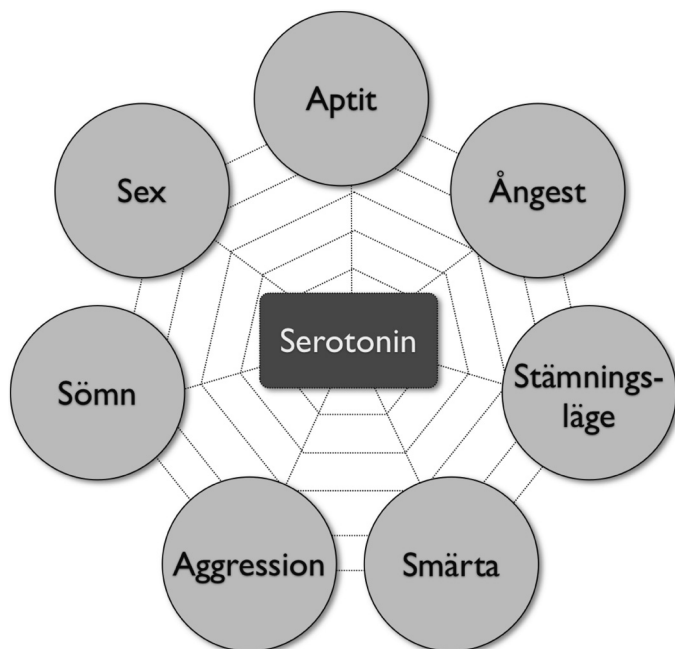
Independent T-test (Paper II)

Levels of significance were corrected for multiple comparisons using a step-down permutation procedure (Paper IV).

Hardy-Weinberg equilibrium for the polymorphisms investigated was checked in all control samples by comparing the observed genotype frequencies with the expected ones using chi-square analyses (Paper I-IV).

For details on data analysis, the reader is referred to the indicated papers, as well as the references cited in these papers.

SWEDISH SUMMARY/SVENSK SAMMANFATTNING



Figur 1. Ett urval av beteenden som serotonin är med och reglerar

Exempelvis kalkonkött och bananer är rikt på tryptofan. För att utöva sin effekt binder serotonin till sina receptorer, mottagarmolekyler, vilka i sin tur utövar påverkan på intelligande nervceller. Femton olika subtyper av receptorer för serotonin har identifierats, men funktionen av alla dessa är ännu långt ifrån utredd. 5-HTT transporterar tillbaka serotonin in i nervterminalen; denna mekanism är den enda som reglerar halten av serotonin i synapsklyftan. Det är 5-HTT som är målet för vissa tricykliska antidepressiva farmaka (TCA) och för de selektiva serotonin-återupptagshämmarna (SSRI). Dessa läkemedel hämmar 5-HTT så att ingen återtransport kan ske, vilket ökar halten signalämne i synapsen och härmed aktiveringen av de serotonerga receptorerna. Serotoninets livscykel avslutas genom att det bryts ner till metaboliten 5-HIAA av enzymet MAO.

SSRI har visats utöva goda effekter vid behandling av depression, ångestsjukdomar, tvångssyndrom, bulimia nervosa och premenstruell dysfori (PMD). Detta har lett till att störningar i den serotonerga transmissionen föreslagits vara av betydelse för dessa tillstånd, och denna hypotes har också vunnit stöd från andra typer av studier. Exempelvis har man visat att man kan förvärpa symptomen vid depression, bulimi och PMD genom att på experimentell väg hämma syntesen av serotonin.

SEROTONIN

Serotonin är en signalsubstans i hjärnan som är inblandad i regleringen av beteenden som t ex aptit, aggression, stämning-läge, sexualdrift och impulsivitet. Dessutom regleras funktioner som sömn, kroppstemperatur och smärta av detta ämne.

Det finns ett antal molekyler som är viktiga för den serotonerga funktionen: *tryptofanhydroxylas* (TPH), de serotonerga receptorerna (5-HT₁-5-HT₇), *serotonin transportören* (5-HTT) och *monoaminoxidas* (MAO). TPH katalyserar bildningen av serotonin (via den intermediära substansen 5-HTP) från aminosyran *tryptofan* som vi får i oss via kosten.

