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2

SINGLE-NUCLEOTIDE POLYMORPHISM PROFILING OF 22 CANDIDATE GENES IN MOOD AND ANXIETY DISORDERS

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CONTENTS

LIST OF ORIGINAL PUBLICATIONS

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ABBREVIATIONS

ACTH	adrenocorticotropin hormone
ANKK1	ankyrin repeat and kinase domain containing 1
APEX	arrayed primer extension
AVP	arginine vasopressin
BDNF	brain-derived neurotrophic factor
BPA	bipolar disorder with comorbid anxiety disorders analysis group
BPD	bipolar disorder; bipolar disorder extended analysis group
CCK	cholecystokinin
CCKAR	cholecystokinin A receptor
CCKBR	cholecystokinin B receptor
CNS	central nervous system
COMT	catechol-O-methyltransferase
CRH	corticotropin releasing hormone
DA	dopamine
DIDMOAD	diabetes insipidus, diabetes mellitus, optic atrophy, deafness
DNA	deoxyribonucleic acid
DRD1	dopamine receptor D1
DRD2	dopamine receptor D2
DRD3	dopamine receptor D3
DRD4	dopamine receptor D4
DRD5	dopamine receptor D5
DSM-IV	Diagnostic and Statistical Manual of Mental Disorders, 4th edition
dNTP	deoxyribonucleotide triphosphate
dTTP	deoxythymidine triphosphate
dUTP	deoxyuridine triphosphate
DZ	dizygotic
GABA	gamma-aminobutyric acid
GABRA5	gamma-aminobutyric acid (GABA) A receptor, alpha 5
GAD	generalized anxiety disorder
HPA axis	hypothalamic-pituitary-adrenal axis
HT	haplotype
HTR1A	5-hydroxytryptamine (serotonin) receptor 1A
HTR1B	5-hydroxytryptamine (serotonin) receptor 1B
HTR2A	5-hydroxytryptamine (serotonin) receptor 2A
HTR2C	5-hydroxytryptamine (serotonin) receptor 2C
HTR3A	5-hydroxytryptamine (serotonin) receptor 3A
HTR7	5-hydroxytryptamine (serotonin) receptor 7
HWE	Hardy-Weinberg equilibrium
LC	locus coeruleus
LC-NA	locus coeruleus-noradrenaline

LD	linkage disequilibrium
LOD score	logarithm base 10 of the likelihood ratio
MAOA	monoamine oxidase A
MD	major depressive disorder without any comorbidity analysis group
MDA	major depressive disorder with comorbid anxiety disorders analysis group
MDD	major depressive disorder; major depressive disorder extended analysis group
M.I.N.I.	Mini International Neuropsychiatric Interview
mRNA	messenger ribonucleic acid
MZ	monozygotic
NA	noradrenaline
OCD	obsessive-compulsive disorder
OPRD1	opioid receptor, delta 1
OPRK1	opioid receptor, kappa 1
OPRM1	opioid receptor, mu 1
PCR	polymerase chain reaction
PD	panic disorder; panic disorder without any comorbidity analysis group
PDA	panic disorder extended analysis group
PDC	panic disorder with comorbid major depressive disorder analysis group
PENK	proenkephalin
PNS	peripheral nervous system
POMC	proopiomelanocortin
RE-MDD	recurrent early-onset major depression
SLC6A2	solute carrier family 6 (neurotransmitter transporter, noradrenaline), member 2
SLC6A3	solute carrier family 6 (neurotransmitter transporter, dopamine), member 3
SLC6A4	solute carrier family 6 (neurotransmitter transporter, serotonin), member 4
SNP	single-nucleotide polymorphism
TH	tyrosine hydroxylase
TPH1	tryptophan hydroxylase 1
WFS1	Wolfram syndrome 1 (wolframin)
3’-UTR	3’-untranslated region
5-HT	serotonin
5’-UTR	5’-untranslated region

1. INTRODUCTION

Mood disorders are common psychiatric diseases. These disorders are among the most prominent causes of disability and the second leading source of disease burden (Murray and Lopez, 1996). The dramatic impact of mood disorders on distress to the affected individual and his or her family, lifetime disability, and suicide highlights the importance of etiologic research to inform treatment and prevention (Merikangas et al., 2002). Major depressive disorder is the most common form out of the mood disorders. The other mood disorders are bipolar disorder, dysthymia, and cyclothymia (Kalia, 2005). Another group of common psychiatric disorders are anxiety disorders which are characterized by inappropriate fear response (increased fearfulness). They are divided into five main categories: panic disorder, social anxiety disorder, generalized anxiety disorder, obsessive-compulsive disorder, and posttraumatic stress disorder (Nutt, 2005). The need to study mood and anxiety disorders is reasoned by relative inefficiency of available drug treatment. There is evidence that only one third of patients respond favourably to antidepressant drugs. One third does not respond at all, and in clinical trials, at least one third responds to placebo (Costa E Silva JA, 2005). Additionally, even the newer generation of antidepressants has side effects, and response to treatment is slow (Nemeroff and Owens, 2002). This situation reflects our limited understanding of the fundamental mechanisms of psychiatric diseases. Therefore new targets for drug development are needed. Molecular genetic approach could be helpful in defining susceptibility genes for mood and anxiety disorders.

Both mood and anxiety disorders are complex disorders caused by genetic and environmental factors and interactions between them. Genetic dissection of psychiatric disorders started already in the middle of the last century, but few causative genes are known nowadays, and pathophysiological mechanisms need further investigation. Determining chromosomal loci harbouring susceptibility genes for psychiatric disorders and establishing robust associations between them and new candidate genes could give new targets in antidepressant and anxiolytic treatment and helps to describe genetic background of psychiatric diseases. The purpose of this study was to find associations between three different psychiatric disorders: major depressive disorder, bipolar disorder, and panic disorder, and genetic markers from 22 candidate genes.

2. REVIEW OF LITERATURE

2.1. Mood disorders

Mood disorders are divided into two large distinct categories according to clinical diagnosis: unipolar major depressive disorder (mainly characterized by depressed mood) and bipolar disorder (periods of elevated mood are also presented).

2.1.1. Major depressive disorder (MDD)

2.1.1.1. Characteristics of MDD

Major depressive disorder (MDD) is considered to be a heterogeneous complex disease resulting from both genetic and environmental factors (Sullivan et al., 2000). MDD is often recurrent, tends to be chronic in course, and comorbidity with other psychiatric disorders and also physical illnesses is common (Hamet and Tremblay, 2005; Costa E Silva JA, 2005). MDD is characterized by one or more major depressive episodes without a history of manic, mixed, or hypomanic episodes. A major depressive episode is defined by two or more weeks of depressed mood or impaired enjoyment, with symptoms such as disturbed sleep and appetite, psychomotor changes, reduced concentration, excessive guilt, and suicidal thoughts or actions. The episode must be accompanied by distress or impairment in social, occupational, or other important areas of functioning (Zubenko et al., 2002).

2.1.1.2. Epidemiology of MDD

Epidemiologic studies of MDD have revealed a population prevalence of 2%–19% and a risk for first-degree relatives of MDD probands 5%–25% (Lesch, 2004). Meta-analysis of family studies provided strong evidence in support of MDD familiality and showed that the relative risk of an association between MDD patients and MDD first-degree relatives is 2.84 (Sullivan et al., 2000). Two clinical features of the probands can predict a greater MDD risk in first-degree relatives: recurrent episodes and early age of onset (Hamet and Tremblay, 2005). Women are affected twice as likely as men (Zubenko et al., 2002). Gender differences in MDD are probably not caused by differences in genetic heritability. Twin studies have shown that the concordance rate between monozygotic (MZ) twin pairs is 50% for MDD (Oswald et al., 2004). Overall heritability of MDD is likely to be in the range of 31–42% (Sullivan et al., 2000), but could be as high as 70% (Lesch, 2004). Suicide has been reported to

occur in 10–15% of patients previously hospitalized for major depressive disorder (Angst et al., 1999).

2.1.1.3. Pathogenesis of MDD

Despite decades of research on psychiatric disorders, the etiology and precise biological mechanisms that underlie mood disorders, including MDD, and normal mood states are still poorly understood (Fuchs et al., 2004). Major depressive disorder appears to have a multi-factorial etiology (Joffe et al., 1999), and these factors are biological, psychological, and sociocultural (Kalia, 2005). From the biological point of view, there is increasing evidence that psychiatric disorders not only have a neurochemical basis but are also associated with morphological alterations in the central nervous system (CNS) neuronal and/or glial cells (Fuchs et al., 2004). Studies using neuroimaging techniques have revealed changes in volumes of frontal cortex, caudate nucleus, putamen, pituitary gland, hippocampus, and the core nuclei of amygdala in the case of recurrent early-onset major depression (RE-MDD) (Sheline, 2000). Imaging studies have provided support for the neural network hypothesis. This hypothesis proposes that mood disorders reflect problems in information processing within particular neural networks in the brain, and that antidepressant drugs and other treatments that alleviate depression gradually improve information processing within these networks (Castren, 2005). A key aspect of the network view is the recognition that the principal role of the nervous system is not to handle chemicals but to store and process information. It is noted that the chemical and network hypotheses are not mutually exclusive but complementary (Castren, 2005). Still, the stress diathesis model, in which genetic vulnerability, early stressors, and immediate acute stressors interact in the pathophysiology of MDD, has gained increased acceptance (Joffe et al., 1999). Longer descriptions of neurochemical hypotheses can be found in Chapter 2.3.

2.1.1.4. Genetics of MDD

The first genome-wide linkage survey identified nineteen chromosomal regions which reached genome-wide statistical significance in the case of unipolar mood disorders. The following eight loci exceeded the criterion of high significance: 1p36–35, 2q35–36, 5q15–21, 5q21–23, 10q11–21, 11pter-p15, 11q13–14, 18q21 (Zubenko et al., 2003). Genome-wide linkage analysis for identifying chromosomal loci of genes that influence the risk of suicidal behaviour in the context of mood disorders revealed that the highly significant regions were 2p12, 6q12, 8p22-p21, and Xq25–26.1. These findings provide evidence for suicide risk loci that are independent of susceptibility loci for

mood disorders (Zubenko et al., 2004). Linkage studies on MDD have revealed that the following loci are related with disease: 2q35 with recurrent early-onset MDD in women (Zubenko et al., 2002; Philibert et al., 2003) and 15q25–26 in RE-MDD without sex-specificity (Holmans et al., 2004). Another sex-specific region has been discovered with genome-wide linkage analysis, namely 12q22-q23.2 in males with MDD (Abkevich et al., 2003).

Association studies in MDD have mainly focused on genes of monoaminergic pathways, but other candidate genes are also studied. The results are still contradictory and do not yield robust associations. Shortly, meta-analysis suggested the study of possible association between MDD and the tyrosine hydroxylase (TH) gene further in larger subject samples (Furlong et al., 1999). The comprehensive European multicentre study failed to find an association with a polymorphism in tryptophan hydroxylase gene (TPH) (Souery et al., 2001). The monoamine oxidase A (MAOA) gene has been studied in several association studies with conflicting results (Kunugi et al., 1999; Ho et al., 2000; Lin et al., 2000; Syagailo et al., 2001). The opposite results have also been revealed by studies of catechol-O-methyltransferase (COMT) gene (Kunugi et al., 1997; Ohara et al., 1998; Frisch et al., 1999; Henderson et al., 2000; Massat et al., 2005). Various studies support a relative influence of serotonin transporter (SLC6A4) in MDD (Battersby et al., 1996; Ogilvie et al., 1996; Hoefgen et al., 2005), but lack of association is also shown (Mendlewicz et al., 2004). Of serotonin receptors genes, HTR2C is indicated in MDD (Lerer et al., 2001). HTR2A (Oswald et al., 2003), HTR3A, and HTR7 genes were studied in several association studies, but no definitive positive association was found. In most studies dopamine receptor D1, dopamine receptor D3, dopamine receptor D4, and dopamine receptor D5 genes (DRD1, DRD3, DRD4 and DRD5) are not implicated in MDD (Serretti et al., 2000). However, gamma-aminobutyric acid (GABA) A receptor, alpha 5 gene (GABRA5) is implicated (Oswald et al., 2004; Oruc et al., 1997).

2.1.2. Bipolar disorder (BPD)

2.1.2.1. Characteristics of BPD

Bipolar disorder (also known as manic depressive illness) (BPD) is a complex genetic disorder where the core feature is a pathological disturbance in mood (affect) ranging from extreme elation or mania to severe depression usually accompanied by disturbances in thinking and behaviour, which may include psychotic symptoms, such as delusions and hallucinations (Craddock and Jones, 1999). It has been suggested that bipolar disorder is a heterogeneous set of diseases with a high variation in symptomatology and course (Ackenheil, 2001). The complex nature of BPD has evoked discussion about the categorization of mood disorders and differentiation of bipolar disorder from unipolar major

depressive disorder. Joffe and colleagues proposed that bipolar disorder constitutes two separate but inter-related disorders: depression and mania (Joffe et al., 1999). The first illness, which is a component of bipolar disorder, depression, is a common disorder. It is heterogeneous in nature and is not different from the broad range of depressive disorders that constitute unipolar depression. The second illness, mania, is a much rarer disorder, which is also more homogeneous in its manifestation than depression (Joffe et al., 1999). The other view is that unipolar major depressive disorder and bipolar disorder are distinctive disorders in the case of which depressions within unipolar and bipolar disorders are qualitatively different in etiology and phenomenology. Drawing on the strong evidence that mania is biologically driven, bipolar depression has been seen as more endogenous than unipolar depressive disorder (Cuellar et al., 2005). There is evidence for both views, but it is not unequivocal and conclusive as yet.

2.1.2.2. Epidemiology of BPD

The lifetime prevalence of BPD is 1%, and it is similar in males and females. Family, twin, and adoption studies provide robust evidence for a major genetic contribution to risk (Craddock and Jones, 1999). Relatives of affected individuals have an elevated risk for the disorder, rising from the 1% population risk to 5–10% in first-degree relatives, 15–20% in co-twins of affected DZ (dizygotic) twins and to 75–80% in co-twins of affected MZ twins (Evans et al., 2000; Craddock and Jones, 1999). At least 25% to 50% of patients with bipolar disorder also attempt suicide at least once (Jamison, 2000).

2.1.2.3. Pathogenesis of BPD

The etiology of bipolar disorder could be described by a model with structural and functional components, which also addresses the role of stressors, coping mechanisms, and psychophysical disposition (Baumann et al., 2003). Several studies have identified distinct biological correlates for mania. These include increased dopamine (DA) activity, hyperpolarization in transmembrane potentials, and changes in dopamine DRD3 receptor mechanisms (Cuellar et al., 2005). According to neuroanatomical research, the underlying functional correlate of cognitive deficits in the case of BPD may be white matter lesions (‘signal hyperintensities’) in the frontal lobes and basal ganglia — regions that are critical for executive function, attention, accelerated information processing, learning and memory, and regulation of affect (Bearden et al., 2001). Decreased prefrontal cortex activity, as well as changes in amygdala activity, has been found both in the case of bipolar depression and unipolar depression (Cuellar et

al., 2005). Neurochemical research has focused mainly on regulatory deficits involving monoamines; other neurotransmission circuits are also studied.

2.1.2.4. Genetics of BPD

According to genome-wide scans and reviews of linkage studies, the susceptibility regions of BPD include 1q31–32, 2p13–16, 4p16–p15, 4q31, 6pter–p24, 9p22.3–21.1, 10p14, 10q21–26, 12q23, 13q32, 14q24.1–32.12, 16p, 17q, 18p11, 18q12–q22, 21q22, 22q11–q12 and Xq26–28 (Maziade et al., 2004; Liu et al., 2003; Segurado et al., 2003; Dick et al., 2003; Oswald et al., 2004). Meta-analysis of eleven whole-genome linkage scans obtained the most significant results for 13q and 22q (Badner and Gershon, 2002).

Association studies in BPD have so far mainly focused on genes of monoaminergic pathways as in the case of MDD. No robust associations have been found between different candidate genes and BPD. Two meta-analyses failed to confirm the implication of tyrosine hydroxylase (TH) gene in the case of BPD (Furlong et al., 1999; Turecki et al., 1997). No association was found between polymorphism in tryptophan hydroxylase gene (TPH) (Souery et al., 2001). The polymorphisms of monoamine oxidase A (MAOA) gene have been studied in many association studies with conflicting results (Lim et al., 1995; Rubinsztein et al., 1996; Kunugi et al., 1999; Ho et al., 2000; Lin et al., 2000; Preisig et al., 2000; Syagailo et al., 2001). Many studies have failed to show an implication of COMT gene (Gutierrez et al., 1997; Kunugi et al., 1997; Ohara et al., 1998; Massat et al., 2005). A few studies have concluded that serotonin transporter has no major role in the etiology of BPD (Craddock et al., 2001; Mendlewicz et al., 2004). Studies on dopamine transporter and noradrenaline transporter are largely negative (Craddock et al., 2001). Of serotonin receptor genes, HTR2C is indicated in BPD (Lerer et al., 2001); HTR2A is not associated with BPD (Mahieu et al., 1997). HTR3A and HTR7 genes were studied in several association studies, but no definitive positive association was found (Potash and DePaulo, Jr., 2000). In the case of extensively studied dopamine receptor 2 gene (DRD2) most studies are negative, but a recent large study showed association (Massat et al., 2002b). In most studies dopamine receptor 1, dopamine receptor 3, dopamine receptor 4, and dopamine receptor 5 genes (DRD1, DRD3, DRD4 and DRD5) were not implicated in BPD (Asherson et al., 1998; Lim et al., 1994; Savoye et al., 1998; Souery et al., 1996; Elvidge et al., 2001). Gamma-aminobutyric acid (GABA) A receptor, alpha 3 (GABRA3) (Massat et al., 2002a) and gamma-aminobutyric acid (GABA) A receptor, alpha 5 genes (GABRA5) are implicated in BPD (Papadimitriou et al., 1998) (Oswald et al., 2004). There was no association between CCK gene polymorphisms and BPD (Bowen et al., 1998).

