GENE – ENVIRONMENT FACTORS IN DEPRESSIVE DISORDERS WITH A FOCUS ON CIRCADIAN GENES

Louise Sjöholm

Stockholm 2010
All previously published papers were reproduced with permission from the publisher.

The image on the front page was painted by Therese Sjöholm.

Published by Karolinska Institutet. Printed by LarsErics Digital Print AB.

© Louise Sjöholm, 2010
ISBN 978-91-7409-962-1
To my family, Pappa, Mamma,
Tess, Andreas,
Mormor and Milli
ABSTRACT

Depressive disorders have a multifactorial etiology, where both environmental and genetic risk factors contribute. Depression is characterized by a depressed mood and accompanied by e.g. loss of interest and pleasure, disturbed sleep and appetite and difficulties in concentrating. A disturbed sleep-wake pattern as well as disruptions of other biological (circadian) rhythms is a hallmark of depression. This fact has led researchers to believe that disruptions of biological rhythms generated by the suprachiasmatic nucleus in the hypothalamus is one possible mechanism behind depressive disorders. A guiding hypothesis in this thesis is that there are protective and vulnerability variations in circadian clock genes regarding development of depressive disorders and that their identification will facilitate improvement of diagnosis and treatment. The main goal of this thesis was to identify circadian clock gene variants associated with depressive disorders in relation with environmental factors.

In paper I, two proposed etiological models for major depression, one model for females and one for males, were applied on the Swedish population-based material (PART) to test the ability of the models to predict major depression and other depressive disorders. Path- and correlation analyses were performed using 16 risk factors. The model was successfully replicated in both genders and predicted two-third of the liability to develop major depression as well as other subtypes of unipolar depression. These results support a similar etiology in major depression and other depressive disorders (Bipolar Disorder, Dysthymia, Mixed Anxiety Depression and Minor Depression). In paper II, the Leu7Pro polymorphism in preproNPY was investigated in PART. The Pro7 allele was shown to be protective against major depression and dysthymia in a dominant model, and this was true also in an environment-induced vulnerable state in the depressed individuals. In paper III, genetic variants in 18 circadian genes were investigated for association to major depression and dysthymia in PART. Genetic variations in PER2 were found to be associated with depression vulnerability in two of three sample sets used. This genetic risk did not seem to require exposure to the potential sleep disturbance factors stressful life events and financial strain. In paper IV, we investigated the involvement of circadian clock genes in comorbidity between major depression and alcohol abuse or dependence in the Finnish population-based cohort Health2000. This comorbid condition was found to be associated with variants in the CLOCK gene. Together with previous reports, our results indicate that CLOCK may be a vulnerability factor for depression in individuals with alcohol use disorders. In paper V the circadian gene CRY2 was investigated with regard to mRNA levels in healthy controls and patients with depression, before, during and after one night of sleep deprivation. We also aimed at identifying variations in the CRY2 gene associated with depression in Swedish and Finnish samples with seasonal affective disorder. CRY2 mRNA levels were reduced in blood from bipolar disorder patients in depressive state and mRNA levels were non-responsive to total sleep-deprivation. Furthermore, CRY2 SNPs were found to associate with seasonal affective disorder in both sample sets. To further investigate CRY2’s role in affective disorders, Swedish bipolar patients with the severe phenotype rapid cycling were investigated in paper VI. Risk and protective CRY2 haplotypes were identified that were similar to, and had similar effect sizes as, those in paper V.

In conclusion, results in this thesis support the involvement of circadian genes in depression, seasonal affective disorder and bipolar disorder. The associations found with NPY and PER2 did not seem to be affected by exposure to the depression risk factors that were identified in paper I. The associations found here need to be replicated and investigated further.
LIST OF PUBLICATIONS

I. A multifactorial developmental model for the etiology of major depression in a population based sample.

II. PreproNPY Pro7 protects against depression despite exposure to environmental risk factors.

III. PER2 variation is associated with depression vulnerability.

IV. CLOCK is suggested to associate with comorbid alcohol use and depressive disorders.

V. CRY2 is Associated with Depression.

VI. CRY2 is associated with risk for rapid cycling in bipolar disorder patients.
ADDITIONAL PUBLICATIONS

I. A role for VAV1 in experimental autoimmune encephalomyelitis and multiple sclerosis.

II. Examining the public refusal to consent to DNA biobanking: empirical data from a Swedish population-based study.
Melas PA, Sjöholm LK, Forsner T, Edhborg M, Juth N, Forsell Y, Lavebratt C.