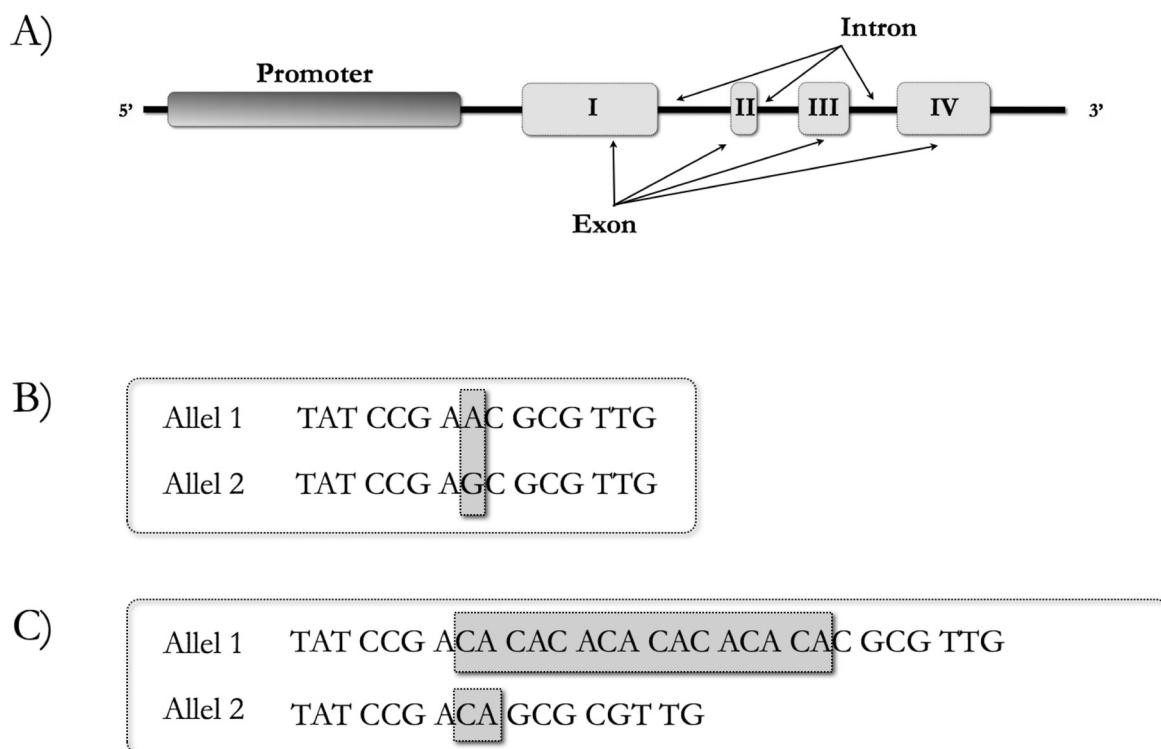
De aptitsänkande effekterna av serotonin medieras sannolikt av 5-HT_{2C}-receptorn. Man har således kunnat konstatera att substanser som binder till och stimulerar denna receptor, 5-HT_{2C}-agonister, minskar födointaget. Man har också påvisat att bantning gör 5-HT_{2C}-receptorerna mer reaktiva, dvs att de utövar en än mer uttalad aptitsänkande effekt. Det finns vissa typer av läkemedel som har kraftig viktuppgång som biverkan, vilka också binder till 5-HT_{2C}-receptorn, men som blockerar denna receptor.

GENETIK

Vårt genetiska material, *DNA*, är en syra som utformas av två komplementära strängar av s k nukleotider eller baser: A (adenosin), T (tymin), C (cytosin) och G (guanin). DNA är mallen utifrån vilken alla *proteiner* i kroppen bildas. Varje protein kodas av en eller flera *gener*. Alla gener består av kodande (exoner) och icke-kodande (introner) sekvenser. Ett s k *kodon* är en sekvens bestående av tre baser som kodar för en speciell aminosyra.

All genetisk variation har sitt ursprung i mutationer som uppstått under evolutionens gång. Mutationer som förekommer i mer än 1% av populationen kallas polymorfismer. Det finns olika typer av genetiska varianter. I avhandlingen har jag studerat två av dessa: *single nucleotide polymorphisms* (SNPs) och repetitions-polymorfismer (=dels mikrosatelliter, dels s k *variable number of tandem repeats*; VNTR). En SNP består i att en bas är utbytt mot en annan (Fig 2); detta utbyte kan leda till att aminosyrasekvensen ändras, och härmed proteinets funktion. Dessutom kan denna typ av polymorfism leda till att mängden bildat protein påverkas. Repetitions-polymorfismer innebär att sekvenser från en enda bas up till 100 baser upprepas olika antal gånger. Beroende på hur repetitionerna är belägna i sekvensen kan de leda till förlängda protein, avsaknad av protein eller till bildning av nonsensprotein.