2.2. Panic disorder (PD)

2.2.1. Characteristics of PD

Panic disorder (PD) is an anxiety disorder characterized by recurrent unprovoked anxiety attacks distinguished by such symptoms as palpitations, chest pain, dyspnoea, choking, tremors, faintness, and sweating, in addition to fears of dying, losing control, or going crazy (van West and Claes, 2004). The first attacks are frequently triggered by physical illnesses, psychosocial stress, certain drug treatments or drugs of abuse that increase the activity of neural systems involved in fear responses. Attacks can be pharmacologically precipitated by carbon dioxide, caffeine, sodium lactate, yohimbine, fenfluramine, m-chlorophenylpiperzine, noradrenaline (NA), adrenaline, and analogues of cholecystokinin (CCK) (Lesch, 2001; Gorman et al., 2000).

2.2.2. Epidemiology of PD

The lifetime prevalence in different countries has been estimated to be 1.6%–2.2%, and females are twice more affected than men. The heritability is between 30% and 62% based on two twin studies (van West and Claes, 2004). Meta-analysis of combined family and twin data indicated that additive genetics and individual environment account for liability to panic disorder with a heritability estimate of 48% (Hettema et al., 2001). The risk ratio for relatives of affected individuals is 3–8% (Merikangas and Risch, 2003). Relative risks of the co-occurrence of PD with agoraphobia and major depression range from 7.5 to 21.4 and from 3.8 to 20.1, respectively (Lesch, 2001).

2.2.3. Pathogenesis of PD

Evidence suggests that both heritable factors and stressful life events, particularly in early childhood, are responsible for the onset of panic disorder. According to a recent neuroanatomical hypothesis, patients with panic disorder inherit an especially sensitive fear mechanism of the central nervous system that has at its centre the central nucleus of the amygdala and includes the hippocampus, thalamus, and hypothalamus, as well as the periaqueductal gray region, locus coeruleus (LC), and other brainstem sites. Amygdala receives direct sensory input from brainstem structures and the sensory thalamus, enabling a rapid response to potentially threatening stimuli; it also receives afferents from cortical regions involved in the processing and evaluation of sensory information. Potentially, a neurocognitive deficit in these cortical processing pathways could result in the misinterpretation of sensory information (bodily

cues) known to be the hallmark of panic disorder, leading to an inappropriate activation of the 'fear network' via misguided excitatory input to the amygdala (Gorman et al., 2000).

The complex fear network operates through many neurotransmitters, and therefore perturbation of mutual modulation ('cross talk') between key brain transmitter systems (serotonin (5-HT), noradrenaline, gamma-aminobutyric acid (GABA)) and several neuropeptides, such as adrenocorticotropic hormone (ACTH), corticotropin releasing hormone (CRH), cholecystokinin, neuropeptide-Y, may underlie the pathogenesis of panic-anxiety (Coplan and Lydiard, 1998; Bergink et al., 2004).

2.2.4. Genetics of PD

So far the results of the molecular genetic studies of panic disorder have been quite modest. Linkage findings are diverse, and the findings of association studies need further confirmation. The number of linkage studies in PD has been limited compared to the number of genome-wide linkage studies done on bipolar disorder and schizophrenia (van West and Claes, 2004). The first complete genome scan for PD revealed six loci with highest LOD scores between 1 and 2 (1p, 20p, 7p, 17p, 20q, X/Y) (Knowles et al., 1998). Further linkage studies have revealed mostly suggestive linkages for the following chromosomal locations: 1q, 7p15, 11p, 12q13, 13q (Smoller et al., 2001; Crowe et al., 2001; Gelernter et al., 2001; Hamilton et al., 2003; Weissman et al., 2000). Association studies have yielded contradictory results about associations between the selected candidate genes and panic disorder. Associations with PD have been found with genes of $alpha_1$- and $alpha_{2A}$-adrenergic receptors, HTR2A, MAOA, COMT, CCK, CCKBR (van West and Claes, 2004; Kennedy et al., 1999b).

2.3. Neurochemical substrates for mood and anxiety disorders

The proposed etiological models for both mood and anxiety disorders involve neurochemical substrates, and interactions between the latter serve as a cause for their development.

2.3.1. Hypothalamic-Pituitary-Adrenal axis

Clinical and preclinical studies have gathered substantial evidence that stress response alterations play a major role in the development of major depressive

disorder, panic disorder, and post-traumatic stress disorder (Strohle and Holsboer, 2003). There is also evidence demonstrating abnormalities of the hypothalamic-pituitary-adrenal (HPA) axis in bipolar disorder. Hypercortisolism may be central to the pathogenesis of depressive symptoms and cognitive deficits, which may in turn result from neurocytotoxic effects of elevated cortisol levels. Manic episodes may be preceded by increased ACTH and cortisol levels, leading to cognitive problems and functional impairments (Daban et al., 2005). Essential to stress response is the activation of hypothalamic-pituitary-adrenal axis (Muller et al., 2002). The HPA axis consists of a feedback loop including the hypothalamus, pituitary, and adrenal glands. In addition to these structures, the axis receives important regulation from the hippocampus, amygdala, bed nucleus of the stria terminalis, and paraventricular nuclei. During stress the HPA axis is activated, and the hypothalamus secretes two hormones – corticotropin-releasing hormone (also called corticotropin-releasing factor (CRF)) and arginine vasopressin (AVP), which act on the pituitary to increase adrenocorticotropin hormone release. ACTH is carried in the blood to the adrenal cortex and interacts with receptors on adrenocortical cells that stimulate the production and release of cortisol. Cortisol is the adrenal glucocorticoid stress hormone; it binds to at least two types of receptors and acts as a negative feedback to the pituitary and hypothalamus (Varghese and Brown, 2001). The end product of the HPA axis, cortisol, arouses the body to cope with a challenging situation by increasing the rate and the strength of heart contractions, sensitizing the blood vessels to the actions of noradrenaline, and affecting many metabolic functions, mainly to provide energy that might be necessary for reacting to the stressor (van Duinen et al., 2004). The HPA axis functions in close concert with the locus coeruleus-noradrenaline (LC-NA) system, which is involved in extensive reciprocal innervation of regions throughout the CNS (Mello et al., 2003). However, detailed regulation of HPA axis is obscure.

Frequently reported findings include elevated level of cortisol and CRH, non-suppression in the dexamethasone suppression test, a blunted ACTH response to CRH, and hippocampal volume reduction in major depressive disorder patients (Varghese and Brown, 2001). Data show that the cortisol response to the combined dexamethasone/corticotrophin-releasing hormone test is abnormal in patients with bipolar disorder (Watson et al., 2004). Also patients with BPD show a significantly enhanced salivary cortisol response to waking (Deshauer et al., 2003), and smaller pituitary volumes (Sassi et al., 2001) compared with control subjects. Panic disorder patients reveal elevated basal salivary, plasma-free and total levels of cortisol (Wedekind et al., 2000) and a subtle elevation of the cortisol level during spontaneous panic attacks (Bandelow et al., 2000). However, the results of different studies are inconclusive. Other interruptions in the HPA system may also lead to depression.

2.3.2. Monoamine hypothesis

There is evidence that the pathology of depression involves dysfunction of monoamine neurotransmitter circuits in the central nervous system, particularly serotonin, noradrenaline, and dopamine. Drugs that selectively antagonize 5-HT, NA, and possibly DA transporters are proven to be clinically effective antidepressants (Nemeroff and Owens, 2002). As drugs that alleviate depression increase extracellular monoamine concentrations, it was proposed that depression might be produced by a serotonin or noradrenaline deficiency at functionally important receptor sites in the brain, a proposal that is now known as the monoamine hypothesis of depression (Castren, 2005). To date the monoamine hypothesis has evolved into what could be called a chemical or molecular hypothesis of depression. This hypothesis presumes that mood disorders are produced by long-term changes in the production or activity of molecules in the brain and that antidepressants function by counteracting these molecular changes. (Castren, 2005). The monoamine hypothesis has remained insufficient to explain the pathogenesis of mood disorders, and new targets have emerged during research.

2.3.2.1. Serotonin system

Serotonin (5-hydroxytryptamine) is a classical neurotransmitter and has been implicated in the etiology of numerous disease states including depression, anxiety, social phobia, schizophrenia, and obsessive–compulsive and panic disorders. In addition to migraine, hypertension, pulmonary hypertension, eating disorders, vomiting and, more recently, irritable bowel syndrome. 5-HT produces its effects through 13 distinct heptahelical, G-protein-coupled receptors and one ligand-gated ion channel. These receptors are divided into seven distinct classes (HTR1 to HTR7) largely on the basis of their structural and operational characteristics. 5-HT and its receptors are found both in the central and peripheral nervous systems, as well as in a number of non-neuronal tissues in the gut, cardiovascular system, and blood (Hoyer et al., 2002). In the brain serotonergic neuron clusters may be allocated, on the basis of their distribution and main projections, into two groups: the rostral group, confined to the mesencephalon and rostral pons, with major projections to the forebrain, and the caudal group, extending from the caudal pons to the caudal portion of the medulla oblongata, with major projections to the caudal brainstem and to the spinal cord (Hornung, 2003). Several researchers have emphasized the importance of deficits in 5-HT regulation of dopamine and/or noradrenaline in the etiology of mood disorders. There is substantial evidence for abnormalities in 5-HT functioning in both unipolar and bipolar depressions (Cuellar et al., 2005). The importance of serotonergic neurotransmission for the pathogenesis of depression is suggested clinically by the efficacy of serotonin re-uptake

inhibitors, the first-line treatment of depression, most related anxiety disorders, and by induction of depression by tryptophan depletion in susceptible individuals (Pezawas et al., 2005).

2.3.2.2. Dopamine system

Dopamine is the catecholamine neurotransmitter, that controls a variety of functions including locomotor activity, cognition, emotion, positive reinforcement, food intake, and endocrine regulation. This catecholamine also plays multiple roles in the periphery as a modulator of cardiovascular function, catecholamine release, hormone secretion, vascular tone, renal function, and gastrointestinal motility. DA receptors are classified as D_1-like receptor subtypes (DRD1 and DRD5) and D_2-like receptor subtypes (DRD2, DRD3, and DRD4) (Missale et al., 1998). Four main dopaminergic pathways have been identified in the central nervous system. The ventral tegmental area is the place of origin of two projection pathways towards the cortex (the mesocortical pathway) and the limbic area (the mesolimbic pathway); the hypothalamus is the place of origin of a projection towards the pituitary gland that controls prolactin secretion (the tuberoinfundibular pathway) and a projection extending from the substantia nigra to the striatum (the nigrostriatal pathway) (Dailly et al., 2004). Experimental studies with animal models of depression and human studies implicate the role of the dopamine system in depression (Dailly et al., 2004). It has been proposed that increased dopaminergic activity either induced by a high release of dopamine or a reduced puffer capacity of the synaptic vesicles, or a higher sensitivity of dopamine receptors, will cause manic symptoms, whereas a decrease in dopaminergic activity results in depressive symptoms (Ackenheil, 2001). Comparison of depressive episodes in unipolar and bipolar depression shows that dopamine activity does not differ between them (Cuellar et al., 2005). Evidence in support of decreased dopamine activity in the case of major depressive disorder has shown that the brains of MDD patients exhibited reduced concentration gradients of venoarterial homovanillic acid, a dopamine metabolite, compared to healthy controls (Lambert et al., 2000). McLean and colleagues found that depletion of tyrosine, precursor of dopamine synthesis, in healthy volunteers resulted in the reduction in tyrosine availability in the brain. Their neuropsychological findings were similar to those reported in previous studies of major depressive disorder (McLean et al., 2004). Tyrosine depletion has been shown to specifically attenuate dopaminergic effects of methamphetamine administration and symptoms of acute mania (McTavish et al., 2001).

2.3.2.3. Noradrenaline system

The neurotransmitter noradrenaline is found in most brain regions. Mapping studies have indicated that most noradrenergic neurons arise either in the LC of the pons or in neurons of the lateral tegmental portion of the reticular formation. The most important noradrenergic projections with regard to psychological functions arise from the LC and ascend from the brainstem to innervate the thalamus, dorsal hypothalamus, hippocampus, and cortex. Adrenergic responses and receptors are classified into two overarching categories, alpha and beta. Considerable experimental and clinical evidence supports the role of NA in the etiology of depression (Elhwuegi, 2004). The brains of major depressive patients exhibited reduced venoarterial NA concentration gradients compared to healthy controls (Lambert et al., 2000). One of the most robust changes noted in BPD is elevated NA levels during acute mania (Joffe et al., 1999). Although NA is presumably an important factor in mood disorders, due to limited technical possibilities the genes of the adrenergic system remained beyond the scope of the present study.

2.3.3. Cholecystokinin system

Cholecystokinin is a brain/gut peptide. It is one of the most abundantly distributed neuropeptides in cerebral cortex, striatum and hippocampus. CCK is present in many important neuronal pathways and co-localized with several classic neurotransmitters, such as DA, GABA, 5-HT, and opiates (Beinfeld, 2001; Rotzinger and Vaccarino, 2003). CCK peptide, initially characterized as a 33-amino-acid sequence, is present in a variety of biologically active molecular forms derived from a 115-amino-acid precursor molecule (prepro-CCK), such as CCK-58, CCK-39, CCK-33, CCK-22, sulphated CCK-8 and CCK-7, unsulphated CCK-8 and CCK-7, CCK-5, and CCK-4 (Noble et al., 1999). There are two types of CCK receptors: CCKAR and CCKBR. CCKBR are widely distributed throughout the central nervous system, whereas CCKAR are only found in certain regions, such as the nucleus tractus solitarius, area postrema, interpenduncular nucleus, posterior hypothalamus, and the nucleus accumbens (Ise et al., 2003). CCK and its receptors have been extensively studied as involved in the pathogenesis of emotional disorders, especially anxiety and panic disorders (Ise et al., 2003; Kennedy et al., 1999b; Hattori et al., 2001). In humans, administration of CCK-4 and other CCKBR agonists produces panic attack in healthy volunteers and in patients with panic disorders (Carrasco and Van de Kar, 2003; Bradwejn, 1992; Bradwejn and Koszycki, 1994). PD patients are hypersensitive to CCKBR receptor stimulation compared to healthy volunteers and patients with other anxiety disorders, and they differ from healthy subjects in CCK metabolism and genetic characteristics of the CCKBR receptor system (Bradwejn and Koszycki, 2001). Patients with major depressive

disorder with no history of panic attacks do not exhibit any augmented behavioural or cardiovascular response to CCK-4 compared to normal controls. The finding that CCK-4 did not exacerbate depressive symptoms in MDD patients provides additional support that the effects of CCK-4 are specific to panic attacks and is in keeping with the idea that CCK-4 is an ideal panicogenic agent (Koszycki et al., 2004).

2.3.4. Opioid system

The endogenous opioid system is composed of three families of opioid peptides – enkephalin, dynorphin, β-endorphin, and three receptor types, respectively: delta-1 (OPRD1), kappa-1 (OPRK1), and mu-1 (OPRM1) opioid receptors. This neuromodulatory system has been implicated in the control of behaviours that are essential for self and species survival, including responses to noxious information and stress, reward, and motivation. Opioid peptides and their receptors also control autonomic functions, including respiration, thermo-regulation, and gastrointestinal motility, and they also modulate immune responses (Kieffer, 1999). It is suggested that the endogenous opioid system is possibly directly involved in the pathogenesis of major depressive disorder. One hypothesis is that MDD may arise from underactivity of the opioid system (Lichtigfeld and Gillman, 2003). There is also evidence from animal experiments that the mu-1 and delta-1 opioid receptors may play a role in anxiety and depression. One might therefore expect that polymorphisms of these genes in humans are associated with anxiety and depression (Jorm et al., 2002). The indirect effect of opioids in mood and anxiety disorders may be related with regulation of the HPA axis by endogenous opioids (Szeto, 2003). In rats opioids stimulate ACTH and corticosterone secretion while an inhibition of ACTH and cortisol levels has been observed in man. In both species naloxone, an opiate antagonist, stimulates the release of ACTH and produces a significant dysphoric effect in depressed patients suggesting a tonic suppression by endogenous opioids (Pfeiffer and Herz, 1984; Martin del Campo et al., 2000). As proopiomelanocortin (POMC) is a common precursor for adrenocortico-tropin hormone and β-endorphin, and patients with mood and anxiety disorders have disturbances in hypothalamic-pituitary-adrenal system, POMC is a good target for association studies (Galard et al., 2002). In animals dynorphin exerts action on the HPA axis via activation of hypothalamic OPRK1 receptors leading to the release of CRH and AVP (Szeto, 2003); in rats β-endorphin is involved in the restraint stress-induced secretion of ACTH, and CRH mediates the β-endorphin-induced secretion of ACTH (Yamauchi et al., 1997). It has been also shown that administration of opioid antagonist naltrexone may precipitate panic attacks (Maremmani et al., 1998). Still the exact modulating nature of endopioid system on HPA axis remains unclear.

2.3.5. Intracellular mechanisms

In recent years researchers have taken an interest in intracellular mechanisms and second messenger systems in relation to mood disorders, especially to BPD. Hypotheses concerning the pathophysiology of bipolar disorders on second messenger systems are based on the effects of mood-stabilizing drugs that interfere with these systems. Many neurotransmitter receptors are G-protein coupled receptors which stimulate or inhibit mainly three second messenger systems: adenylyl cyclase, phospholipase C, and ion channels (Ackenheil, 2001). Some research provides evidence for differences in the intracellular signal transduction system in bipolar disorder compared to unipolar depression (Suzuki et al., 2001). Further research is needed, however.

2.4. Comorbidity of mood and anxiety disorders

The close relationship between anxiety and depressive disorders has long been recognized (Dindo and Coryell, 2004). Data suggest that patients with MDD and comorbid anxiety diagnoses have worse depressive symptoms, a worse clinical course, and a higher risk of suicide (Young et al., 2004; Brown et al., 1996; Schaffer et al., 2000; Lydiard and Brawman-Mintzer, 1998). Disturbance in HPA axis has been detected in patients with comorbid MDD and anxiety disorder but not in 'pure' phenotypes suggesting interactive presence of both depressive and anxiety symptoms (Young et al., 2004). Findings indicate the importance of temporal sequencing when panic disorder and major depressive disorder coexist. Subjects who had relatives with primary MDD and secondary PD were substantially more likely to have MDD themselves than subjects whose relatives had primary PD and secondary depression. These subjects, in turn, were not more likely to have MDD than the subjects who did not have any first-degree relatives with any major psychiatric illness (Dindo and Coryell, 2004).