III. Variations in FKBP5 and BDNF genes are suggestively associated with depression in a Swedish population-based cohort.
Lavebratt C, Aberg E, Sjöholm LK, Forsell Y.
Journal of Affective Disorders. 2010 Mar 11. [Epub ahead of print].
LIST OF ABBREVIATIONS

ABD Anonymous blood donors
ACTH Adrenocorticotropin
ARNTL Aryl hydrocarbon receptor nuclear translocator-like
APA The American Psychiatric Association
AUD Alcohol abuse or dependence
AUDIT Alcohol Use Disorders Identification Test
BDNF Brain derived neurotrophic factor
BP Bipolar Disorder
BP-NOS Bipolar disorder
BP-RC Bipolar disorder with rapid Cycling
CCGS Clock-controlled genes
CIDI Composite International Diagnostic Interview
CKI, δ Casein kinase 1ε, δ
CLOCK Clock homolog (mouse)
CRH Corticotrophin-releasing hormone
CRF Corticotrophin-releasing factor
CRY Cryptochrome (photolyase-like)
DEC deleted in esophageal cancer
DSM-IV Diagnostic and Statistical Manual of Mental Disorders edition IV
DZ Dizygotic twins
GABA Gamma-aminobutyrik acid
GR Glucocorticoid receptor
HPA Hypothalamic-pituitary-adrenal
HWE Hardy-Weinberg equilibrium
ICD International Classification of Diseases, Version 10
LD Linkage disequilibrium
MAF Minor allele frequency
MALDI-TOF MS Matrix-assisted laser desorption-ionization-time of flight mass spectrometry
MAOA Monoamine Oxidase A
MAOIs Monoamine oxidase inhibitors
MD Major Depression
MDI Major Depression Inventory
MZ Monozygotic twins
nonRC-BP Non rapid cycling Bipolar Disorder
NOS Not Otherwise Specified
NPAS Neuronal PAS domain protein 2
NPY Neuropeptide Y
NRIs Selective noradrenaline reuptake inhibitors
OR Odds ratio
PACAP Pituitary adenylate cyclase-activating polypeptide
PART Psykisk hälsa, arbete och relationer
PER Period homolog 1 (Drosophila)
PVN Paraventricular nucleus
qPCR Quantitative PCR
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>REV-ERBα</td>
<td>Nuclear receptor subfamily 1, group D, member 1</td>
</tr>
<tr>
<td>RHT</td>
<td>Retinohypothalamic tract</td>
</tr>
<tr>
<td>RORα</td>
<td>RAR-related orphan receptor A</td>
</tr>
<tr>
<td>SAD</td>
<td>Seasonal affective disorders</td>
</tr>
<tr>
<td>SCAN</td>
<td>Schedules for Clinical Assessment in Neuropsychiatry</td>
</tr>
<tr>
<td>SCN</td>
<td>Suprachiasmatic nuclei</td>
</tr>
<tr>
<td>SSRI</td>
<td>Selective serotonin reuptake inhibitors</td>
</tr>
<tr>
<td>SEM</td>
<td>Structural equation modeling</td>
</tr>
<tr>
<td>TCA</td>
<td>Tricyclic antidepressants</td>
</tr>
<tr>
<td>TIMELESS</td>
<td>Timeless homolog (Drosophila)</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organization</td>
</tr>
<tr>
<td>WHO-DAS-S</td>
<td>The World Health Organization Short Disability Assessment Schedule</td>
</tr>
<tr>
<td>5-HTT</td>
<td>Serotonin transporter</td>
</tr>
<tr>
<td>5-HTTLPR</td>
<td>Serotonin transporter length polymorphism</td>
</tr>
</tbody>
</table>
1 INTRODUCTION

The focus of my thesis has been to investigate the etiology behind depressive disorders, by studying in particular the influence of variation in circadian rhythm related genes on depression vulnerability. In the studies of non-seasonal unipolar depression, the analysis considered also the individuals’ exposure to certain environmental risk factors. Depressive disorders are clinically heterogeneous and both gene and environmental factors predispose. In paper I, environmental risk factors previously shown to contribute to depression were verified with the Swedish population based material PART. In paper II-VI association of circadian rhythm related genes to depression were investigated. Circadian clock gene products constitute the body’s biological clock. The endogenous master clock, located in the suprachiasmatic nucleus (SCN) of the brain, produces, maintains and synchronizes a variety of biological rhythms such as the sleep-wake pattern, blood pressure and hormone release. Some of these rhythms are disturbed in depressed individuals. Our hypothesis was that genetic vulnerability mediated by these clock genes predisposes for depression and that environmental stressors, both during childhood and later on in life, may also predispose the depressive symptoms. These clock genes were investigated in two large population bases materials, the Swedish PART and the Finnish Health2000 study, as well as in four clinical case-control materials with seasonal affective disorder patients and bipolar disorder patients from Sweden, Finland and the USA. Our studies showed association between certain circadian genes variants and depressive disorders. Thus, indicating that rhythm disturbances seen in patients may to some extent depend on variation in circadian clock genes.