När det visats i t ex tvillingstudier att en sjukdom är ärftlig börjar sökandet efter de gener som skulle kunna vara av betydelse för risken att drabbas av sjukdomen i fråga. Vanliga sjukdomar, som t ex flertalet psykiska sjukdomar, hjärt-kärlsjukdom och diabetes, orsakas antagligen av ett flertal olika gener, i kombination med olika miljöfaktorer, vilket gör dem komplexa att utreda.



Figur 2. En schematisk bild över hur en gen är uppbyggd (A) samt exempel på hur en SNP (B) och en repetitions-polymorfism (C) kan se ut. De grå fälten (B-C) markerar vart polymorfismen i fråga sitter.

En metod för att identifiera potentiella sjukdomsgener är den så kallade associationsstudien. Man studerar härvid förekomsten av en genvariant, *allel*, i en grupp som har den sjukdom som man vill studera, och jämför med förekomsten av samma allel i en kontrollgrupp. Om grupperna skiljer sig åt vad avser den studerade allelen kan man dra slutsatsen att denna gen påverkar benägenheten att drabbas av sjukdomen i fråga.

SYFTE MED AVHANDLINGEN

De studier som ligger till grund för denna avhandling har haft till syfte att besvara följande frågor:

1. Utövar varianter av de gener som kodar för 5-HT_{2C}-receptorn (*HTR2C*) och serotonintransportören (*SLC6A4*) någon påverkan på kroppsvikt? (Artikel I och II)
2. Kan polymorfismer i *SLC6A4*, som alltså är den gen som kodar för 5-HTT, påverka mängden av detta protein i hjärnan, och föreligger det härvidlag någon skillnad mellan personer som försökt begå självmord och friska kontroller? (Artikel III)
3. Är serotoninrelaterade gener av betydelse för uppkomsten av PMD? (Artikel IV)

RESULTAT OCH KONKLUSION

Resultaten i denna avhandling visar följande:

1. De undersökta polymorfismerna i *HTR2C* (Cys23Ser och -759C>T) och i *SLC6A4* (5-HTTLPR) visade sig vara olika fördelade mellan underviktiga och överviktiga individer. Våra resultat utgör starkt stöd för hypotesen att 5-HT_{2C}-receptorn påverkar födointag, och att variation i den gen som kodar för denna receptor härmed kan påverka kroppssvikt. Vad gäller associationen mellan *SLC6A4* och vikt får våra fynd däremot ses som preliminära, och bör tolkas med försiktighet till dess att de blivit replikerade i en oberoende studie.

2. Genetiska varianter av två polymorfismer i *SLC6A4* var associerade med lägre mängd serotonintransportör i hjärnan, framför allt hos individer som hade försökt begå självmord. Då de studerade grupperna var små får denna observation ses som preliminär, men den är av intresse *dels* såtillvida att den studerade polymorfismen *bör* påverka mängden av detta protein, och *dels* såtillvida att det länge föreslagits att det föreligger skillnader mellan personer som utför självmordsförsök och kontroller vad avser serotonerg transmission i hjärnan.

3. Alleler i de gener som kodar för transkriptionsfaktorn GATA2 och för en subenhet till den serotonerga 5-HT₃-receptorn, kallad 5-HT_{3B}, visade sig vara olika fördelade mellan kvinnor med PMD och kontroller från normalpopulationen. Studien är den största associationsstudie som hittills utförts vad avser PMD, och resultaten ter sig robusta. Sambandet mellan 5-HT₃-receptorn och PMD är av särskilt intresse eftersom denna receptor sedan tidigare ansetts vara involverad i regleringen av ilska och aggression, och eftersom irritabilitet är kardinalsymptomet vid PMD. Betydelsen av en variant av GATA2-receptor-genen för uppkomst av PMD illustrerar det potentiella värdet av att analysera de gener som kodar för transkriptionsfaktorer av betydelse för de serotonerga neuronens utveckling. Denna grupp av kandidatgener har till nu ägnats alltför litet intresse i studier av gener av möjlig betydelse för serotoninrelaterade psykiska sjukdomar.

Sammantaget utgör resultaten av våra studier stöd för synsättet att variationer i gener av betydelse för den serotonerga transmissionen kan medverka till inter-individuella skillnader i beteende.

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