2.5. Wolfram syndrome

Wolfram syndrome (MIM 222300) is a rare autosomal recessive neuro-degenerative disorder, characterized by diabetes insipidus, diabetes mellitus, optic atrophy, and deafness (acronym DIDMOAD). The characteristic symptoms include juvenile onset diabetes mellitus and progressive bilateral optic atrophy (Kinsley et al., 1995). Patients may later develop diabetes insipidus and deafness, as well as a range of neurological and psychiatric abnormalities, including dementia, psychosis, and affective disorder (Swift et al., 1990). The gene for Wolfram syndrome, wolframin (WFS1), has been

identified in chromosomal region 4p16 (Inoue et al., 1998; Strom et al., 1998). WFS1 gene is a challenging target for psychiatric research because linkage studies indicate 4p16 region as harbouring a putative susceptibility gene for bipolar disorder (Blackwood et al., 1996; Detera-Wadleigh et al., 1999; Ewald et al., 1998; Als et al., 2004). Heterozygous carriers of the gene for the Wolfram syndrome are predisposed to psychiatric disorders as shown by the 26-fold psychiatric hospitalization among them than among non-carriers (Swift et al., 1998; Swift and Swift, 2005a). Function of WFS1 protein is unknown, but due to its proposed impact on the mood disorders, it remains an important target for association studies.

2.6. Genetics of complex diseases

2.6.1. Characteristics of complex diseases

It is acknowledged that most common diseases that have a genetic component are likely to have a complex etiology. There are also hereditary diseases that are in nature monogenic simple mendelian diseases, such as Huntington's disease, cystic fibrosis and early-onset Alzheimer's disease (Thornton-Wells et al., 2004). Mendelian diseases are typically caused by mutation of a single gene that results in an identifiable disease state, the inheritance of which can readily be traced through generations (Chakravarti and Little, 2003). Mutational diversity at each locus is high; each mutation is rare, having occurred in recent human history (no older than 2,000 years) and each mutation is necessary and sufficient to cause the phenotype of interest (Chakravarti, 1999). Mendelian disorders, however, are uncommon (Conneally, 2003).

In the case of complex diseases the underlying genes are likely to be numerous, with no single gene having a major role, and mutations within these genes are common and impart small genetic effects (none of which are either necessary or sufficient) (Chakravarti and Little, 2003). Disease phenotype probably arises in individuals who lie above some biological threshold of risk (Chakravarti, 1999).

The mapping of susceptibility loci for complex diseases may be complicated due to any or all of the following phenomena:

- high population frequency,
- penetrance (i.e. probability of phenotypic expression among individuals with a susceptibility gene),
- variable expressivity (i.e. variation in clinical expression associated with a particular gene),
- gene-gene interaction (i.e. interaction between two or more DNA variations either directly (DNA-DNA or DNA-mRNA interactions) to change

transcription or translation levels, or indirectly by way of their protein products, to alter disease risk separate from their independent effects),

- gene-environment interaction (i.e. expression of genotype only in the presence of particular environmental exposures),
- phenocopies (i.e. presence of a disease phenotype that has a non-genetic basis),
- genetic heterogeneity (i.e. different genes leading to indistinguishable phenotypes),
- epistasis (i.e. masking of the phenotypic effect of alleles at one gene by alleles of another gene), and
- pleiotropy (i.e. capacity of genes to manifest several different phenotypes simultaneously) (Altmuller et al., 2001; Merikangas and Risch, 2003; Thornton-Wells et al., 2004; Altmuller et al., 2001).

2.6.2. Mapping strategies of complex diseases

2.6.2.1. Linkage analysis

The traditional approach to locating a disease gene is linkage analysis, which tests the association between DNA polymorphic markers and affected status within families. After linkage is detected with an initial marker, many other nearby markers may also be examined. Markers showing the strongest correlation with disease in families are assumed to be closest to the disease locus (Merikangas and Risch, 2003). Disease gene regions that are identified by linkage are often large and can encompass hundreds or even thousands of possible genes across many megabases of DNA (Cardon and Bell, 2001).

2.6.2.2. Association study

Linkage analysis has not proven successful in identifying genes for most complex diseases, presumably because the effects of the underlying genes are not strong enough to be detected by linkage. Therefore, genome-wide association studies have been offered as a more powerful approach. Association studies examine candidate genes among affected individuals and unrelated unaffected control subjects (Merikangas and Risch, 2003). An alternative family based, approach tests for preferential transmission of one allele of the marker from heterozygous parents to affected offspring. This is known as the transmission disequilibrium test (Mathew, 2001).

Both approaches are based on the assumption that the single-nucleotide polymorphism (SNP) being tested is the actual sequence variant that causes the

genetic susceptibility or that it is in linkage disequilibrium (LD) with the true susceptibility allele (Mathew, 2001).

The search for association may be random (testing SNPs at regular intervals across the critical region) or use a candidate gene approach, which tests SNPs within genes of particular interest. Candidate genes are selected on the basis of having a known or predicted function and expression profile that is consistent with the disease phenotype because they result from positional cloning, or because they are homologous with animal genes proven relevant in animal models of the disease (Mathew, 2001; Slagboom and Meulenbelt, 2002).

One approach to perform association studies involves testing each putative causal variant for correlation with the disease (the 'direct' approach). An alternative approach (the 'indirect' approach) has been proposed, whereby a set of sequence variants in the genome could serve as genetic markers to detect association between a particular genomic region and the disease, whether or not the markers themselves had functional effects. The idea is that these SNPs are in linkage disequilibrium with disease causing variations, and knowing LD patterns and haplotypes across the genome would reduce the number of genotyping needed SNPs (2003).

2.6.3. DNA markers used for gene mapping

Earlier linkage studies employed restriction fragment length polymorphisms as DNA markers, whereas subsequent studies examined short tandem repeat markers or 'microsatellites' — DNA sequences that show considerable variability among people but have no functional consequences. More recently, linkage and association studies have examined SNPs to track diseases in families (Merikangas and Risch, 2003). SNP is a common DNA sequence variant that alters only one base in a particular sequence of DNA and has an allele frequency of at least 1% in the population (Mathew, 2001; Tabor et al., 2002). Most human sequence variation is attributable to SNPs; SNPs occur (on average) every 1,000–2,000 bases when two human chromosomes are compared. SNPs are mostly biallelic; SNPs have a low rate of recurrent mutation, making them stable indicators of human history. The total number of SNPs has been estimated to be 10 million or more common (>20% minor allele frequency) SNPs in the human population (Sachidanandam et al., 2001; Lai, 2001).

When selecting SNPs for association study, the following points should be considered: functionality of SNP; minor allele frequency should be at least 5% to detect more common variants that probably have less severe effects (Tabor et al., 2002). Polymorphisms with functional consequences are expected to have lower allele frequencies. In fact, the majority of coding region SNPs that change an amino acid have allele frequencies below 5% (Kruglyak and Nickerson, 2001).

2.6.4. Linkage disequilibrium and haplotype blocks

If marker allele and disease allele are close to each other, they may co-segregate through many generations. These alleles are said to be in linkage disequilibrium; they co-occur at frequencies higher than predicted on the basis of their individual allele frequencies (Slagboom and Meulenbelt, 2002).

There are several measures of linkage disequilibrium, one of the earliest was D. The two most common measures are the absolute value of D', and $r2$. The case of $D' = 1$ is known as complete LD. Values of $D' < 1$ indicate that the complete ancestral LD has been disrupted (Ardlie et al., 2002).

In some cases determination of haplotypes or combinations of SNPs that are in LD might offer more power to detect associations than simply measuring individual SNPs (Tabor et al., 2002).

3. AIMS OF THE STUDY

The general aim of the present study was to find possible genetic factors influencing different psychiatric disorders: major depressive disorder, bipolar disorder, and panic disorder. The specific aims were as follows:

1. To find associations between 118 SNPs from 22 candidate genes and major depressive disorder and its comorbid phenotypes.
2. To detect associations between 118 SNPs from 22 candidate genes and bipolar disorder and its comorbid phenotypes.
3. To examine associations between 118 SNPs from 22 candidate genes and panic disorder and its comorbid phenotypes.
4. To compare three different psychiatric disorders and to find SNP profiles specific for the analysed disorder groups.

4. MATERIALS AND METHODS

4.1. Ethical considerations

Studies were conducted in accordance with the principles of the Declaration of Helsinki. The study protocol was approved by the Ethics Review Committee on Human Research of the University of Tartu. Each subject provided written informed consent.

4.2. Subjects and psychiatric assessment

Unrelated patients (N = 269) with mood and anxiety disorders were recruited in the study along with healthy control individuals (N = 160) from the Estonian population. The number of controls used in analyses of PD was 146. The diagnoses of patients were substantiated by psychiatric interview and verified by Mini International Neuropsychiatric Interview (M.I.N.I. 5.0.0) based on DSM-IV (Sheehan et al., 1998). Controls were evaluated using M.I.N.I. to exclude those with psychiatric morbidity and with a family history interview to exclude those with a known history of major psychiatric disorders in first-degree relatives. There were no significant demographic differences between patients and healthy volunteers in terms of age and sex. Table 1 presents clinical demographic characteristics of the study subjects.

Patients were divided into subgroups because of the high rate of comorbidity of mood and anxiety disorders and to find possible subgroup-specific genetic markers. Psychiatric subjects were divided into diagnostic categories as follows:

MDD – major depressive disorder extended; all cases with major depressive disorder, includes pure phenotype as well as phenotypes with comorbid anxiety disorder [panic disorder, generalized anxiety disorder (GAD), obsessive-compulsive disorder (OCD), social phobia] (N = 177);

MDA – major depressive disorder with comorbid anxiety disorder (GAD, OCD, social phobia) except panic disorder (N = 48);

MD – major depressive disorder without any comorbid disorder (N = 69);

BPD – bipolar disorder extended; includes 12 patients with bipolar disorder without any comorbid disorder and 35 bipolar disorder patients with comorbid anxiety disorder (panic disorder, GAD, OCD, social phobia) (N = 47);

BPA – bipolar disorder with comorbid anxiety disorder (panic disorder, GAD, OCD, social phobia) (N = 35);

PDA – panic disorder extended; all cases with panic disorder, includes pure phenotype as well as phenotypes comorbid with mood disorders (major depressive disorder, bipolar disorder) and other anxiety disorders (N = 127);
PDC – panic disorder comorbid with major depressive disorder (N = 60);
PD – pure panic disorder phenotype (N = 42).

Table 1. Demographic and clinical characteristics of subjects

	Total number of individuals	Sex (male/female)	Age (years), mean ± SD	Range (years)
MDD	177	39/138	40.3±13.5	18–73
MDA	48	14/34	41.2±12.2	18–63
MD	69	16/53	40.3±15.0	18–73
BPD	47	21/26	35.4±12.7	17–65
BPA	35	12/23	35.5±11.9	17–61
PDA	127	23/104	38.4±13.0	17–73
PDC	60	9/51	39.7±12.9	18–69
PD	42	6/36	37.9±12.9	20–73
Control	160	51/109	38.2±14.1	18–71

Psychiatric subjects were divided into diagnostic categories as follows:

MDD – major depressive disorder extended; all cases with major depressive disorder, includes pure phenotype as well as phenotypes with comorbid anxiety disorder [panic disorder, generalized anxiety disorder (GAD), obsessive-compulsive disorder (OCD), social phobia];

MDA – major depressive disorder with comorbid anxiety disorder (GAD, OCD, social phobia) except panic disorder;

MD – major depressive disorder without any comorbid disorder;

BPD – bipolar disorder extended; includes 12 patients with bipolar disorder without any comorbid disorder and 35 bipolar disorder patients with comorbid anxiety disorder (panic disorder, GAD, OCD, social phobia);

BPA – bipolar disorder with comorbid anxiety disorder (panic disorder, GAD, OCD, social phobia);

PDA – panic disorder extended; all cases with panic disorder, includes pure phenotype as well as phenotypes comorbid with mood disorders (major depressive disorder, bipolar disorder) and other anxiety disorders;

PDC – panic disorder comorbid with major depressive disorder;

PD – pure panic disorder phenotype.

Patients were recruited among consecutive out-patients and in-patients at the Clinic of Psychiatry of Tartu University Clinics and controls by newspaper advertisement in Tartu, Estonia. All subjects were unrelated individuals of Caucasian origin living in Estonia.

4.3. Selection of single-nucleotide polymorphisms

Initial SNP selection was made on the basis of literature where results of association and linkage studies were reported. Originally the number of selected polymorphisms was 273. Many polymorphisms were omitted because of technical problems with polymerase chain reaction (PCR) or arrayed primer extension (APEX) reaction. Some of the selected SNPs turned out to be monomorphic or very rare and were excluded from further analysis. The number of genotyped SNPs on microchip varied on different stages of elaboration the microchip, but the final number of analysed SNPs was 90 from 21 genes (Papers II and III, list of original publications) plus 28 SNPs from WFS1 gene (Paper I) summing up 118 SNPs from 22 genes.

Choosing missense SNPs for genotyping, was reasoned by that at least some of them are likely causative mutations affecting function of the encoded protein associated with the underlying phenotype. Common synonymous SNPs were included in the present study on the assumption that silent SNPs, being in linkage disequilibrium with unknown functional polymorphism, can reveal an association with the actual disease-causing SNP(s). SNPs in regulatory sequences are thought to have potential to control the level of gene expression, therefore in some genes polymorphisms in 5' or 3' untranslated regions and intronic SNPs were included. Table 2 presents detailed information about studied polymorphisms.

Table 2. Description of single-nucleotide polymorphisms analysed in the study

Gene name (abbreviation)	Gene and SNP	Position from ATG	Location	db SNP rs #	Allele 1	Allele 2	Function	Allele 1 frequency
Cholecystokinin (CCK)	CCK -45	CCK -1172	3p22-p21.3	rs1799923	C	T	5'-UTR	0.89
	CCK 1270	CCK -9	3p22-p21.3	rs754635	C	G	5'-UTR	0.85
	CCK 6662	CCK 5386	3p22-p21.3	rs3774396	C	T	intron	0.98
Cholecystokinin A receptor (CCKAR)	CCKAR -128	CCKAR -333	4p15.1-p15.2	rs1800908	G	T	5'-UTR	0.96
	CCKAR 201	CCKAR -286	4p15.1-p15.2	rs1799723	A	G	5'-UTR	0.94
	CCKAR 246	CCKAR -241	4p15.1-p15.2	rs # n.a.	G	A	5'-UTR	0.97
	CCKAR 608	CCKAR 122	4p15.1-p15.2	rs1800856	G	A	intron	0.96
	CCKAR 1260	CCKAR 773	4p15.1-p15.2	rs1800855	T	A	intron	0.71
	CCKAR 1266	CCKAR 779	4p15.1-p15.2	rs1800857	T	C	intron	0.76
	CCKAR 3849	CCKAR 8231	4p15.1-p15.2	rs1805037	C	T	I296I	0.99
Cholecystokinin B receptor (CCKBR)	CCKBR -215	CCKBR -216	11p15.4	rs1799721	C	A	5'-UTR	0.95
	CCKBR 109	CCKBR 109	11p15.4	rs1805000	C	T	L37F	0.93
	CCKBR 1550	CCKBR 9962	11p15.4	rs1805002	G	A	V125I	0.92
	CCKBR 2491	CCKBR 10907	11p15.4	rs1800843 rs8192470	C	A	intron	0.88
Dopamine receptor D1 (DRD1)	DRD1 -2218	DRD1 -2218	5q35.1	rs # n.a.	T	C	5'-UTR	0.94
	DRD1 -2102	DRD1 -2102	5q35.1	rs # n.a.	C	A	5'-UTR	0.93
	DRD1 -2030	DRD1 -2030	5q35.1	rs # n.a.	T	C	5'-UTR	0.97
	DRD1 -1251	DRD1 -1252	5q35.1	rs # n.a.	G	C	5'-UTR	0.86
	DRD1 -800	DRD1 -800	5q35.1	rs265981	T	C	5'-UTR	0.38
	DRD1 -94	DRD1 -94	5q35.1	rs5326	G	A	5'-UTR	0.84
	DRD1 -48	DRD1 -48	5q35.1	rs4532	G	A	5'-UTR	0.44

Gene name (abbreviation)	Gene and SNP	Position from ATG	Location	db SNP rs #	Allele 1	Allele 2	Function	Allele 1 frequency
Dopamine receptor D2 (DRD2)	DRD2 -241	DRD2 -50978	11q23	rs1799978	A	G	5'-UTR	0.78
	DRD2 -141	DRD2 -50878	11q23	rs1799732	C	del	5'-UTR	0.84
	DRD2 -7054	DRD2 -7053	11q23	rs # n.a.	C	A	5'-UTR	0.92
	DRD2 -913	DRD2 -913	11q23	rs1079597	A	G	5'-UTR	0.32
	DRD2 -901	DRD2 -901	11q23	rs1079598	C	T	5'-UTR	0.32
	DRD2 286	DRD2 287	11q23	rs # n.a.	T	C	intron	0.93
	DRD2 3625	DRD2 3626	11q23	rs2734834	A	T	intron	0.49
	DRD2 3785	DRD2 3786	11q23	rs1800498	C	T	intron	0.39
	DRD2 11924	DRD2 11890	11q23	rs1801028	C	G	S311C	0.93
	DRD2 11997	DRD2 11915	11q23	rs6277	T	C	P319P	0.94
	DRD2 16893	DRD2 16891	11q23	rs2234689	C	G	3'-UTR	0.72
	DRD2 24470	DRD2 24546	11q23	rs1800497	C	T	K713E (in ANKK1 gene)	0.80
Dopamine receptor D3 (DRD3)	DRD3 -707	DRD3 -710	3q13.3	rs1800828	G	C	5'-UTR	0.71
	DRD3 -343	DRD3 -346	3q13.3	rs1800827	G	A	5'-UTR	0.96
	DRD3 25	DRD3 25	3q13.3	rs6280	A	G	G9S	0.69
Dopamine receptor D4 (DRD4)	DRD4 -1217	DRD4 -1216	11p15.5	rs # n.a.	G	del	5'-UTR	0.62
	DRD4 -809	DRD4 -808	11p15.5	rs936461	G	A	5'-UTR	0.80
	DRD4 -768	DRD4 -767	11p15.5	rs4987058	G	A	5'-UTR	0.86
	DRD4 -616	DRD4 -615	11p15.5	rs747302	C	G	5'-UTR	0.68
	DRD4 -521	DRD4 -521	11p15.5	rs1800955	C	T	5'-UTR	0.41
	DRD4 -376	DRD4 -376	11p15.5	rs916455	C	T	5'-UTR	0.96
Dopamine receptor D5 (DRD5)	DRD5 1481	DRD5 1481	4p16.1	rs1967551	C	T	3'-UTR	0.65
Tyrosine hydroxylase (TH)	TH 241–243	TH 2066	11p15.5	rs6356	G	A	V81M	0.61
	TH 614	TH 3891	11p15.5	rs # n.a.	T	C	L205P	0.96
5-hydroxytryptamine (serotonin) receptor 1A (HTR1A)	HTR1A –1018	HTR1A -1019	5q11.2-q13	rs6295	C	G	5'-UTR	0.43
	HTR1A –480	HTR1A –480	5q11.2-q13	rs # n.a.	A	del	5'-UTR	0.91