"From the brain and the brain alone arise our pleasures, joys, laughter and jests, as well as our sorrows, pains and griefs"

Hippocrates
2 BACKGROUND

2.1 DEPRESSION

Depression is a common disorder in today's society and the numbers of affected are increasing. In 2003 the World Health Organization (WHO) ranked depression in third place on the top-list of global burden of disease and depression is expected to still have a top rank in 2030 (WHO, 2004). Depressive disorders cause great suffering not only for the affected person but also for the relatives as well as a substantial economical burden for the society. The study "Health Economics of Depression in Sweden" (HEADIS) carried out in 2004 and 2005, investigated the quality of life and costs of patients treated for depression in primary care. The estimated cost was €5100 to treat a patient for a depressive episode. Furthermore, the patients followed were on average absent from work 1.5 months during a six-month period and the quality of life was reduced by 50% as compared to the general population (Sobocki, 2006). Moreover, Sobocki et al. estimated that the total cost for depression in 2005 was €3.5 billion, were the sum included among other things the costs for sick leave and early retirement (Sobocki et al., 2007).

Core symptoms of depression include depressed mood accompanied by additional symptoms such as loss of interest and pleasure, difficulties in concentrating and disturbed sleep and appetite (APA, 1994). Environmental risk factors together with genetic vulnerability constitute the complex background of depression, making the pathogenesis heterogeneous and the prognosis difficult to predict. If depression is not treated it can become chronic and lead to cognitive impairment (Airaksinen et al., 2006; Camp and Cannon-Albright, 2005; Nestler et al., 2002). Depressive disorders are also highly associated with increased mortality and morbidity, not only because it is the main cause for suicide but also because it is often comorbid with other disorder, such as substance use disorders and anxiety (Camp and Cannon-Albright, 2005; Nestler et al., 2002), type 2 diabetes, coronary artery disease diseases (Krishnan and Nestler, 2008).

2.2 CLINICAL DIAGNOSES

A diagnosis of depression is set after a clinical examination, where different interview tools are being used. To help in diagnosis different self-report instruments are used. The Composite International Diagnostic Interview (CIDI) from the WHO is often used in research (WHO, 2007). The diagnoses are then made according to diagnostic criteria. The two most commonly used diagnostic manuals are Diagnostic and Statistical Manual of Mental Disorders edition IV (DSM-IV) and the International Classification of Diseases, Version 10 (ICD-10). The DSM-IV is proposed by the American Psychiatric Association (APA) and classifies mental disorders. DSM-IV is the manual predominantly used in both research and at psychiatric clinics. In 2013 a fifth edition of DSM will be released (APA, 1994). The ICD-10, proposed by the WHO, is being used for classification of diseases and other related health problems in health care (WHO, 2007).
Depression and bipolar disorders are in DSM-IV classified as an Axis 1 disorders, under the section affective (mood) disorders. Included in the DSM-IV classification of depressive disorders are Major Depressive (MD) Disorder, Dysthymic Disorder and Depressive Disorder Not Otherwise Specified. The Bipolar Disorder (BP) classification comprises of Bipolar Disorder 1, Bipolar Disorder 1, Cyclothymic Disorder and Bipolar Disorder Not Otherwise Specified (BP-NOS). The main difference between Depressive Disorders and Bipolar Disorder is the absence of manic, mixed or hypomanic episodes in depressive disorders. Several specifications can be made to the diagnoses, such as severity and the course of the disorder (APA, 1994). In ICD-10 depression and bipolar disorders are described in chapter V under the affective disorder heading (WHO, 2007).

A short description of the diagnoses being used in this thesis will now follow.

### 2.2.1 Depression

To fulfill the criteria for MD, or Major Depressive Episode, at least five of the following symptoms must have occurred within the same two-week period. One of the first two symptoms though needs to be present.

1. Depressed mood
2. Loss of interest and pleasure
3. Depressed mood most of the day, nearly every day
4. Markedly diminished interest or pleasure in all or almost all activities
5. Significant weight gain/loss or a decrease/increase in appetite
6. Insomnia or hypersomnia
7. Psychomotor agitation or retardation
8. Fatigue or loss of energy
9. Feelings of worthlessness or excessive or inappropriate guilt
10. Diminished ability to think or concentrate, or indecisiveness
11. Recurrent thoughts of death or a suicide attempt or a specific plan for committing suicide

The symptoms above (except the last one) should be experienced most of the day, nearly every day. Also, the symptoms for Mixed Episodes should not be met and the symptoms should cause clinically significant distress or impairment in social, occupational or other important aspects. Symptoms that are clearly due to somatic illness, delusions or hallucinations should not be accounted for. Also, the symptoms should not be due to direct physiological effects of any substance (drug of abuse or medication). Furthermore, the symptoms should not be better accounted for by mourning, that is, the symptoms persist for more than two months (APA, 1994).