Gene name (abbreviation)	Gene and SNP	Position from ATG	Location	db SNP rs #	Allele 1	Allele 2	Function	Allele 1 frequency
5-hydroxytryptamine (serotonin) receptor 1B (HTR1B)	HTR1B	HTR1B -1089	6q13	rs1778258	T	C	5'-UTR	0.24
	HTR1B	HTR1B -700	6q13	rs1228814	C	A	5'-UTR	0.55
	HTR1B -511	HTR1B –511	6q13	rs130056	G	T	5'-UTR	0.995
	HTR1B -161	HTR1B –161	6q13	rs130058	A	T	5'-UTR	0.78
	HTR1B 129	HTR1B 129	6q13	rs6298	C	T	S43S	0.74
	HTR1B 276	HTR1B 276	6q13	rs130059	G	A	A92A	0.96
	HTR1B 371	HTR1B 371	6q13	rs130060	T	G	F124C	0.99
	HTR1B 705	HTR1B 705	6q13	rs130062	C	T	A235A	0.80
	HTR1B 861	HTR1B 861	6q13	rs6296	G	C	V287V	0.74
	HTR1B	HTR1B 1180	6q13	rs6297	G	A	3'-UTR	0.23
5-hydroxytryptamine (serotonin) receptor 2A (HTR2A)	HTR2A -1438	HTR2A –1437	13q14-q21	rs6311	A	G	5'-UTR	0.42
	HTR2A 73	HTR2A 74	13q14-q21	rs1805055	C	A	T25N	0.98
	HTR2A 102	HTR2A 102	13q14-q21	rs6313	T	C	S34S	0.37
	HTR2A 1354	HTR2A 61008	13q14-q21	rs6314	C	T	H452Y	0.94
5-hydroxytryptamine (serotonin) receptor 2C (HTR2C)	HTR2C 68	HTR2C 4390	Xq24	rs6318	G	C	C23S	0.83
5-hydroxytryptamine (serotonin) receptor 3A (HTR3A)	HTR3A 1302	HTR3A -507	11q23.1-q23.2	rs1150226	T	C	5'-UTR	0.31
	HTR3A 1596	HT3A 14378	11q23.1-q23.2	rs1176713	G	A	L459L	0.26
Solute carrier family 6 (neuro-transmitter transporter, serotonin), member 4 (SLC6A4)	SLC6A4	SLC6A4 18784	17q11.1-q12	rs6352	A	C	K605N	0.96
	SLC6A4	SLC6A4 10647	17q11.1-q12	rs6353	G	A	T439T	0.92
	SLC6A4	SLC6A4 167	17q11.1-q12	rs6355	G	C	G56A	0.77
Tryptophan hydroxylase 1 (tryptophan 5-monooxygenase) (TPH1)	TPH1 218	TPH1 14494	11p15.3-p14	rs1800532	A	C	intron	0.29
	TPH1 779	TPH1 15055	11p15.3-p14	rs1799913	A	C	intron	0.27

Gene name (abbreviation)	Gene and SNP	Position from ATG	Location	db SNP rs #	Allele 1	Allele 2	Function	Allele 1 frequency
Opioid receptor, mu 1 (OPRM1)	OPRM1 31	OPRM1 50665	6q24-q25	rs # n.a.	G	A	intron	0.92
	OPRM1 118	OPRM1 118	6q24-q25	rs1799971	A	G	N40D	0.78
	OPRM1 440	OPRM1 50431	6q24-q25	rs # n.a.	C	G	S147C	0.84
	OPRM1 691	OPRM1 51325	6q24-q25	rs2075572	C	G	intron	0.54
Opioid receptor, delta 1 (OPRD1)	OPRD1 80	OPRD1 80	1p36.1-p34.3	rs1042114	T	G	C27F	0.91
	OPRD1 921	OPRD1 50702	1p36.1-p34.3	rs2234918	T	C	G307G	0.63
Opioid receptor, kappa 1 (OPRK1)	OPRK1 36	OPRK1 36	8q11.2	rs1051660	G	T	P12P	0.84
	OPRK1	OPRK1 10807	8q11.2	rs1365097	A	G	intron	0.69
	OPRK1	OPRK1 10915	8q11.2	rs1365098	G	T	intron	0.66
	OPRK1	OPRK1 11220	8q11.2	rs997917	A	G	intron	0.54
	OPRK1 459	OPRK1 16128	8q11.2	rs7815824	C	T	S153S	0.90
	OPRK1 843	OPRK1 21441	8q11.2	rs702764	A	G	A281A	0.72
	OPRK1 846	OPRK1 21444	8q11.2	rs # n.a.	C	T	V282V	0.97
Proopiomelano-cortin (POMC)	POMC 18	POMC 18	2p23.3	rs8192605	C	T	C6C	0.99
	POMC 282	POMC 3170	2p23.3	rs # n.a.	C	T	S94S	0.92
	POMC 313	POMC 3201	2p23.3	rs # n.a.	G	T	E105Stop	0.96
	POMC 346	POMC 3234	2p23.3	rs # n.a.	C	T	L116L	0.98
	POMC 585	POMC 3473	2p23.3	rs2071345	C	T	A195A	0.94
	POMC 866	POMC 3755	2p23.3	rs1042571	C	T	3'-UTR	0.85
Proenkephalin (PENK)	PENK 28	PENK -588	8q23-q24	rs2609999	C	A	5'-UTR	0.57
	PENK 808	PENK 4686	8q23-q24	rs3839874	C	del	3'-UTR	0.67
Wolfram syndrome 1 (wolframin) (WFS1)	WFS1 406	WFS1 11622	4p16	rs # n.a.	C	T	Q136X	0.99
	WFS1 460	WFS1 11676	4p16	rs # n.a.	G	A	5' splice signal	0.97
	WFS1 505	WFS1 13786	4p16	rs # n.a.	G	A	E169K	0.89

Gene name (abbreviation)	Gene and SNP	Position from ATG	Location	db SNP rs #	Allele 1	Allele 2	Function	Allele 1 frequency
	WFS1 676	WFS1 14506	4p16	rs # n.a.	C	T	Q226X	0.99
	WFS1 684	WFS1 14514	4p16	rs7672995	C	G	R228R	0.54
	WFS1 874	WFS1 23214	4p16	rs # n.a.	C	T	P292S	0.99
	WFS1 887	WFS1 23227	4p16	rs # n.a.	T	G	I296S	0.98
	WFS1 935	WFS1 23275	4p16	rs # n.a.	T	G	M312R	0.80
	WFS1 997	WFS1 23337	4p16	rs1801212	A	G	I333V	0.68
	WFS1 1023	WFS1 23363	4p16	rs # n.a.	C	T	F341F	0.90
	WFS1 1185	WFS1 23525	4p16	rs1801206	C	T	V395V	0.48
	WFS1 1287	WFS1 23627	4p16	rs # n.a.	C	T	C429C	0.99
	WFS1 1294	WFS1 23634	4p16	rs # n.a.	C	G	L432V	0.95
	WFS1 1321	WFS1 23661	4p16	rs # n.a.	G	A	V441M	0.89
	WFS1 1367	WFS1 23707	4p16	rs1801208	G	A	R456H	0.94
	WFS1 1549	WFS1 23889	4p16	rs # n.a.	del	C	del517fs/ ter521	0.99
	WFS1 1645	WFS1 23985	4p16	rs # n.a.	C	T	L549L	0.96
	WFS1 1832	WFS1 24172	4p16	rs734312	G	A	R611H	0.53
	WFS1 2206	WFS1 24546	4p16	rs # n.a.	G	A	G736S	0.91
	WFS1 2254	WFS1 24594	4p16	rs # n.a.	G	T	E752X	0.99
	WFS1 2314	WFS1 24654	4p16	rs # n.a.	C	T	R772C	0.98
	WFS1 2322	WFS1 24662	4p16	rs2230721	G	A	K774K	0.93
	WFS1 2433	WFS1 24773	4p16	rs1046314	A	G	K811K	0.56
	WFS1 2565	WFS1 24905	4p16	rs1046316	G	A	S855S	0.63
	WFS1 2596	WFS1 24936	4p16	rs3821945	G	A	D866N	0.99
	WFS1 2611	WFS1 24951	4p16	rs # n.a.	G	A	V871M	0.93
	WFS1 2642	WFS1 24982	4p16	rs # n.a.	del	TC	del882fs/ ter937	0.95
	WFS1 2763	WFS1 25103	4p16	rs # n.a.	G	A	3'-UTR	0.92

db SNP rs # – accession number of SNP in NCBI dbSNP database; allele frequency is based on controls of the study.
rs # n.a. – SNP is not listed in NCBI dbSNP database (http://www.ncbi.nlm.nih.gov/snp/).

4.4. Template preparation and genotyping

Standard high-salt extraction method was used to isolate genomic DNA from 9 ml venous blood samples. Two different PCR programs were used to amplify the genomic regions containing the whole set of studied 118 polymorphisms with 72 individual PCR reactions (single or multiplex). The first program contained the following cycles:

initial denaturation:	95°C 5 min	
denaturation:	95°C 30 sec	} 34 cycles
primer annealing:	55°C 40 sec	
extension:	72°C 40 sec	
final extension:	72°C 6 min	

In program 2 (*touchdown*) the amplification reactions were as follows:

initial denaturation:	95°C 5 min	
denaturation:	95°C 25 sec	} 2 cycles (decreasing 2°C per cycle)
primer annealing:	68°C 30 sec	
extension:	72°C 30 sec	
denaturation:	95°C 25 sec	} 11 cycles
primer annealing:	64°C 30 sec	
extension:	72°C 30 sec	
denaturation:	95°C 25 sec	} 3 cycles (decreasing 1°C per cycle)
primer annealing:	63°C 30 sec	
extension:	72°C 30 sec	
denaturation:	95°C 25 sec	} 22 cycles
primer annealing:	60°C 30 sec	
extension:	72°C 30 sec	
final extension:	72°C 6 min	

Samples were processed in a PTC-200 (MJ Research Inc., Watertown, MA, USA) and Mastercycler Gradient thermal cyclers (Eppendorf-Netheler-Hinz GmbH, Hamburg, GER). Table 3 shows primer sequences and PCR conditions used for amplification.

Table 3. Primers used to amplify genomic regions for APEX analysis

	Dopamine	Forward primer 5'→3'	Reverse primer 5'→3'	Product size bp	PCR program
1	DRD1 P1	GCGGTGAGTTAAAAGAACAG	CATGACTGCCAGAACCTGAA	417	2
2	DRD1 P22	GCTTACTTGAGGTTCTGACA	CGTTTGGGGAAAGGATCCCA	589	2
3	DRD1 P3	GATTGCAACTGACTAGCAGA	TTCAGATCCTCATCTTCCT	376	1
4	DRD1 3/4	GGGCTAATTCATCCTTGAAC	TGTGTTGGAAAGCAGCAGAG	512	2
5	DRD2 P1	TCAAAGGAGAAGACTGGCGA	CACTGAAGCTGGACAGCTCT	289	2
6	DRD2 1	CAGAAGCTGCTGAGGTTGGA	CCTGCATGTCAAGTTCTTCCT	179	2
7	DRD2 2	ATCCTGCCAAACCTCATCATC	GAGGTTGCAATAGGCAAGATC	222	2
8	DRD2 3	CAGACCACCACCAACTACCT	GGAAGCACCAGGAAACTCAT	162	2
9	DRD2 4	GTGTCAGGGAAGACTTTCAG	GTGACAAGTACTTGGTAAGC	510	1
10	DRD2 5	GAGTCTTCAGAGGGTGAAAG	ACAATGGCGAGCATCTGAGT	420	1
11	DRD2 7	GACGAGTTGTCTAGCAGACT	TGCTGTGTGACACGTCATCT	291	2
12	DRD2 8	TCTAGGAAGGACATGATGCC	CTTCCTGAGTGTCATCAACC	222	2
13	DRD3 E1	GTCTCCTCACAGGAAGCCCCTT	CCGCTCCTTCAGCACAGCCATGC	220	2
14	DRD3 1/2	TGGACTAAGATAGATGGGTT	GAGTCTGGTGAGGCTGGAGC	249	1
15	DRD3 5	TCTCCTCCAGGTCAAGACTCAAT	GACTCTTTGGGGCTTGGTTGCTT	352	2
16	DRD4 1	CTGCACAAGAGGGACTGAGCCTG	GACAGGGCGTGCGTTAAAGGG	165	2
17	DRD4 Neg3/4	CAGGTCACAGGTCACCCCTCTT	TTGCTCATCTTGGAATTTTGCG	792	1
18	DRD4 1	CATCCTGGGAGAGAAGAAAC	CATCCTGATGCTCTAGTCTG	363	2
19	DRD5 3	GTCCCTTTCTGCAGTGGACACCCTG	CAGCACCATATCTCTTCTCTCATAGGAT	740	2
20	TH 1	GATGAGTGACACAGCGTCTC	GCAGCTGCACCTCTGCTATA	628	2
21	TH 205	GACCCTGACCTGGACTTGGAC	GCCCCTCACTGCCTGTACTGG	284	2
	Opioid				
22	OPRD 1	CGCCGGCGCCGAGCTGCAGC	CAGCAGCCCCACGGCGCACA	172	1
23	OPRD 3	GCGCATCACGCGCATGGTGC	CGCGGGCGCGGCTGAAGCTG	277	2
24	OPRM 1	GAGAATGTCAGATGCTCAGC	ACCAGGAAGTTTCCGAAGAG	461	1
25	OPRM 2	CAGATGCCTTAGCCACCAGT	GAGGATCCAGTTGCAGACAT	244	2

		Forward primer 5'→3'	Reverse primer 5'→3'	Product size bp	PCR program
26	OPRM IVS2	TGCAACTGGATCCTCTCTTC	GTACAATCTATGGAACCTAG	858	1
27	OPRK 1	AAAGGCAGCGAGAAGTCCGT	ACGAACATGACCAGCGAGTT	445	2
28	OPRK 2	TAACCTGGCTTTGGCAGATG	CTGGACTTGCCAGGTTGTAC	635	2
29	OPRK 3	TGTCATTGAGTGCTCCTTGC	AATAGCTGGAGAGAGCAGCT	319	2
30	OPRK IVS1	TGGCATTTGATCACACTTGT	TCCTGCTGTTGTTACTGTTA	676	1
31	POMC 2	TCAAGGTCCTTCCTGGTGAG	GTTGCTTTCCGTGGTGAGGT	222	2
32	POMC 3	AAGTACGTCATGGGCCACTT	AGAGGCTGATTATCTGCCAC	696	2
33	PENK IVS1	TATAAAGTGGCTCCAGCAGC	GTTGACGCTGTTCGGATGGA	247	2
34	PENK 4	TCATGAGAAGAGTAGGTCGC	ACTGTCCCTGAGTCTAGGAT	563	2
	Serotonin				
35	HTR1A 1018	GCTGGACTGTTAGATGATAG	ACTCACTTACACACACCAGG	420	1
36	HTR1A BC	CAGAGGAAAGAGGCACTCCTC	AGTTCTTACTGCTTCGGCGAA	531	2
37	HTR1B 1	TGTGACCATGGCTAAGGACA	TGAAGTCTAGGAGCAGCGCT	651	2
38	HTR1B 2	AGTGGCCAGAGAGTGAAAAG	CAGGTTTGTCCCCAGTTGAT	569	2
39	HTR1B 3	AACTTATCCTCTGCTCCCTC	GTCCTTTTAGCTGAGTACTCC	415	2
40	HTR1B 4	TGTGGGTCTTCTCCATCTCT	AAGGGTAGCCAACACACAAT	471	2
41	HTR1B 5	TGCAAAGATGCCTGCTGGTT	GATTCGACCTACCTGTGGAA	231	2
42	HTR2A P2	CTAGCCACCCTGAGCCTATG	TTGTGCAGATTCCCATTAAGG	200	2
43	HTR2A 1	CATCTGCTACAAGTTCTGGC	CTACGGCTGTCAGTAAAGCA	276	2
44	HTR2A 3	GCCTACAAGTCTAGCCAACT	TCAGTGTGCCTTCCACAGTT	203	2
45	HTR2C 1	TGCATGAGCAACGTATTGTG	CATGCTTACTGCCATGATCA	276	2
46	HTR3A 2	ATGGGAAACCACTGCAGCCA	GTTCAGACCTTGGCTTGTGA	470	2
47	HTR3A 4	AGATGAGGACAAAAGGGCAGAC	TGCAGAAGCCCATGAGACAA	251	2
48	TPH1 1	CTCCATGGGACTCAACACCA	AGAATGGTACCTGGCATGAA	240	2
49	TPH1 2	CAAGAGAAGCCAAGCCAATT	GTGTGAGTCTGAGTGGCCAA	282	2
50	5HTT T1	ACTGCATAGGAACCTCATCT	GTGCACCCAAATGATCAGCT	317	2
51	5HTT T2	TAGGACAGGTCTTGTCAACC	TGGTAAATGCCGAGGAGTCA	320	2
52	5HTT T3	AGCGTGTGAAGATGGAGAAG	TCTACTCGCAGCCTGTGATA	330	2

		Forward primer 5'→3'	Reverse primer 5'→3'	Product size bp	PCR program
	CCK				
53	CCK 45	GATTAACTCCACCCACTAGAC	TTGGCGTTTCCAACCGGAGCAG	238	2
54	CCK 1270	CTCTGTTGCCCAGCCTTTCAG	CTGGGAACAAGGGCAGAAGTG	314	2
55	CCK 6662	GATGCTTTTAGATGCAATGTC	ACAGACAATGAGTTATGAGTG	278	2
56	CCK 1 P1	TTGTTCCTGTCTCACACAACC	CATTCCTAAAGGCGACTTCAG	300	1
57	CCK1R P2	TAAGGAGGTAGAACACAGTCC	GCTTTCCACCAAGTGCTGG	540	2
58	CCK1R E1	ATTCACCAGCTCTCCAGCAC	CTCCCACAACTCAATAGTTTC	220	2
59	CCK1R E2	TTAGCATTCTGCTGTCATCAC	TGAAATCCTTGAGCAGATTGG	250	2
60	CCK1R E5	CCAGAAAGGAAACCTAGCACC	GGATGAAGGAAATGGGGGTTC	300	2
61	CCK2R P	GCAGGAGAGAAATCTCTAAGAG	ACCTGCTCACGCTGCGATTC	273	2
62	CCK2R E1	CCGGGTCGAGCTGAGTAAG	GTACAGTGAGAAATAGCTTGTG	157	2
63	CCK2R E2	GATTTGACTGAATGAAGGCTG	ACATCCACAAGAGCTTTAGGC	370	2
64	CCK2R E4	TCTGTGATTACAGCTGGACAG	TCTGTGATTACAGCTGGACAG	327	2
	WFS1				
65	WFS 4	TCGGAGAATCTGGAGGCTGA	CATTACAAGCTGCTCAACCC	253	2
66	WFS 5	ACAAGGCCTTTGACCACATC	GTGCCCAGGGTGAATCCTC	225	2
67	WFS 6	CTATGATCCCCAGAACGTAGGA	CAGAACACTGAGCCCCAAAC	419	2
68	WFS 8A	CCTCGTTCCCACGTACCATC	GTAGCAGTAGGTGCCCTTGA	766	2
69	WFS 8B	CCTGGTCGTCCTCAATGTCA	CATAGAACCAGCAGAACAGC	447	2
70	WFS 8CD	TGGTTCACGTCTCTGGAGCT	GAACTTCTTGATGTGGCAGG	549	2
71	WFS 8E	CTGGATGCGCTGCCTCTACG	TCAGGCCGCCGACAGGAATG	350	2
72	WFS 8H	GAGTTCAGCACCATCCTGGAG	ACAGCAGCCTTCCCTTTGTCG	381	2

A 20% fraction of the dTTP in the amplification mixture was substituted by dUTP, allowing later fragmentation of PCR products with uracil-N-glycosylase. Pooled amplification products were concentrated and purified, followed by fragmentation and functional inactivation of the unincorporated dNTPs as described in (Tõnisson et al., 2002). Production of oligonucleotide microchips and APEX reactions were performed as described earlier (Tõnisson et al., 2002). Slides were imaged with Genorama Quattroimager detector (Asper Biotech Ltd., Tartu, Estonia) and polymorphisms were identified by Genorama™ 4.1 genotyping software by using signal patterns from a wild-type DNA sequence as the reference.

4.5. Statistical analysis

Association analysis statistics was performed using GENEPOP Version 3.3 software (Raymond and Rousset, 1995). P-values for allelic and genotypic association were calculated using Fisher's exact test. The significance level for all statistical tests was 0.05. Haplotype analysis was performed using the maximum likelihood method for estimating simultaneously haplotype frequencies and haplotype-phenotype association as described in (Tregouet et al., 2002). Pairwise LD was estimated by a log-linear model, and the extent of disequilibrium was expressed in terms of standardized D´ characteristic. The Bonferroni correction was used after association and haplotype analysis to adjust for multiple testing.

5. RESULTS

Altogether 118 polymorphisms (112 SNPs and 6 insertions/deletions) were genotyped in 22 candidate genes in 269 unrelated patients and 160 healthy controls. Given the relatively small number of subjects with MDD, BPD, and PD phenotypes without any comorbity, overlapping analyses served to maximize the likelihood of finding differences by diagnostic subcategory, if they existed in the studied population. On the other hand, such stratification helped to define subtype-specific SNPs or SNPs reflecting the general risk of mood and anxiety disorders.

5.1. Results of association analysis

Allele frequencies of SNPs were compared between control and patients groups. Comparisons were done between major depressive disorder, bipolar disorder, panic disorder pure and comorbid phenotype groups and the healthy control group. Altogether 27 polymorphisms in 15 genes displayed association with mood and anxiety disorders. There was no deviation from Hardy-Weinberg equilibrium (HWE) expectations at any of the genotyped loci for any of the diagnostic subcategories. P-values for informative SNPs for all groups are presented in Table 4. Polymorphisms from CCKAR (246G/A, 1266T/C), DRD1 (-2102C/A), DRD2 (-7054C/A), DRD3 (-707G/C, 25A/G), DRD5 (1481C/T), HTR2C (68G/C), SLC6A4 (10647G/A), OPRM1 (118A/G), POMC (282C/T), and WFS1 (684C/G, 935T/G, 1023C/T, 1185C/T, 1645C/T, 2206G/A, 2565G/A) genes were associated with major depressive disorder and its comorbid phenotypes.

Polymorphisms from CCK (-45C/T, 1270C/G), CCKAR (246G/A), DRD1 (-800T/C), DRD5 (1481C/T), HTR2A (73C/A), OPRM1 (118A/G), and WFS1 (684C/G, 1023C/T, 1185C/T, 2565G/A) genes were associated with bipolar disorder and its comorbid phenotypes. Polymorphisms from CCK (1270C/G), CCKAR (246G/A), CCKBR (-215C/A), DRD1 (-94G/A), DRD4 (-1217del/G), HTR1A (-1018C/G), HTR2A (102T/C), HTR2C (68G/C), and WFS1 (684C/G, 1023C/T, 1185C/T, 2206G/A) genes were associated with panic disorder and its comorbid phenotypes.

Table 4. P-values of association analysis of 118 polymorphisms in mood and anxiety disorders

SNP	**Allele**		**Gene**	**Allelic P**							
	1	2		MD	MDA	MDD	BPA	BPD	PDA	PDC	PD
				N=69	N=48	N=177	N=35	N=47	N=127	N=60	N=42
-45	C	T	CCK				**0.05**				
1270	C	G	CCK				**0.05**		**0.03**	**0.05**	
246	G	A	CCKAR		**0.015**	**0.006**		**0.05**		**0.02**	
1266	T	C	CCKAR		**0.03**						
-215	C	A	CCKBR						**0.05**		
-2102	C	A	DRD1			**0.008**					
-800	T	C	DRD1				**0.04**				
-94	G	A	DRD1								**0.02**
-7054	C	A	DRD2			**0.03**					
-707	G	C	DRD3	**0.01**							
25	A	G	DRD3	**0.05**							
-1217	del	G	DRD4						**0.03**		
1481	C	T	DRD5		**0.016**		**0.01**	**0.05**			
-1018	C	G	HTR1A						**0.05**	**0.05**	
73	C	A	HTR2A					**0.05**			
102	T	C	HTR2A								**0.01**
68	G	C	HTR2C	**0.03**		**0.02**			**0.03**		
10647	G	A	SLC6A4		**0.05**						
118	A	G	OPRM1	**0.03**			**0.009**	**0.007**			
282	C	T	POMC	**0.01**							
684	C	G	WFS1			**0.007**	**0.02**	**0.005**		**0.04**	
935	T	G	WFS1		**0.01**						
1023	C	T	WFS1		**0.02**	**0.02**	**0.05**		**0.04**		
1185	C	T	WFS1	**0.04**		**0.01**		**0.05**		**0.02**	
1645	C	T	WFS1		**0.05**						
2206	G	A	WFS1	**0.02**		**0.04**			**0.01**		**0.01**
2565	G	A	WFS1			**0.04**		**0.05**			

Only $P < 0.05$ are indicated.

SNP – single-nucleotide polymorphism. MD – major depressive disorder without any comorbid disorder; MDA – major depressive disorder with comorbid anxiety disorder (GAD, OCD, social phobia) except panic disorder; MDD – major depressive disorder extended; all cases with major depressive disorder, includes pure phenotype as well as phenotypes with comorbid anxiety disorder [panic disorder, generalized anxiety disorder (GAD), obsessive-compulsive disorder (OCD), social phobia]; BPA – bipolar disorder with comorbid anxiety disorder (panic disorder, GAD, OCD, social phobia); BPD – bipolar disorder extended; includes 12 patients with bipolar disorder without any comorbid disorder and 35 bipolar disorder patients with comorbid anxiety disorder (panic disorder, GAD, OCD, social phobia); PDA – panic disorder extended; all cases with panic disorder, includes pure phenotype as well as phenotypes comorbid with mood disorders (major depressive disorder, bipolar disorder) and other anxiety disorders; PDC – panic disorder comorbid with major depressive disorder; PD – pure panic disorder phenotype.

5.1.1. Results of association analysis of MDD

In the screening set, 18 genetic variations in altogether 10 genes displayed association with broadly-defined major depressive disorder. Allelic frequencies and P-values for informative SNPs are presented in Table 5.

Table 5. Results of association analysis of 118 polymorphisms in major depressive disorder

SNP	Allele		Gene	Allelic P			Allele 2 frequencies			
	1	2		MD	MDA	MDD	MD	MDA	MDD	Controls
246	G	A	CCKAR	0.09	**0.015**	**0.006**	0.07	0.10	0.09	0.03
1266	T	C	CCKAR	0.91	**0.03**	0.64	0.25	0.14	0.22	0.24
-2102	C	A	DRD1	0.06	0.11	**0.008**	0.02	0.02	0.02	0.07
-7054	C	A	DRD2	0.09	0.24	**0.03**	0.14	0.12	0.14	0.08
-707	G	C	DRD3	**0.01**	0.52	0.06	0.16	0.24	0.22	0.29
25	A	G	DRD3	**0.05**	0.37	0.16	0.23	0.26	0.26	0.31
1481	C	T	DRD5	0.91	**0.016**	0.27	0.34	0.49	0.39	0.35
68	G	C	HTR2C	**0.03**	0.26	**0.02**	0.09	0.12	0.10	0.17
10647	G	A	SLC6A4	0.85	**0.05**	0.21	0.07	0.02	0.05	0.08
118	A	G	OPRM1	**0.03**	1	0.11	0.13	0.22	0.17	0.22
282	C	T	POMC	**0.01**	0.12	0.06	0.18	0.03	0.13	0.08
684	C	G	WFS1	0.08	0.09	**0.007**	0.50	0.52	0.52	0.41
935	T	G	WFS1	0.62	**0.01**	0.19	0.20	0.11	0.18	0.22
1023	C	T	WFS1	0.11	**0.02**	**0.02**	0.07	0.04	0.07	0.12
1185	C	T	WFS1	**0.04**	0.15	**0.01**	0.58	0.56	0.58	0.47
1645	C	T	WFS1	1	**0.05**	0.55	0.04	0	0.03	0.04
2206	G	A	WFS1	**0.02**	0.38	**0.04**	0.04	0.08	0.06	0.10
2565	G	A	WFS1	0.08	0.63	**0.04**	0.32	0.38	0.33	0.41

P < 0.05 are highlighted.

SNP – single-nucleotide polymorphism. MD – major depressive disorder without any comorbid disorder; MDA – major depressive disorder with comorbid anxiety disorder [generalized anxiety disorder (GAD), obsessive-compulsive disorder (OCD), social phobia] except panic disorder; MDD – major depressive disorder extended; all cases with major depressive disorder, includes pure phenotype as well phenotypes with comorbid anxiety disorder (panic disorder, GAD, OCD, social phobia).

SNPs specific for MD group were in the DRD3, OPRM1, and POMC genes. Namely, the SNPs at positions –707G/C and 25A/G at DRD3, 118A/G at OPRM1 and 282C/T at POMC genes were associated with the presence of major depressive disorder without comorbidity. On the other hand, SNPs in HTR2C at position 68G/C and in WFS1 at positions 1185C/T and 2206G/A were associated with ‘narrow’ MD and ‘wide’ MDD phenotype while no

association between MDA group and these SNPs were found. Excess of minor allele was found in the case of POMC marker 282C/T (T) and in WFS1 1185C/T (T) in the affected group. On the contrary, the minor alleles of DRD3, OPRM1, HTR2C, and WFS1 2206G/A markers were more frequent in the control subjects.

In the MDA group (major depressive disorder with comorbid anxiety disorders except panic disorder) five SNPs (CCKAR 1266T/C, DRD5 1481C/T, 10647G/A SLC6A4, WFS1 935T/G and 1645C/T) were associated only with MDA phenotype and therefore represent potential subtype-specific markers.

Two SNPs (CCKAR 246G/A and 1023C/T) were significantly associated both with MDA and MDD phenotype.

Minor allele frequencies of CCKAR 1266C, SLC6A4 10647A, WFS1 935G, 1023T, and 1645T were higher in the control group and probably have a protective effect. Minor allele frequencies for other markers (CCKAR 246A and DRD5 1481T) were found to be in excess in patients compared to control subjects, thus presenting a potential risk allele for major depressive disorder with comorbid anxiety phenotype.

In the MDD group (in addition to the MD group major depressive disorder with comorbid anxiety disorders including panic disorder) four SNPs showed phenotype-specific associations — DRD1 -2102C/A, DRD2 -7054C/A, WFS1 684C/G, and 2565G/A. Minor allele frequencies of two SNPs (DRD1 -2102A and WFS1 2565A) were higher in the control group and two SNPs (DRD2 -7054A and WFS1 684G) were higher in the patient group. However, other significant associations in this group (CCKAR, HTR2C, and WFS1) were also valid for MD or MDA groups. As MDD represents the broadest phenotype (all cases where major depressive disorder is involved), SNPs associated with this phenotype could be interpreted as general markers of major depressive disorder.

5.1.2. Results of association analysis of BPD

In the case of bipolar disorder a slightly different strategy was used for stratification. Patients were divided into two subgroups — BPA (bipolar disorder with comorbid anxiety disorders) and BPD (includes group BPA and also 12 patients with only bipolar disorder). Significant associations were found between 11 SNP markers from seven genes and both subgroups of bipolar disorder. Table 6 presents allelic frequencies and P-values for informative SNPs.

The BPA group revealed a phenotype-specific association with SNPs in CCK (-45C/T and 1270C/G), DRD1 (-800T/C) and WFS1 (1023C/T). In the case of the extended group (BPD, contains also 12 patients with pure bipolar disorder) specific associations with SNPs in CCKAR (246G/A), in HTR2A (73C/A), and in WFS1 (1185C/T and 2565G/A) genes were identified. An association was found with both groups between SNP 1481C/T in DRD5 gene, 118A/G variant in OPRM1 gene and polymorphism 684C/G in WFS1 gene.

Four minor alleles (DRD1 –800C, OPRM1 118G, WFS1 1023T and 2565A) suggest a protective effect to BPD as their frequencies were higher in the control group. Minor allele frequencies of the rest of significantly associated markers were higher in the patient group.

Table 6. Results of association analysis of 118 polymorphisms in bipolar disorder

SNP	Allele		Gene	Allelic P		Allele 2 frequencies		
	1	2		BPA	BPD	BPA	BPD	Controls
-45	C	T	CCK	**0.05**	0.08	0.21	0.18	0.11
1270	C	G	CCK	**0.05**	0.14	0.24	0.21	0.15
246	G	A	CCKAR	0.09	**0.05**	0.09	0.09	0.03
-800	T	C	DRD1	**0.04**	0.12	0.48	0.53	0.62
1481	C	T	DRD5	**0.01**	**0.05**	0.51	0.47	0.35
73	C	A	HTR2A	0.06	**0.05**	0.06	0.05	0.02
118	A	G	OPRM1	**0.009**	**0.007**	0.09	0.10	0.22
684	C	G	WFS1	**0.02**	**0.005**	0.58	0.59	0.41
1023	C	T	WFS1	**0.05**	0.12	0.04	0.06	0.12
1185	C	T	WFS1	0.12	**0.05**	0.58	0.59	0.47
2565	G	A	WFS1	0.40	**0.05**	0.35	0.29	0.41

P < 0.05 are highlighted.

SNP – single-nucleotide polymorphism. BPA – bipolar disorder with comorbid anxiety disorder [panic disorder, generalized anxiety disorder (GAD), obsessive-compulsive disorder (OCD), social phobia]; BPD – bipolar disorder extended; includes 12 patients with bipolar disorder without any comorbid disorder and 35 bipolar disorder patients with comorbid anxiety disorder (panic disorder, GAD, OCD, social phobia).

5.1.3. Results of association analysis of PD

Twelve SNP markers from 9 genes displayed significant association with broadly-defined panic disorder. Allelic frequencies and P-values for informative SNPs are presented in Table 7.

Table 7. Results of association analysis of 118 polymorphisms in panic disorder

SNP	Allele		Gene	Allelic P			Allele 2 frequencies			
	1	2		PDA	PDC	PD	PDA	PDC	PD	Controls
1270	C	G	CCK	**0.03**	**0.05**	0.23	0.21	0.22	0.19	0.14
246	G	A	CCKAR	0.10	**0.02**	1	0.06	0.09	0.02	0.03
-215	C	A	CCKBR	**0.05**	0.42	0.21	0.02	0.03	0.01	0.05
-94	G	A	DRD1	0.13	0.55	**0.02**	0.11	0.13	0.06	0.16
-1217	G	del	DRD4	**0.03**	0.11	0.07	0.29	0.30	0.27	0.38
-1018	C	G	HTR1A	**0.05**	**0.05**	0.17	0.47	0.45	0.47	0.56

SNP	Allele		Gene	Allelic P			Allele 2 frequencies			
	1	2		PDA	PDC	PD	PDA	PDC	PD	Controls
102	T	C	HTR2A	0.43	1	**0.01**	0.67	0.63	0.78	0.63
68	G	C	HTR2C	**0.03**	0.23	0.07	0.10	0.09	0.08	0.17
684	C	G	WFS1	0.08	**0.04**	0.80	0.48	0.52	0.39	0.41
1023	C	T	WFS1	**0.04**	0.49	0.07	0.07	0.09	0.05	0.12
1185	C	T	WFS1	0.07	**0.02**	0.71	0.55	0.60	0.50	0.47
2206	G	A	WFS1	**0.01**	0.29	**0.01**	0.06	0.08	0.03	0.12

P < 0.05 are highlighted.