### 2.2.2 Bipolar Disorder

Bipolar Disorder, previously known as manic-depressive disorder, includes Bipolar Disorder 1 and Bipolar Disorder 2 and BP-NOS. The essential feature of Bipolar Disorder 1 is the occurrence of one or more Manic Episodes or Mixed Episodes and for Bipolar Disorder 2 it is the occurrence of one or more Major Depressive Episodes accompanied by at least one Hypomanic Episode. Bipolar Disorder is characterized by the occurrence of three (four if the mood is only irritable) of the following seven symptoms of Mania or Hypomania. To have a Manic Episode the symptoms should last at least a week, whereas to have a Hypomanic episode the symptoms should last throughout at least four days.
1. Inflated self-esteem or grandiosity
2. Decreased need for sleep, e.g., feels rested after only three hours of sleep
3. More talkative than usual or pressure to keep talking
4. Flight of ideas or subjective experience that thoughts are racing
5. Distractibility, i.e., attention too easily drawn to unimportant or irrelevant external stimuli
6. Increase in goal-directed activity (either social, at work or school, or sexually) or psychomotor agitation
7. Excessive involvement in pleasurable activities which have a high potential for painful consequences, e.g., the person engages in unrestrained buying sprees, sexual indiscretions, or foolish business investments

As for depression, a Bipolar Diagnosis will not be made if it’s a direct consequence of any medication, drug of abuse or any other general medical condition (APA, 1994).

2.2.3 Seasonal Affective Disorders

Seasonal Affective Disorder (SAD) is not a separate psychiatric diagnosis, but a specifier to the recurrent Depression and bipolar disorder diagnoses. The DSM-IV criteria for SAD are the following:

1. There has been a regular temporal relationship between the onsets of a Major Depressive Episodes in Bipolar Disorder 2, Bipolar Disorder 2 or Recurrent Major Depressive Disorder and at a particular time of the year
2. Full remission (or a change from depression to hypomania or mania) should also occur at a specific time of the year
3. At least two Major Depressive Episodes have occurred the last two years, fulfilling the criteria above and not separated by any non-seasonal episodes
4. Moreover, the number of seasonal episodes should outnumber the non-seasonal episodes in the individual’s life

The course of the disorder, where the depression starts in late fall or winter and remits during spring is the most common form of SAD, hence the name winter depression. These seasonal associations seen should not be due to any seasonal-related psychosocial stressors (APA, 1994).

2.2.4 Alcohol Abuse and Dependence

Common for both Alcohol Abuse and Alcohol dependence is the use of alcohol that significant leads to impairment or distress and is manifested by one or more of the following criteria, occurring within a 12-month period.

**Alcohol Abuse**

- Repeated alcohol use resulting in failure to fulfill obligations at work, school, or home
- Repeated alcohol use in situations in which it is physically hazardous
- Repeated alcohol-related legal problems
- Continued use of alcohol despite persistent or recurrent social or interpersonal problems caused or worsens by the effects of the alcohol

These symptoms have not met the criteria for alcohol dependence (below).

**Alcohol Dependence**

1. Tolerance, as defined by either of the following criteria:
   - A need for markedly increased amounts of alcohol to achieve desired effect or intoxication
   - Continued use of the same amount of alcohol has a markedly diminished effect
2. Withdrawal, as defined by either of the following criteria:
   - The characteristic withdrawal syndrome for alcohol
   - Alcohol is taken to avoid or relieve withdrawal symptoms
3. Alcohol is often taken over longer periods than was intended or in larger amounts
4. There are unsuccessful efforts or a persistent desire to cut down or control the use of alcohol.
5. A big part of the individual’s time is spent in activities necessary to obtain alcohol, use alcohol or recover from its effects.
6. Important activities, such as social, recreational or occupational activities are given up or being reduced due to alcohol use.
7. Alcohol use is continued despite knowledge of having a recurrent or persistent physical or psychological problem that is likely to have been caused or exacerbated by the alcohol use (APA, 1994).

2.3 THE COMPLEXITY OF DEPRESSIVE DISORDERS

Depression is a complex disorder, meaning that the etiology of depression is due to multiple genetic loci interacting with several environmental factors (Burmeister et al., 2008; Nestler et al., 2002; Krishnan and Nestler, 2008). As for other disorders there are no physiological or biochemical tests for affective disorders in clinical use today. The diagnoses, as previously mentioned, are made by a clinical examination as an interview and observations (Burmeister et al., 2008; Nestler et al., 2002). The manifestation of depression is considered to be clinically heterogeneous, that is several disorders with distinct causes and pathophysiology (Mann and Currier, 2006; Krishnan and Nestler, 2008; Nestler et al., 2002). Depression includes a spectrum of symptoms of psychological, behavioral, cognitive and neuroendocrine manifestations. Moreover, depressive symptoms overlap with symptoms of other affective and psychiatric disorders, further complicating the dissection of the etiology (Krishnan and Nestler, 2008; Nestler et al., 2002; Burmeister et al., 2008). Several medical conditions are also associated with depression, such as endocrine disturbances, cancers, stroke, diabetes, side effects of drugs and immunological mediators, by mentioning a few, further adding to the complexity (Akil et al., ; Belmaker and Agam, 2008; Camp and Cannon-Albright, 2005; Krishnan and Nestler, 2008; Nestler et al., 2002). Most probably the individual’s genetic sequence variants set a vulnerability level that together with events that induce epigenetic changes can result in a depression (Akil et al., ; Burmeister et al., 2008; Krishnan and Nestler, 2008; Mann and Currier, 2006; Nestler et al., 2002).
2.3.1 Genetics and depression

There is a considerable hereditary basis for depression, as well as for other psychiatric disorders established through twin, family and adoption studies (Burmeister et al., 2008; Mann and Currier, 2006). First-degree family members of individuals with depression are at higher risk of developing depression than the general population, moreover depression tends to cluster in some families. The concordance rate between monozygotic (MZ) and dizygotic (DZ) twins differs and is greater between monozygotic twins (Mill and Petronis, 2007). For MZ twins the concordance is 46% and for DZ twins are 20% (Burmeister et al., 2008; Mann and Currier, 2006). The degree of genetic contribution seems to vary between different subtypes of depression. For recurrent and early onset depression the genetic contribution is estimated to be higher than for other subtypes of depression. This is thought to be the consequence of a more genetically homogenous sub-group of individuals (Camp and Cannon-Albright, 2005). Of the major psychiatric disorders MD is the disorder with the lowest heritability whereas Bipolar Disorder is with the highest genetic contribution (Burmeister et al., 2008).

Genetic epidemiology

The life time prevalence of MD is estimated to ~17% and the one-year prevalence is estimated to 4-9% with two onset-peaks in the mid-twenties and mid-forties. Depression is two- to three-times more prevalent in women than in men. The genetic contribution in major depression is estimated to be approximately 40% (Burmeister et al., 2008; Camp and Cannon-Albright, 2005; Mann and Currier, 2006; McGuffin et al., 2003; Nestler et al., 2002). The reported prevalence for season dependent depression, SAD, varies from 1-10% in the population and women are overrepresented. Of the persons diagnosed with depression 10-20% has seasonal variation (Howland, 2009; Magnusson, 2000; Partonen and Lonqvist, 1998).

The population prevalence for Bipolar Disorder is 1-5%, with Bipolar Disorder 1 being more prevalent than Bipolar Disorder 2 (Mann and Currier, 2006; Nestler et al., 2002). Bipolar Disorder has higher heritability than depression, 60-85% (Burmeister et al., 2008) or even up to 93% (Kieseppa et al., 2004). The concordances rates for MZ and DZ twins are similar to those of depression, 70% and 19% respectively (Burmeister et al., 2008). The age of onset for Bipolar Disorder is generally in the late teens or early adulthood (Burmeister et al., 2008; Mann and Currier, 2006; Martinowich et al., 2009)

A person with depression is very likely to have other comorbid disorder such as anxiety or alcohol related problems. Studies clearly show that persons with depression are at an increased risk for developing alcohol abuse or dependence (AUD) and vise versa (Lynskey, 1998). Approximately 30% of the persons with depression also have alcohol problems (Davis et al., 2008; Watts, 2008) and 12% have the comorbid condition (Pirkola et al., 2005). The majority (~80%) of the individuals with AUD report symptoms of depression and 20% have the comorbid condition (Pirkola et al., 2005). The cause of this co-occurrence has been debated. Is it two disorders with overlapping clinical manifestations and criteria, or is it one disorder? Prescott and colleagues concluded that the causes behind depression and alcoholism overlap and they estimated that the shared overlap of genetic and
environmental factors influencing depression and AUD was only 9-14% (Prescott et al., 2000). Or, does one of the disorders increase the vulnerability for the other disorder? Nevertheless, having both depression and AUD is associated with an even higher risk of morbidity and mortality then having only one of the disorders (Schuckit, 2009; Sher, 2005).