SNP – single-nucleotide polymorphism. PDA – panic disorder extended; all cases with panic disorder, includes pure phenotype as well as phenotypes comorbid with mood disorders (major depressive disorder, bipolar disorder) and other anxiety disorders; PDC – panic disorder comorbid with major depressive disorder; PD – pure panic disorder phenotype.

Markers -94G/A in DRD1 gene and 102T/C in HTR2A gene were specifically associated with the PD pure phenotype. SNP WFS1 2206G/A was associated with PD pure phenotype and with extended PD phenotype. Minor alleles of these free SNPs were more frequent in control subjects. In PDC (panic disorder with comorbid major depressive disorder) three markers (CCKAR 246G/A, WFS1 684C/G and 1185C/T) were associated only with this phenotype. CCK 1270C/G and HTR1A -1018C/G were significantly associated both with PDC and PDA groups. Minor alleles of all these five markers were present in a higher proportion of affected subjects. The PDA group included patients with only panic disorder as well PD patients with comorbid major depressive disorder and bipolar disorder. CCKBR -215C/A, DRD4 -1217G/del, HTR2C 68G/C, and WFS1 1023C/T SNPs were uniquely associated with the PDA group.

All these SNPs had higher minor allele frequencies in control subjects. Other significant associations in this group (CCK, HTR1A, and WFS1) were also valid for PD or PDC groups.

5.2. Results of haplotype analysis

Haplotype (HT) analysis was performed according to the particular pairwise LD pattern for each gene in different disorder phenotype groups. Only these genes that were genotyped for two or more SNPs, showed the presence of LD in both the affected and the control groups, and had preliminary evidence of marker-disease association were included in haplotype analysis. Markers were also tested for absence of Mendelian inheritance errors.

5.2.1. Results of haplotype analysis of MDD

Haplotype analysis was performed according to the particular pairwise LD pattern for each gene in broadly-defined major depressive disorder (cases+controls, N = 337). Haplotypes for major depressive disorder extended (MDD) dataset were constructed from SNPs in CCKAR and WFS1 genes. Additionally, haplotype analysis was done for POMC gene with major depressive disorder pure phenotype (MD, major depressive disorder without any comorbid disorders, cases+controls, N = 229).

5.2.1.1. CCKAR haplotypes

In the case of CCKAR (Table 8) six haplotypes (HT) were found. Reference haplotype combined the major alleles at each locus, which together with the other common haplotype constituted almost 90% of all alleles. Both haplotypes were nearly equally represented in cases and control subjects. Haplotype 3 (GAGT) was significantly overrepresented in the affected group, reflecting the higher frequency of the rare 246A allele by comparison with the reference haplotype (GGGT). This haplotype (GAGT) was associated with a higher risk of MDD (OR=7.42; P=0.002) by comparison with the reference haplotype (GGGT). A significant individual SNP effect (OR=7.40; P=0.002) was detected for 246G/A in a haplotype context HT1 (GGGT) vs HT3 (GAGT). The test of a global CCKAR haplotypic association with MDD was significant in the population studied (χ^2=17.60, df=5; P=0.004).

Table 8. Estimated haplotype frequencies and HT effects in CCKAR gene, MDD (N = 177)

HT	Single-nucleotide polymorphism				Haplotype frequency		Haplotypic OR (95% C.I.)	P
	-128G/T	246G/A	608G/A	1266T/C	Controls	Patients		
1	G	G	G	T	67.5	65.6	*	
2	G	G	G	C	21.6	20.0	0.905 (0.611–1.338)	0.625
3	G	A	G	T	1.2	7.5	7.418 (2.129–25.85)	**0.002**
4	T	G	G	T	3.8	2.6	0.517 (0.203–1.320)	0.168
5	G	G	A	T	2.4	1.3	0.588 (0.137–2.523)	0.475
6	G	A	G	C	2.4	1.3	0.588 (0.137–2.523)	0.475

P < 0.05 highlighted

5.2.1.2. WFS1 haplotypes

In the case of WFS1 gene eight haplotypes were found based on three most polymorphic SNPs (684C/G, 1185C/T, 1832G/A) (Table 9). Six haplotypes were present with probabilities higher than 2%; the global P-value for

haplotypic association with major depressive disorder was 0.027 (χ^2=12.63, df=5). The reference haplotype (HT1) combined the major alleles at each locus while another major haplotype (HT2) combined the minor alleles. Taken together with haplotype HT3, these three common haplotypes constituted more than 80% of the alleles in MDD patients and controls. HT1 (CCG) was more frequent in control subjects (44.3%) compared to cases (33.7%), whereas HT2 (GTA) was overrepresented in the affected group. Haplotype 3 (CTA) was the only one that was almost equally represented both in cases and controls. Haplotypes HT4, HT5 and HT6 were enriched in affected individuals, expressing the haplotype effect associated with an increased risk of depression (OR≥2). Haplotypes HT7 and HT8 were rare. Haplotype 2 (GTA) was significantly associated with a higher risk of MDD (OR=1.59; P=0.01) by comparison with the reference haplotype (CCG). Other haplotypes (HT4–HT6) showed only tentative associations with MDD. With HT4 (GCG) the higher relative risk was found for individuals carrying the 684G allele (OR=2.02; P=0.06) by comparison with the reference haplotype (CCG) while with HT5 (CTG) a higher relative risk for individuals carrying the 1185T allele (OR=2.01; P=0.07) by comparison with the reference haplotype was established.

Table 9. Estimated haplotype frequencies and HT effects in WFS1 gene, MDD (N = 177)

HT	Single-nucleotide polymorphism			Haplotype frequency		Haplotypic OR (95% C.I.)	P
	684C/G	1185C/T	1832G/A	Controls	Patients		
1	C	C	G	44.3	33.7	*	
2	G	T	A	31.0	38.9	1.587 (1.116–2.255)	**0.010**
3	C	T	A	9.3	8.3	1.216 (0.666–2.223)	0.530
4	G	C	G	5.1	7.4	2.024 (0.970–4.223)	0.060
5	C	T	G	3.6	5.9	2.015 (0.937–4.386)	0.072
6	G	T	G	2.7	4.1	2.107 (0.935–4.811)	0.075
7	G	C	A	2.1	0.7	–	–
8	C	C	A	1.9	1.0	–	–

P < 0.05 highlighted

5.2.1.3. POMC haplotypes

For POMC gene six haplotypes were found (Table 10), with a reference haplotype (CCC), which combines the most frequent alleles at each polymorphic site and a common haplotype HT2 (CCT). Three most frequent haplotypes accounted for nearly 90% of all alleles. The reference haplotype and HT2 were found in a higher proportion of control subjects compared to the affected group. Three haplotypes – HT3, HT4, and HT5 – were enriched in affected individuals,

expressing the haplotype effect associated with an increased risk of depression (OR>2). With HT3 (TCC) a significant association was found with the greater relative risk for individuals carrying the 282T allele (OR=3.18; P=0.007) by comparison with the reference haplotype (CCC). Testing a global POMC haplotypic association with MD did not reveal any significant evidence of association in the population studied (χ^2=9.39, df=5; P=0.094). These results provide additional support to data from association analysis suggesting an interaction between POMC gene variants and major depressive disorder, where markers 282C/T and 585C/T, being in LD with 866C/T, define haplotypes associated with an increased risk of development of major depressive disorder.

Table 10. Estimated haplotype frequencies and HT effects in POMC gene, MD (N = 69)

HT	Single-nucleotide polymorphism			Haplotype frequency		Haplotypic OR (95% C.I.)	P
	282C/T	585C/T	866C/T	Controls	Patients		
1	C	C	C	74.0	66.1	*	
2	C	C	T	13.9	11.6	0.865(0.430–1.736)	0.681
3	T	C	C	3.8	10.5	3.179 (1.381–7.317)	**0.007**
4	C	T	C	3.4	5.6	2.177 (0.695–6.816)	0.182
5	T	T	C	3.0	5.2	2.004 (0.641–6.271)	0.232
6	T	C	T	1.9	1.0	0.646 (0.017–24.64)	0.814

P < 0.05 highlighted

5.2.2. Results of haplotype analysis of BPD

Haplotypes for bipolar disorder dataset (based on BPD group, cases+controls, N = 207) were constructed from SNPs in 4 genes — CCKAR, HTR2A, OPRM1, and WFS1.

5.2.2.1. CCKAR haplotypes

Six haplotypes were found in CCKAR gene (Table 11), with a major reference haplotype combining the major alleles at each locus, which together with the other common haplotype constituted almost 90% of all alleles. Haplotype 5 (GAGT) was overrepresented in the affected group, reflecting a higher frequency of the rare 246A allele in cases than in controls compared to the reference haplotype. This haplotype was associated with a higher risk of BPD (OR=7.55; P=0.005). Also, a significant individual SNP effect (OR=7.53; P=0.005) was detected for 246G/A in a haplotype context HT1 (GGGT) vs HT5

(GAGT). The testing of global CCKAR haplotypic association with BPD established a significant association in the population studied (χ^2=12.66, df=5; P=0.027).

Table 11. Estimated haplotype frequencies and HT effects in CCKAR gene

HT	Single-nucleotide polymorphism				Haplotype frequency		Haplotypic OR (95% C.I.)	P
	-128G/T	246G/A	608G/A	1266T/C	Controls	Patients		
1	G	G	G	T	67.1	68.9	*	
2	G	G	G	C	22.0	14.7	0.661 (0.354–1.233)	0.192
3	T	G	G	T	3.8	4.3	1.030 (0.321–3.308)	0.912
4	G	G	A	T	3.5	3.3	0.836 (0.191–3.668)	0.808
5	G	A	G	T	1.2	8.1	7.554 (1.815–31.43)	**0.005**
6	G	A	G	C	2.4	0.7	0.764 (0.004–163.3)	0.921

P < 0.05 highlighted

5.2.2.2. HTR2A haplotypes

SNPs in the HTR2A gene formed seven haplotypes (Table 12). The major reference haplotype (GCCC) combines the most frequent alleles at each polymorphic site. Taken together with HT2 and HT3, these haplotypes constituted more than 85% of all alleles. The reference haplotype was found in a significantly higher proportion in controls compared to the affected group, whereas haplotypes 3, 4, and 6 were significantly more frequent in cases than in controls. The relative greater risk for individuals carrying the -1438A allele (OR=2.26; P=0.028) was established with HT3. An individual SNP effect (OR=0.44; P=0.03) was found for -1438A/G in a haplotype context HT3 (ACCC) vs HT1 (GCCC). The test of a global HTR2A haplotypic association with BPD was not significant in the population studied (χ^2=6.84, df=6; P=0.34).

Table 12. Estimated haplotype frequencies and HT effects in HTR2A gene

HT	Single-nucleotide polymorphism				Haplotype frequency		Haplotypic OR (95% C.I.)	P
	-1438A/G	73C/A	102T/C	1354C/T	Controls	Patients		
1	G	C	C	C	49.8	38.0	*	
2	A	C	T	C	28.3	31.6	1.517(0.807–2.853)	0.196
3	A	C	C	C	9.2	16.7	2.263 (1.089–4.699)	**0.028**
4	G	C	T	C	3.8	5.0	1.372 (0.385–4.902)	0.627
5	A	C	T	T	3.7	2.6	0.754 (0.136–5.097)	0.742
6	G	A	C	C	2.0	4.1	2.310 (0.552–9.660)	0.252
7	G	C	C	T	2.7	0.8	0.799 (0.004–149.4)	0.933

P < 0.05 highlighted

5.2.2.3. OPRM1 haplotypes

In the OPRM1 gene revealed five haplotypes (Table 13) with two major haplotypes and two relatively common haplotypes. The reference haplotype, which combines the major alleles at each SNP, was more frequent in the affected group compared to controls, reflecting the higher frequency of the 691C allele in cases than in controls. Haplotypes 3 and 5 were significantly more frequent in the control group. HT3 (GGC) was associated with a lower risk of BPD (OR=0.37; P=0.019) compared to the reference haplotype. This association mainly reflects the higher frequency of the minor 118G allele in controls than in cases. Test of a global OPRM1 haplotypic association with BPD was not significant in the studied population (χ^2=8.20, df=4; P=0.084). Haplotypes carrying protective 118G allele appear to be associated with a lower risk of bipolar disorder, supporting the data from association analysis concerning significant association between marker 118A/G and bipolar disorder.

Table 13. Estimated haplotype frequencies and HT effects in OPRM1 gene

HT	Single-nucleotide polymorphism			Haplotype frequency		Haplotypic OR (95% C.I.)	P
	31G/A	118A/G	691C/G	Controls	Patients		
1	G	A	C	37.7	44.8	*	
2	G	A	G	32.1	34.9	0.859 (0.486–1.517)	0.600
3	G	G	C	16.1	8.4	0.368 (0.160–0.848)	**0.019**
4	A	A	G	8.6	10.6	0.978 (0.425–2.248)	0.958
5	G	G	G	5.5	1.2	0.371 (0.002–61.79)	0.704

P < 0.05 highlighted

5.2.2.4. WFS1 haplotypes

In the case of WFS1 gene eight haplotypes were found based on three most polymorphic SNPs (684C/G, 1185C/T, 1832G/A) (Table 14). Seven haplotypes were present with probabilities higher than 2%; the global P-value for haplotypic association with bipolar disorder was 0.034 (χ^2=15.17, df=7). The reference haplotype (HT1) combined the major alleles at each locus while another major haplotype (HT2) combined the minor alleles. Together with haplotype HT3 these three haplotypes constituted more than 80% of all alleles in controls but only 75% of all alleles in cases. HT1 was overrepresented in control subjects (44.3%) compared to cases (28.9%), whereas HT2 was more frequent in the affected group. Unlike the major depression group, HT3 (CTA) and HT4 (GCG) were overrepresented in controls similarly to the reference haplotype. Haplotypes HT5, HT6, and HT7 were more frequent in affected individuals, expressing the haplotype effect associated with an increased risk of bipolar disorder (OR>>2). HT6 and HT7 were clearly more frequent in BPD

patients compared to the major depression study group. Haplotype 2 (GTA) was associated with a higher risk of BPD (OR=1.89; P=0.03) by comparison with the reference haplotype (CCG). Unlike with major depression, HT4 (GCG) did not show any association with relative risk of BPD. With HT5 (CTG) tentative evidence of a higher relative risk for individuals carrying the 1185T allele (OR=2.48; P=0.09) by comparison with the reference haplotype (CCG) was established. Interestingly, HT6 (GTG) and HT7 (GCA) were quite common in cases, both clearly indicating associations with a higher risk of BPD: with OR of 3.80 (P=0.03) for HT6 and with OR of 4.25 (P=0.02) for HT7.

Table 14. Estimated haplotype frequencies and HT effects in WFS1 gene

HT	Single-nucleotide polymorphism			Haplotype frequency		Haplotypic OR (95% C.I.)	P
	684C/G	1185C/T	1832G/A	Controls	Patients		
1	C	C	G	44.3	28.9	*	
2	G	T	A	31.0	39.5	1.890 (1.074–3.325)	**0.027**
3	C	T	A	9.3	5.5	0.732 (0.214–2.531)	0.626
4	G	C	G	5.1	3.3	0.834 (0.196–3.537)	0.803
5	C	T	G	3.6	5.8	2.477 (0.892–7.139)	0.092
6	G	T	G	2.7	7.7	3.797 (1.123–12.43)	**0.033**
7	G	C	A	2.1	8.0	4.251 (1.225–14.75)	**0.023**
8	C	C	A	1.9	1.3	1.076 (0.112–10.38)	0.950

P < 0.05 highlighted

5.2.3. Results of haplotype analysis of PD

Haplotype analysis was performed in the whole data set (cases+controls, N=273). Haplotypes for PDA group were constructed from SNPs in two genes — CCK and DRD1. Additional haplotype analyses were done for DRD1 and HTR2A gene in the PD pure phenotype subgroup (cases+controls, N=188).

5.2.3.1. CCK haplotypes

Three haplotypes were found for CCK gene in the PDA group with a major haplotype and a relatively common haplotype 2 (Table 15). The reference haplotype, which combines the wild-type alleles at each locus, was slightly more frequent in controls, whereas haplotype 2 was significantly more frequent in patients. Haplotype 2 (TG) was associated with a higher risk of PD (OR=1.77; p=0.04) by comparison with the reference haplotype. This association reflects a higher frequency of both SNP -45T and 1270G alleles in cases compared to controls. The global haplotypic effect of CCK gene did not reach the level of statistical significance (χ^2=5.23, df=2; p=0.073).

Table 15. Estimated haplotype frequencies and HT effects in CCK gene, PDA (N = 127)

HT	Single-nucleotide polymorphism		Haplotype frequency		Haplotypic OR (95% C.I.)	P
	-45C/T	1270C/G	Controls	Patients		
1	C	C	85.5	78.9	*	
2	T	G	10.7	16.6	1.77 (1.03–3.04)	**0.04**
3	C	G	3.1	4.4	1.47 (0.60–3.60)	0.40

P < 0.05 highlighted

5.2.3.2. DRD1 haplotypes

Five haplotypes were formed for DRD1 gene in the PD pure group (Table 16) with two major haplotypes: the reference haplotype 1, which combines the most frequent alleles at each polymorphic site, and a common haplotype 2 combining the wild-type alleles at each locus. Together with haplotype 3 these haplotypes constituted more than 90% of all alleles. The reference haplotype was found in a significantly higher proportion of affected individuals, whereas haplotype 3 was significantly more frequent in controls. Haplotype 5 was not represented in the cases. Haplotype 3 (CAA) was associated with a lower risk of PD pure phenotype (OR=0.25; p=0.03) as compared to the reference haplotype. This association mainly reflects a higher frequency of -94A allele in controls indicating an individual SNP -94G/A effect of marginal significance. The test of the global DRD1 haplotypic effect demonstrated association with pure PD phenotype (χ^2=9.02, df=3; P=0.029). The haplotype count and distribution in the PDA group were similar to the PD pure group, except for haplotypes 1 and 3, where differences in haplotype frequencies between cases and controls were much less remarkable (40.6% vs. 44.4% and 14.5% vs. 9.5% respectively). Therefore, the haplotype-phenotype effect for haplotype 3 (CAA) was not significant (OR=0.58; P=0.089). Thus, in the case of DRD1 gene, the haplotype-based strategy detected an involvement of -94G/A in the genetic predisposition to PD.

Table 16. Estimated haplotype frequencies and HT effects in DRD1 gene, PD (N = 42)

HT	Single-nucleotide polymorphism			Haplotype frequency		Haplotypic OR (95% C.I.)	P
	-800T/C	-94G/A	-48G/A	Controls	Patients		
1	C	G	A	40.3	51.1	*	
2	T	G	G	35.7	39.2	0.88 (0.50–1.57)	0.67
3	C	A	A	14.9	4.8	0.25 (0.07–0.90)	**0.03**
4	C	G	G	6.5	3.6	0.47 (0.13–1.75)	0.26
5	T	G	A	1.9	0.1	–	–

P < 0.05 highlighted

5.2.3.3. HTR2A haplotypes

Four two-marker haplotypes were found for HTR2A gene in the PD pure group (Table 17) with a major reference haplotype, which combined the most frequent alleles, and a common haplotype 2 combining the wild-type alleles at each locus. These haplotypes constituted more than 85% of all alleles. The reference haplotype (GC) was found in a higher proportion of affected individuals, whereas haplotype 2 (AT) was significantly more frequent in controls. Haplotype 4 was not represented in cases. Haplotype 2 was associated with a lower risk of PD (OR=0.49; P=0.04) by comparison with the reference haplotype. This association reflected in part a higher frequency of 102T allele and 102TT genotype in controls. The individual risk effect for SNP 102T/C by comparison with haplotype 2 (AT) vs 3 (AC) was not statistically significant (OR=1.8; P=0.23). Despite the fact that for SNP -1438A/G the individual haplotype effect remained not significant either an interaction between -1438A/G and 102T/C allelic variants seemed to determine to a large extent the actual gene dose effect for HTR2A. These markers have been consistently reported to be in linkage disequilibrium (D'=0.84 in the population of the present study). The test of the global HTR2A haplotypic association with PD was not significant (χ^2=5.54, df=3; P=0.14).

Table 17. Estimated haplotype frequencies and HT effects in HTR2A gene, PD (N = 42)

HT	Single-nucleotide polymorphism		Haplotype frequency		Haplotypic OR (95% C.I.)	P
	-1438A/G	102T/C	Controls	Patients		
1	G	C	54.1	67.5	*	
2	A	T	31.6	21.0	0.491 (0.246–0.979)	**0.043**
3	A	C	10.1	11.1	0.881 (0.348–2.228)	0.788
4	G	T	4.2	0.4	–	–

P < 0.05 highlighted

None of the described marker-disease associations remained statistically significant after adjustment for multiple testing, except the GAGT haplotype effect of CCKAR gene in the case of MDD (OR=7.42; P=0.002; P=0.04 after the Bonferroni correction).

6. DISCUSSION

Clinical as well as molecular genetic studies indicate that major depressive disorder (MDD), bipolar disorder (BPD), and panic disorder (PD) are complex diseases. Besides environmental factors, many genes, each of minor individual contribution, are likely to be involved in the development of mood and anxiety disorders. In the screening set of 118 polymorphisms in 22 candidate genes, variations in 10 genes displayed an association with MDD, variations in 7 genes were associated with BPD, and variations in 9 genes with PD. These results provide further evidence for the involvement of genes related to monoaminergic and peptidergic neurotransmission in the regulation of mood and anxiety disorders. Stratification of broadly-defined patient samples with regard to comorbidity yielded a specific pattern of association to the particular subphenotypes. The data shows that the genetic variability in candidate genes may have a distinctive influence on pure and comorbid phenotypes of mood and anxiety disorders.

6.1. Genetic associations in MDD

The major depressive disorder group included patients not only with MDD phenotype but also patients who had primary MDD diagnosis with comorbid anxiety disorder, association analysis showed significant relation altogether with ten genes.

The analysis revealed that polymorphisms from two genes: DRD3 and POMC were uniquely associated with pure major depressive disorder (MD subgroup). The last association was confirmed also by haplotype analysis indicating an increased risk of POMC gene TCC haplotype (OR=3.179; P=0.007) carriers for development of MDD (Figure 1).

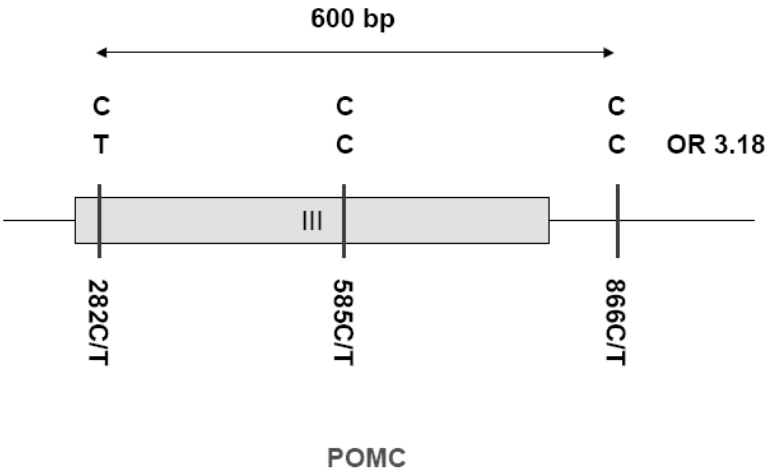

Figure 1. POMC haplotype related to an increased risk of pure MD

SNP in OPRM1 gene was associated with the MD group and with both bipolar disorder subgroups, possibly suggesting a common marker for two mood disorders. Besides MDD, pure phenotype HTR2C gene was associated with major depressive disorder extended phenotype (MDD) and panic disorder extended phenotype (PDA) implicating a common part in major depressive disorder and panic disorder. Wolframin gene (WFS1) polymorphisms were the only ones that gave significant associations with all three studied disorders and their comorbid phenotypes. Haplotype analyses with MDD and BPD gave further support for the role of WFS1 in mood and anxiety disorders. Haplotype GTA (OR=1.58; P=0.01) carriers have an increased risk of MDD (Figure 2). The WFS1 protein is a glycoprotein located in the endoplasmic reticulum membrane, but its function is poorly understood (Takeda et al., 2001). However, it is hypothesized that wolframin is related to the activation of the processing of peptide prohormones (Luuk et al., 2005). Therefore impaired function of WFS1 protein may cause abnormalities in peptide processing leading to several psychiatric disorders.

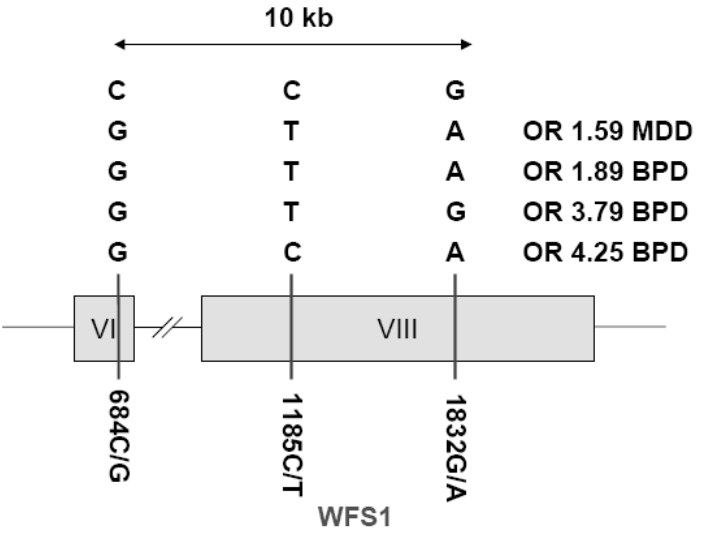

Figure 2. WFS1 haplotype related to an increased risk of MDD and BPD

Analysis of major depressive disorder with comorbid anxiety disorders (generalized anxiety disorder, obsessive-compulsive disorder, social phobia), MDA revealed associations with CCKAR, DRD5, SLC6A4, and WFS1 genes. Significant association with the polymorphism of the serotonin transporter gene only in patients with comorbid anxiety disorder implicates its importance in the regulation of anxiety and anxiety disorders. Indeed, several studies have shown that SLC6A4 polymorphisms are related to anxiety-related traits (Lesch et al., 1996; Murakami et al., 1999). Polymorphism in DRD5 gene (1481C/T) is also a possible specific marker for anxiety disorders which gave similar associations in both bipolar disorder subgroups because most patients in these groups were with comorbid anxiety disorders. CCKAR gene seems to be a general risk factor for mood and anxiety disorders, whereas it was additionally related to major depressive disorder extended (MDD), bipolar disorder (BPD), and panic disorder comorbid with major depressive disorder (PDC) subgroups. Associations with the two first groups were confirmed by haplotype analysis where GAGT haplotype carrying risk for the MDD (OR=7.418; P=0.002) and for the BPD (OR=7.554; P=0.005) was established. The haplotype effect for MDD remained also significant after the Bonferroni correction (P=0.04 after Bonferroni's adjustment) (Figure 3).

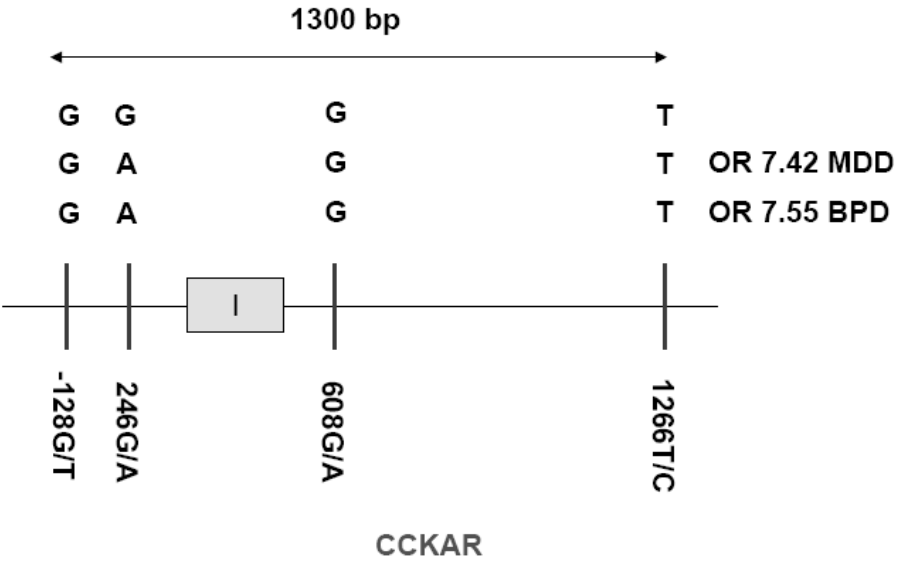

Figure 3. CCKAR haplotype related to an increased risk of MDD and BPD

Polymorphisms from CCKAR, DRD1, DRD2, HTR2C, and WFS1 genes were associated with MDD extended group (MDD), in which MDD patients with comorbid panic disorder were added compared to the MDA group. Interestingly, association with DRD2 gene was unique to this group, possibly being also a comorbid marker of MDD and anxiety disorders. DRD1 gene polymorphisms were also significantly associated with pure panic disorder phenotype (PD) and bipolar disorder (BPA) indicating the impact on mood and panic disorders. Predisposition of DRD1 gene to PD was affirmed by haplotype analysis, where CAA haplotype was associated with a lower risk of panic disorder (OR=0.25; P=0.03).

To sum up, major depressive disorder seems to be most significantly related to POMC, CCKAR, and WFS1 genes.

6.2. Genetic associations in BPD

Eleven polymorphisms from seven genes were significantly associated with bipolar disorder. Differently from major depressive disorder no unique marker for bipolar disorder appeared. On one hand, it can be explained by the small number of subjects with bipolar disorder only. Most patients had also comorbid anxiety disorder, and that is why associations of CCK, CCKAR, DRD1, DRD5, and HTR2A genes in bipolar disorder subgroups were significant also in panic disorder and in major depressive disorder subgroups comorbid with anxiety

disorders. The statistically most significant associations with BPD were with OPRM1 gene polymorphism, which was related also to major depressive disorder without comorbid anxiety disorders (MD). Haplotype analysis with BPD indicated that OPRM1 GGC haplotype is associated with a lower risk of the disorder (OR=0.37, P=0.02) (Figure 4). Although the direct role of endogenous opiates in mediating mental illness has been difficult to establish, their interaction with a number of neurotransmitter systems involved in mood disorders suggests their possible modulatory role (Vaccarino and Kastin, 2001; Tortella et al., 1989).

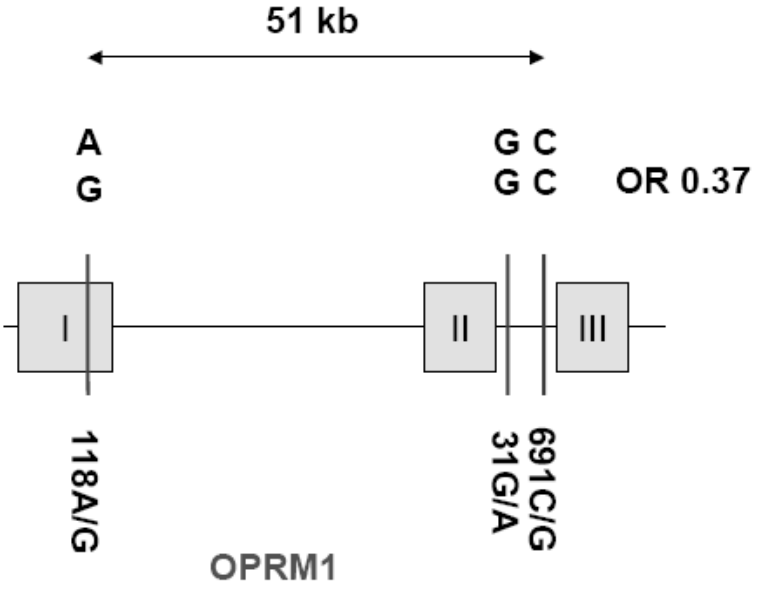

Figure 4. OPRM1 haplotype related to a decreased risk of BPD

Haplotype analysis confirmed also associations of CCKAR GAGT (OR=7.55; P=0.005) haplotype (Figure 3), HTR2A ACCC (OR=2.26; P=0.028) haplotype, and WFS1 GTA (OR=1.89; P=0.027), GTG (OR=3.80; P=0.033), and GCA (OR=4.25; P=0.023) haplotypes (Figure 2) with a higher risk of BPD. Several studies did not find any associations with BPD and HTR2A polymorphisms, but some positive findings do exist (Vincent et al., 1999; Bonnier et al., 2002). Interestingly, HTR2A seems to be quite selective for the BPD, as no associations were found with MDD. This finding is in good line with previously published data, where most positive findings are related to the BPD (Bonnier et al., 2002; Oswald et al., 2003). However, due to the limited sample size one can not draw any definite conclusions related to bipolar disorder.

6.3. Genetic associations in PD

Twelve SNPs from nine genes were associated with panic disorder phenotypes. As in the case of bipolar disorder, no markers were detected that could be specific to panic disorder pure phenotype (PD). In patients with only PD associations with DRD1, HTR2A, and WFS1 genes were found. Associations of DRD1 and HTR2A gene were confirmed by haplotype analyses; haplotypes CAA (OR=0.25; P=0.03) and AT (OR=0.49; P=0.04) indicate a lower risk of panic disorder, accordingly.

The PDC subgroup was formed of patients who had both major depressive and panic disorder. Therefore, markers related to this subgroup (CCK, CCKAR, HTR1A, and WFS1) could be considered markers common to both disorders. As all these genes were associated also with other analysed subgroups including panic disorder extended (PDA), major depressive disorder extended (MDD) groups, they are probably generally related to mood and anxiety disorders.

The extended panic disorder subgroup PDA was composed of patients with only panic disorder and additionally patients with panic disorder with comorbid major depressive and bipolar disorder. CCKBR and DRD4 genes were specifically associated to this group. Interestingly, both these genes are located very closely in 11p15.4-p.15.5 chromosomal region. Suggestive linkage for chromosomal location 11p has been detected at marker CCKBR in pedigrees of panic disorder probands (Gelernter et al., 2001). It gives additional support for the hypothesis that DRD4 and CCKBR genes may be related to the development of panic disorder. These and other genes in this region (e.g. tyrosine hydroxylase) need further analysis in larger sample sizes to confirm their role in the disorder. Polymorphisms in CCK, HTR1A, HTR2C, and WFS1 genes associated with PDA group were additionally significantly related to other studied comorbid subgroups PDC, BPA, MDD, and MD. Involvement of CCK gene in comorbid panic disorder was confirmed with haplotype analysis TG haplotype (OR=1.77; P=0.04) showing a higher risk of the disease. Altogether CCKBR and DRD4 genes are probably related to panic disorder.

6.4. General discussion

Associations of CCKAR and WFS1 gene with all the three studied psychiatric disorders could be taken as the most considerable results of this study. CCKAR polymorphisms have been shown to be involved in schizophrenia and auditory hallucinations (Wang et al., 2002; Wei and Hemmings, 1999), but also in panic disorder (Miyasaka et al., 2004). Preclinical studies suggest that CCKAR regulates directly the release of dopamine in the nucleus accumbens and amygdala (Hamilton and Freeman, 1995). Therefore, CCKAR is implicated in the regulation of emotional behaviour and motivation.

Several previous studies found no associations between SNPs in the WFS1 gene and mood disorders (Middle et al., 2000; Evans et al., 2000; Ohtsuki et al., 2000). Therefore, it is probably not a major susceptibility gene for psychiatric disorders. However, it remains possible that WFS1 variants increase substantially susceptibility to mood disorders. A recent study by Swift et al. estimated that the relative risk of psychiatric hospitalization for depression was 7.1 (Swift and Swift, 2005b). Supportive evidence of CCKAR and WFS1 genes involvement in mood and anxiety disorders is also related to their genomic localization (4p15.1-p15.2 and 4p16 respectively). DRD5 gene, which was associated with major depressive and bipolar disorders, is also located in 4p16.1 region. 4p16 locus has been repeatedly shown to be related to bipolar disorder (Kennedy et al., 1999a). Als et al found that markers in 4p15 region appeared to be associated with schizophrenia, and schizophrenia combined with bipolar disorder (Als et al., 2004). Also, supportive evidence was found that schizophrenia and bipolar disorder are associated with 4p16 region.