**The neurobiology of depression**

Psychiatric disorders, among them depression, are likely to in part be the result of defects disrupting neuronal circuits leading to chemical imbalance and structural alteration. Several brain regions have been implicated in depression etiology. Mainly areas regulating emotions and stress such as prefrontal cortex, cingulate cortex, hippocampus, striatum and amygdala, to mention a few, have been proposed (Akil et al., ; Mann and Currier, 2006; Nestler et al., 2002). These areas are all very interconnected, with projections of GABAergic, glutamatergic, dopaminergic, noradrenergic and serotonergic neurons. Based on the effects of selective serotonin reuptake inhibitors (SSRI) and other drugs on depression, as well as indications from the symptoms of depression, some circuits and neurotransmitter systems have received a special attention (Nestler et al., 2002). A brief description will now follow of three proposed depression-hypotheses, the monoamine hypothesis, the role of the hypothalamic-pituitary axis and the implication of neurotrophic factors in depression.

**The monoamine hypothesis**

The monoamine-deficiency hypothesis postulates that depression is caused by a depletion of monoamines such as serotonin, norepinephrine and dopamine in the central nervous system (CNS). The theory is based on the actions and the clinical effects of antidepressants. SSRI and selective noradrenaline reuptake inhibitors (NRI), or a combination thereof (SNRI), prevent the presynaptic neuronal reuptake of serotonin and noradrenaline, and the monoamine oxidase inhibitors (MAOI) block the degradation of monoamines. Thus, the overall action of these inhibitors is to accumulate the amount of synaptic monoamines, thus to increase the neuronal signal transmission (Belmaker and Agam, 2008; Burmeister et al., 2008; Krishnan, 2008). Furthermore, both up- and downstream actions of the monoamine neurotransmission circuits have been investigated and implicated in depression, both with regard to serotonin, noradrenaline and dopamine, although extensive consistencies between genetic studies are limited. For examples, like tryptophan and tryptophan hydroxylase, involved in the synthesis of serotonin and in turn melatonin have also been associated with depressive disorders (Belmaker and Agam, 2008). Also, the roles of the transporter and receptors of serotonin, and p11 a protein interacting with the serotonin receptor, have been extensively studied in depression (Pezawas et al., 2005; Svenningsson et al., 2006).

**Dysregulation of the Hypothalamic-Pituitary-Adrenal Axis**

When our body is subjected to stress, the body responds by activating the hypothalamic-pituitary-adrenal (HPA) axis. The activity of the HPA axis is under the control of numerous brain regions, including hippocampus and amygdala. Neurons in the hypothalamus, more specific in the paraventricular nucleus (PVN), secrete corticotrophin-releasing factor (CRF) that stimulates the synthesis and release of adrenocorticotropicin (ACTH) from the anterior pituitary. ACTH in turn stimulates the
synthesis and release of glucocorticoids such as cortisol from the adrenal cortex (Nestler et al., 2002; Mann and Currier, 2006). Glucocorticoids exert profound effects on behavior via direct actions on several brain regions (hippocampal and PVN neurons) as well as on the body’s general metabolism. Elevated levels of glucocorticoids under sustained period as seen under conditions of severe stress may damage hippocampal neurons. There are reports showing that depressed patients have elevated cortisol levels in plasma and increased levels of corticotrophin-releasing hormone (CRH) in cerebrospinal fluid (Belmaker and Agam, 2008; Krishnan and Nestler, 2008). Excessive activation of the HPA axis is seen in many, but not all depressed individuals, and can be normalized with antidepressants. Thought, how is the increased activation of the HPA-axis connected to depression? Several hypotheses have been proposed. Increased level of cortisol and CRF over a prolonged time might be toxic to the neurons in hippocampus. A subsequent hippocampal volume reduction might contribute to some of the characteristics in depression such as cognitive abnormalities which have been observed in both animal and human studies (Nestler et al., 2002; Mann and Currier, 2006). Genetic variants involved in the HPA axis have been under investigation for a possible role in depression, such as the glucocorticoid receptor (GR) and the FKBP5 gene, a glucocorticoid receptor-regulating co-chaperone (Belmaker and Agam, 2008; Mann and Currier, 2006).

**Neurotrophins and neurogenesis**

Decreased volume of brain areas such as hippocampus has been observed in depressed patients. A hypothesis for this volume reduction is the proposed mechanism involving decrement in neurotrophic factors, which regulate neuronal plasticity in the adult brain (Krishnan and Nestler, 2008; Nestler et al., 2002). One of these factors is brain derived neurotrophic factor (BDNF), which is part of a growth factor cascade influencing cellular plasticity and resilience. More specific, BDNF is involved in neuronal and synaptic plasticity, neuronal excitability and neurogenesis especially in the hippocampus (Koyama and Ikekaya, 2005; Schmidt-Kastner et al., 1996). Several forms of stress, like stress induced high glucocorticoid levels or other mechanisms such as serotonergic mechanisms have been shown to reduce BDNF-signaling. Treatment with certain antidepressants increases BDNF signaling (Krishnan and Nestler, 2008; Mann and Currier, 2006; Nestler et al., 2002).