Another genomic region that appeared from the analysis is 11p15 locus in the case of panic disorder. CCKBR and DRD4 genes were associated with extended PD subgroup (PDA) and linkage has been shown between this region and panic disorder. CCKBR is a good candidate gene for further confirmation in relation to panic disorder. It has been shown that in humans the administration of CCK-4 and other CCKBR agonists produces panic attack in healthy volunteers and in patients with panic disorders (Carrasco and Van de Kar, 2003; Bradwejn, 1992; Bradwejn and Koszycki, 1994). DRD4 is mainly studied in relation to novelty-seeking and attention-deficit/hyperactivity disorder. Results of these studies are inconsistent, and it is not clear if DRD4 is related to these traits (Leung et al., 2005; Frank et al., 2004). Associations between DRD4 and panic disorder have not been studied extensively. One previous study did not find any significant associations between them (Hamilton et al., 2000). 4p15-p16 and 11p15 regions seem to be good candidate loci for psychiatric disorders and need a further investigation in larger sample sizes.

The results of this study provide further evidence for the involvement of genes related to monoaminergic and peptidergic neurotransmission in the regulation of mood and anxiety disorders. Still one cannot exclude the hypothesis that the described polymorphisms are in LD with other functionally significant polymorphisms, which could actually be involved in mood and anxiety disorders. It has been shown that missense SNP itself does not probably cause disease but is in strong LD with non-functional SNP, which actually may contribute to susceptibility to disease (Handoko et al., 2005). This warrants the study of not only functional polymorphisms but also untranslated SNPs.

This study should be considered as an exploratory study due to the limited sample size. A multi-stage approach is recommended to distinguish false-positive discoveries from real associations (Hirschhorn and Daly, 2005). As many association studies produce unreplicable results due to false-positive findings induced by multiple testing, then it is suggested that at first many

markers should be typed for a subset of individuals. Afterwards the most promising markers are evaluated on a larger sample (van den Oord and Sullivan, 2003). Therefore, replication studies with larger and independent samples are needed.

6.5. Future prospects

Future studies should focus on 4p15-p16 chromosomal region. Besides CCKAR, DRD5, and WFS1 genes, which revealed associations in the current study, in this region or close to it there are other genes with potential relevance to mood and anxiety disorders. For example, huntingtin; alpha-2C-adrenergic receptor; gamma-aminobutyric acid A receptor, alpha-4 (GABRA4); and gamma-aminobutyric acid A receptor, gamma-1 (GABRG1) genes. GABA is a major inhibitory neurotransmitter in the brain and is possibly also related to psychiatric disorders. Of course other genes in this region need to be studied as well. Also, 11p15 chromosomal locus in relation to panic disorder needs more thorough analysis to detect functionally connected genes to panic disorder.

Other potential candidate genes could be included based on animal studies, e.g. LsAMP gene, which has been shown to be overexpressed in animal models related to emotional behaviour (Nelovkov et al., 2003). These further projects need larger sample sizes, and different genetic study designs could be put togther to achieve more powerful results. New targets besides measuring MAOA activity in blood should be taken into consideration to detect gene expression differences in psychiatric disorders.

7. CONCLUSIONS

1. Polymorphisms of CCKAR, DRD1, DRD2, DRD3, DRD5, HTR2C, SLC6A4, OPRM1, POMC, and WFS1 genes were associated with major depressive disorder and its comorbid phenotypes. Supportive evidence of involvement of 4p15-p16 chromosomal region in mood disorders was detected.
2. Polymorphisms of CCK, CCKAR, DRD1, DRD5, HTR2A, OPRM1, and WFS1 genes showed associations with bipolar disorder and its comorbid phenotypes.
3. Panic disorder and its comorbid phenotypes were related to CCK, CCKAR, CCKBR, DRD1, DRD4, HTR1A, HTR2A, HTR2C, and WFS1 genes. In addition, relatedness of 11p15 chromosomal locus to panic disorder was suggested.
4. For major depressive disorder pure phenotype two specific markers appeared: DRD3 and POMC genes. Panic disorder extended phenotype specific markers included CCKBR and DRD4. Other significant associations were largely common to several disorders and their comorbid phenotypes. DRD1, DRD2, HTR1A, and HTR2A genes could be considered as major depressive disorder and panic disorder comorbidity specific markers. SLC6A4 and DRD5 are rather general anxiety markers. Based on this study, CCKAR and WFS1 genes could be considered general markers for mood and anxiety disorders.

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SUMMARY IN ESTONIAN

22 kandidaatgeeni ühenukleotiidsete polümorfismide profiilide seos meeleolu- ja ärevushäiretega

Meeleolu- ja ärevushäired on laialtlevinud ja nii inimesele endale kui lähiperekonnale vaegusi ja toimetulekuraskusi põhjustavad psüühikahäired. Suurima grupi meeleoluhäiretest moodustab unipolaarne depressioon, mille levimuseks hinnatakse 2–19% populatsioonist. Depressiooni peamisteks tunnusteks on alanenud meeleolu, huvi ja elurõõmu kadumine ning väsimus. Teine suurem meeleoluhäirete grupp on bipolaarne meeleoluhäire. Seda iseloomustavad korduvad meeleolu ja aktiivsuse häiritud episoodid, mille käigus mõnel juhul ilmneb meeleolu kõrgenemine ning energia ja aktiivsuse tõus (maania või hüpomaania), teistel juhtudel meeleolu alanemine ning energia ja aktiivsuse alanemine (depressioon). Bipolaarset meeleoluhäiret esineb ligikaudu 1% inimestel. Ärevushäirete alla kuuluvad foobiad, paanikahäire, generaliseerunud ärevushäire, obsessiiv-kompulsiivne häire. Paanikahäire põhiliseks iseärasuseks on korduvad rasked ärevushood e. paanikahood, mis ei ole seotud kindla situatsiooni või muude välistingimustega. Sümptomid võivad varieeruda, kuid tavalised on ootamatu algusega südamekloppimine, valu rindkeres, lämbumistunne, nõrkus- ja ebareaalsustunne. Peaaegu alati on surmahirm, hirm kaotada enesekontroll või hulluks minna. Ligikaudu 1.6%–2.2% elanikkonnast kannatab paanikahäire all. Kõigi kolme psüühikahäire puhul on tegu komplekshaigustega, kus haiguse kujunemises mängivad olulist rolli nii geneetilised kui keskkonnategurid. Perekonna- ja kaksikuteuuringud on näidanud, et suurima pärilikkuse komponendiga on bipolaarne meeleoluhäire, järgnevad depressioon ja paanikahäire. Komplekshaiguste puhul arvatakse, et mõju avaldavad paljud geenid, interakteerudes üksteise ja keskkonnateguritega ning iga geeni osakaal on väike. Komplekshaiguste geneetilisel analüüsil on sarnaselt monogeensete haiguste uurimisele kasutatud aheldusanalüüsi perekonnauuringutes, mille abil tuvastatakse suuremad kromosoomipiirkonnad. Assotsiatsiooniuuring võimaldab täpsemalt analüüsida kandidaatgeenide seotust haigusega. Kandidaatgeenid valitakse lähtuvalt teadaolevast või eeldatavast funktsioonist, aheldusanalüüsiga kindlaks määratud piirkonnast või näiteks loommudelitel saadud oluliste tulemuste põhjal. Psüühikahäirete patogeneesi bioloogilised mudelid on senini keskendunud peamiselt monoamiinide (serotoniin, noradrenaliin, dopamiin) neurotransmissioonisüsteemi erinevate komponentide uurimisele. Lisaks on võetud teisi sihtmärke (neuropeptiidid, endopioidsüsteem jne), kuna monoamiinide ülekande häirumisel põhinevad hüpoteesid ei seleta ära psüühikahäirete patogeneesi ning neil põhinevad meeleolu- ja ärevushäirete ravimid pole piisavalt efektiivsed.

Uurimuse põhieesmärgid

Antud uurimuse peamine eesmärk oli leida depressiooni, bipolaarse meeleoluhäire ja paanikahäirega seotud võimalikud geenid. Täpsemad eesmärgid olid:

1. Kindlaks määrata assotsiatsioonid 22 kandidaatgeeni 118 polümorfismi ja depressiooni ning depressiooni ja temaga kaasnevate ärevushäirete vahel.
2. Kindlaks määrata assotsiatsioonid 22 kandidaatgeeni 118 polümorfismi ja bipolaarse meeleoluhäire ning bipolaarse meeleoluhäire ja temaga kaasnevate ärevushäirete vahel.
3. Kindlaks määrata assotsiatsioonid 22 kandidaatgeeni 118 polümorfismi ja paanikahäire ning paanikahäire ja temaga kaasnevate meeleoluhäirete vahel.
4. Võrrelda kolme erinevat psüühikahäiret ja leida haigusspetsiifiline polümorfismide profiil.

Meetodid

Antud assotsiatsiooniuuringus osalesid suguluses mitteolevad meeleolu- ja paanikahäirega patsiendid (N = 269) ja terved vabatahtlikud kontrollisikud (N = 160) Eesti populatsioonist. Kuna enamusel patsientidest esines mitu psüühikahäiret korraga ja ainult ühe häirega patsiente oli vähe ning et leida nii ühele kui mitmele häirele spetsiifilisi geneetilisi markereid, siis jagasime võrdlusanalüüsideks patsiendid vastavalt diagnoosile mitmetesse gruppidesse. 22 kandidaatgeenist pärit 118 ühenukleotiidse polümorfismi genotüpeerimiseks kasutasime APEX-tehnoloogial (oligonukleotiidmaatriksil põhinev praimerekstensioon) põhinevat geenikiipi. Statistilise analüüsi käigus võrreldi markerite alleelisagedusi patsientide gruppide ja tervete kontrollgrupi vahel. Samuti viidi läbi haplotüübianalüüs.

Tulemused

Assotsiatsioonianalüüsi tulemused näitasid, et meeleolu- ja paanikahäiretega oli statistiliselt oluliselt seotud kokku 27 polümorfismi 15 geenist. Depressiooni grupiga, kus olid nii ainult depressiooniga patsiendid, kui isikud, kellel lisaks depressioonile oli kaasnev ärevushäire, oli statistiliselt oluliselt seotud kokku markerid kümnest geenist: CCKAR, DRD1, DRD2, DRD3, DRD5, HTR2C, SLC6A4, OPRM1, POMC ja WFS1. Spetsiifiliselt depressiooniga oli seotud DRD3 ja POMC geenid. POMC geeni TCC haplotüübi (OR=3.179; P=0.007) kandjatel esineb suurenenud risk haigestuda depressiooni. OPRM1 geeni polümorfism oli lisaks depressioonile oluline bipolaarse meeleoluhäire gruppides. Seega võiks tegemist olla mõlema meeleoluhäire puhul ühist rolli mängiva geeniga. Depressioon ja paanikahäire on sageli üksteisega kaasnevad haigused. Antud uurimuses ilmnesid oletatavad mõlemale haigusele ühised geenid olevat

HTR2C, HTR1A, DRD1 ja DRD2. Nende puhul esinesid olulised assotsiatsioonid mitmes depressiooni ning depressiooniga kaasneva paanikahäire või paanikahäirega kaasneva meeleoluhäire alagrupis. Wolframini geeni polümorfismid olid ainsad, mille puhul esines statistiliselt olulisi assotsiatsioone kõigis analüüsitud depressiooni, bipolaarse meeleoluhäire, paanikahäire ning nendega kaasnevate meeleolu- ja ärevushäirete alagruppides. Lisaks ilmnes haplotüübianalüüsiga, et GTA (OR=1.58, P=0.01) on riskihaplotüüp depressiooni kujunemisel. Wolframin on endoplasmaatilise retiikulumi membraanivalk, mille oletatav funktsioon võiks olla prohormoonide töötlemise aktiveerimine. Vigane wolframini valk võib seega põhjustada kõigile psüühikahäiretele omaseid peptiidide töötlemise häireid. Teine üldine meeleolu- ja ärevushäirete riskigeen näib olevat CCKAR, mis samuti osutus oluliseks mitmetes alagruppides (depressioon koos kaasnevate ärevushäiretega, paanikahäire kaasneva depressiooniga, bipolaarne meeleoluhäire kaasnevate ärevushäiretega). Haplotüübianalüüs kinnitas CCKAR geeni haigusriski seoses depressiooniga, näidates et GAGT (OR=7.42; P=0.002) on riskihaplotüüp. Antud haplotüübiefekt jäi statistiliselt oluliseks ka pärast Bonferroni korrektsiooni (P=0.04). Ärevuse markeriteks võib pidada SLC6A4 ja DRD5 geene, kuna neis asuvad polümorfismid andsid seoseid gruppides, kus valdavalt olid kaasnevate muude ärevushäiretega (generaliseerunud ärevushäire, obsessiiv-kompulsiivne häire, sotsiaalfoobia) meeleoluhäiretega patsiendid. Kokkuvõttes, POMC, CCKAR ja WFS1 geenid osutusid kõige olulisemalt seotuks depressiooniga. Samuti kinnitasime 4p15-p16 kromosoomi piirkonna seotust erinevate psüühikahäiretega. Selles piirkonnas asuvad CCKAR ja WFS1 geenid, mis andsid olulise assotsiatsiooni- ning haplotüübitulemuse depressiooniga, kuid samuti bipolaarse meeleoluhäirega. Bipolaarse meeleoluhäirega patsientide grupis andsid olulise seose CCK, CCKAR, DRD1, DRD5, HTR2A, OPRM1 ja WFS1 geenid. Ükski neist polnud unikaalne sellele häirele, kuna osutusid olulisteks ka depressiooni ja paanikahäire ning nendega kaasnevate häiretega gruppides. Osaliselt on see seletatav katseisikute väikese arvuga nii mittekaasneva kui kaasnevate ärevushäiretega bipolaarse häirega gruppides. OPRM1 geeni seotust bipolaarse meeleoluhäirega kinnitas haplotüübianalüüs, näidates GGC (OR=0.37; P=0.02) haplotüübi protektiivset efekti haiguse suhtes. Seevastu CCKAR geeni GAGT (OR=7.55; P=0.005) ning WFS1 geeni GTA (OR=1.89; P=0.027), GTG (OR=3.80; P=0.033) ja GCA (OR=4.25; P=0.023) haplotüübid osutusid riskihaplotüüpideks bipolaarse meeleoluhäire suhtes. Samuti kinnitavad nad assotsiatsioonianalüüsi tulemusi nende geenide seotusest bipolaarse meeleoluhäirega. Paanikahäire ja temaga kaasnevate meeleoluhäiretega oli seotud kokku üheksa geeni: CCK, CCKAR, CCKBR, DRD1, DRD4, HTR1A, HTR2A, HTR2C ja WFS1. DRD1, HTR2A ja WFS1 geenide polümorfismid andsid olulise seose ilma kaasneva häireta paanikarühmas. DRD1 ja HTR2A geenide seost kinnitas haplotüübianalüüs, näidates et vastavalt CAA (OR=0.25; P=0.03) ja AT (OR=0.49; P=0.04) haplotüübid olid protektiivse efektiga paanikahäire suhtes. Paanikahäire ja kaasnevate meeleoluhäiretega gruppides andsid olulise

seose CCK, CCKAR, CCKBR, DRD4, HTR1A, HTR2C ja WFS1 geenid. CCK geeni assotsiatsiooni kinnitas ka haplotüübianalüüs, osutades et TG (OR=1.77; P=0.04) haplotüüp on samuti protektiivse efektiga paanikahäire suhtes. CCKBR ja DRD4 geenid olid seotud ainult paanikahäire grupiga, kus lisaks olid patsiendid kaasnevate meeleoluhäiretega. Mõlemad geenid asuvad üksteisele lähestikku kromosoomi piirkonnas 11p15.4–p15.5. Varasemalt on ülegenoomsel aheldusanalüüsil leitud aheldumine 11p CCKBR geeni piirkonnas paanikahäirega patsientide perekondades. Need tulemused viitavad, et CCKBR, DRD4 või teised selles regioonis asuvad geenid võiksid olla seotud paanikahäirega.

Järeldused

1. CCKAR, DRD1, DRD2, DRD3, DRD5, HTR2C, SLC6A4, OPRM1, POMC ja WFS1 geenid on seotud depressiooni ning temaga kaasnevate ärevushäiretega. Samuti leidis kinnituse 4p15–p16 kromosoomi piirkonna seotus erinevate psüühikahäiretega.
2. Bipolaarse meeleoluhäirega ja temaga kaasnevate ärevushäiretega olid seotud polümorfismid CCK, CCKAR, DRD1, DRD5, HTR2A, OPRM1 ja WFS1 geenidest.
3. Paanikahäire ja temaga kaasnevate meeleoluhäiretega on seotud CCK, CCKAR, CCKBR, DRD1, DRD4, HTR1A, HTR2A, HTR2C ja WFS1 geenid. Lisaks leidis kinnitust, et kromosoomi piirkond 11p15 võiks olla seotud paanikahäirega.
4. Ilma kaasnevate haigustega depressiooniga olid spetsiifiliselt seotud kaks geeni: DRD3 ja POMC. Kaasnevate meeleoluhäiretega paanikahäire fenotüübiga olid unikaalselt seotud CCKBR ja DRD4 geenid. Teised olulised assotsiatsioonid olid üldiselt ühised mitmele häirele ja nendega kaasnevate häiretega diagnoosidele. Depressiooni ja paanikahäire ühisteks geenideks võiks pidada DRD2, HTR1A ja HTR2A geene. SLC6A4, DRD1 ja DRD5 geene võib lugeda üldisteks ärevusemarkeriteks. Antud uurimuse kohaselt on meeleolu- ja ärevushäirete kõige silmapaistvamad üldised markerid CCKAR ja WFS1 geenid.

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Teadustöö

Teadustöö peamised uurimisvaldkonnad on geneetiliste markerite tuvastamine assotsiatsiooni- ja aheldusanalüüsil ning geenikspressiooni määramine seoses meeleolu- ja ärevushäiretega.