All of the three aforementioned hypotheses can be linked to each other, or unified, by a hypothesis that is based on the functions of the circadian clocks (Fernandez et al., 2008; Hampp and Albrecht, 2008; Hampp et al., 2008; Oster et al., 2006; Son et al., 2008; Wang et al., 2009).

2.3.2 Environmental risk factors and depression

Environmental risk factors contribute significantly in depression predisposition. The fact that the concordance between monozygotic twins is not 100% is partly due to environmental effects. Several environmental risk factors have been proposed and integrated in statistical models with the aim of explain the complex etiology of depression. Some of the risk factors that seem to be of importance are stressful life events such as early parental loss, low social support, marital difficulties and poor financial support but also substance misuse (Kendler et al., 2002; Kendler et al., 1993). A variety of external factors have also been implicated in depression pathogenesis,
such as viral infections but also random processes during brain development (Nestler et al., 2002).

**Gene x environment interactions**

During the last years it has become more common to study the combined effect of gene and environmental risk factors. Investigating the interaction between the two factors is called gene x environment (G x E) analyses. The serotonin transporter (5-HTT) and its promoter polymorphism referred to as 5-HTTLPR is one of the most studied genes in regard to G x E analysis in depression. Caspi and colleagues investigated why some individuals became depressed and others not, despite having experienced stressful life events. They concluded that the risk of becoming depressed after stressful life events was increased if the person had one or two copies of the short low-activity allele of the 5-HTTLP. This points towards that an individual respond differently on environmental stressors depending on genetic predisposition (Caspi et al., 2003). G x E analysis could therefore be considered as an attempt to mimic the true complexity of depression etiology. However, a recent meta-analysis found no support for the 5-HTT x stressful life events association to depression (Risch et al., 2009). Nevertheless, genes involved in the hypothalamic-pituitary-axis, the body’s stress response system have also been subject to G x E analyses (Belmaker and Agam, 2008). GR variants were shown to interact with childhood adversity and increase clinically relevant depressive symptoms (Bet et al., 2009). In another study, Caspi and colleagues could associate the interaction between the low-activity promoter form of the gene monoamine oxidase A (MAOA) and with childhood maltreatment to an increased risk of criminal activity and impulsive-aggressive or violent behavior (Caspi et al., 2002), which has been independently replicated (Kim-Cohen et al., 2006).

**Epigenetics and depression**

A new field of genetics has been advancing, epigenetics. Epigenetics is the modifications on the DNA and histones that can be both inherited and/or acquired. Genome-wide SNP association studies performed on psychiatric disorders have revealed genetic variants explaining only a minor part of the heritability (Cichon et al., 2009; Maher, 2008). Probably epigenetics can explain some of the heritability that does not seem to be explained by DNA sequence variation, and thereby aid in the understanding of the pathophysiology of depression. There are several types of epigenetic modifications: methylation of DNA (primarily in CpGs), modification of histones for example acetylation and methylation, and small interfering RNAs (Mosher and Melnyk). The amount of DNA methylation is strongly negatively correlated with the gene expression, whereas the histone modifications can either open up for transcription to take place, or block transcription (Mill and Petronis, 2007). Epigenetics can be seen as a link between the environment and the genome. McGowan and colleagues showed that epigenetic regulation of the GR in the hippocampus of suicide victims was associated with childhood abuse, compared to either suicide victims with no childhood abuse or controls (McGowan et al., 2009). This provided a mechanism explaining the finding that early adversity or abuse was associated with abnormal lasting HPA axis function in adulthood (Mann and Currier, 2006). Hence, stress early in life can alter gene expression within the brain creating permanent modification on the
HPA axis which later in life may result in abnormal hormonal responses to further stressful stimuli (Mann and Currier, 2006; Meaney).

2.4 THE CIRCADIAN CLOCK SYSTEM

The circadian or the biological clock is an innate time-keeping mechanism controlling a number of biological and physiological processes in the body, such as the sleep-wake cycle, metabolism, body temperature, hormone secretion and cell proliferation (Dardente and Cermakian, 2007; Gachon et al., 2004; Gillette and Sejnowski, 2005; Hastings, 1998; Ko and Takahashi, 2006; Reppert and Weaver, 2002). In addition, the seasonal fluctuations, e.g. in mood and behaviour, are guided by this mechanism (Hofman and Swaab, 1993; VanderLeest et al., 2007). This biological clock governs a rhythmic activity cycle of roughly 24-hour intervals (Dardente and Cermakian, 2007; Gillette and Sejnowski, 2005; Hastings, 1998; Reppert and Weaver, 2002; Shirani and St Louis, 2009). Hence, the name circadian is derived from the latin words *circa diem*, meaning “about a day” (Shirani and St Louis, 2009). The circadian time-keeping is fundamental in organisms from bacteria to all mammals (Gachon et al., 2004; Hastings, 1998; Ko and Takahashi, 2006).

The human “master biological clock” generating these endogenous rhythms is located in the SCN of the anterior hypothalamus. SCN consists of a cluster of approximately 10000 neurons, clock cells (Hastings, 1998; Reppert and Weaver, 2002; Shirani and St Louis, 2009). The circadian timing is organized in a hierarchy of multiple circadian oscillators, the “master clock” in the SCN and numerous slave oscillators throughout the body (various brain regions, e.g. paraventricular nucleus, pineal gland and arcuate nucleus, as well as in peripheral tissues such as liver, heart, muscle and kidney). These peripheral clocks generate oscillations independently. However they are coordinated and synchronized by the “master clock” in SCN (Dardente and Cermakian, 2007; Ko and Takahashi, 2006; Reppert and Weaver, 2002). A critical feature of circadian clocks, both in the SCN and in peripheral tissues is that they can function autonomously (self-sustained) even in dissociated cultured cells (Gachon et al., 2004; Ko and Takahashi, 2006). Only processes continuing to oscillate in the absence of external time cues are considered to be outputs of biological time-keepers (Gachon et al., 2004). A free-running clock oscillates with a periodicity slightly longer than 24 hours, with some intra individual differences.

How are these biological rhythms generated? Many of the proteins encoded by the circadian clock genes are transcription factors. They regulate both their own transcription and the transcription of other downstream genes through transcriptional-translational feedback loops (Gillette and Sejnowski, 2005). The downstream genes controlled by the circadian clock proteins are called clock-controlled genes (CCGS). These clock-controlled genes encode various proteins such as peptides, metabolic enzymes, transcription factors, key regulators for the cell cycle and ion channels (Cermakian and Boivin, 2009; Ko and Takahashi, 2006). The transcriptional-translational regulations generate the periodicity of circadian time by the cycling expression of genes that makes up and drives the timing system (Hastings, 1998; Shirani and St Louis, 2009). Furthermore, these output pathways are the link between the circadian clock and the physiological rhythms that are responsible for the proper
timing of feeding behavior, sleep-wake homeostasis and hormone release (Cermakian and Boivin, 2009; Ko and Takahashi, 2006).

The rhythms are sustained by certain stimuli, both external (e.g. photic) and internal (non-photic) cues. These stimuli are known as zeitgebers, directly taken from the German word time-givers. The zeitgebers exert a synchronizing effect on the circadian rhythm sustaining an in-phase and the progression of the circadian period. This prevents the drift and divergence from the 24-hour periodicity. The process of establishing phase synchronization, setting the biological clock, is referred to as entrainment (Shirani and St Louis, 2009). The main entraining zeitgeber of the endogenous circadian period is the light-dark cycle (Reppert and Weaver, 2002; Shirani and St Louis, 2009). Although, other external cues also have entraining properties, such as ambient temperature, meal time and physical activity, as do intrinsic ones, e.g. circulating factors like glucocorticoids (Dardente and Cermakian, 2007; Hastings, 1998; Takahashi et al., 2008). To avoid misalignment, both environmental and internal time-keeping stimuli have a need for synchronization by the SCN to ensure a proper phase angle between the circadian rhythms and behavior. Among those 31 genes identified to express themselves in rhythms throughout the body, is mPer2 whose expression can be driven by both systemic cues and local oscillators, suggesting a plausible mechanism for the phase entrainment of subordinated clocks in peripheral organs (Kornmann et al., 2007).

2.4.1 The neurobiology of the circadian clock

The discovery of the circadian system and its genes was first done in the fruit fly (Drosophila melanogaster), where the Drosophila protein Period was shown to be required for its own cyclic accumulation of mRNA (Gachon et al., 2004). Since then, almost all clock genes in Drosophila have been found to have orthologs in mammals, including humans (Gachon et al., 2004; Hastings, 1998). Most of the knowledge about the molecular function of the human circadian system has been translated from the Drosophila and mouse circadian clocks (Vitaterna et al., 1994; Yu and Hardin, 2006).

The entraining stimulus from the light-dark cycle on the SCN is mediated through light-sensitive receptors of the retina. A specific subset (0.8%) (Hannibal et al., 2004) of retinal ganglion cells, called intrinsically photosensitive retinal ganglion cells, contain the light sensitive photopigment melanopsin. When light activates this system the melanopsin containing ganglion cells send nerve impulses via the retinohypothalamic tract (RHT) directly to the SCN.
As described above, these retina afferents to SCN which modulates the endogenous pacemaker activity and set the biological clocks in phase with the 24-h light dark cycle. The activation of the SCN neurons by the RHT is done through release of multiple neurotransmitters, mainly pituitary adenylate cyclase-activating polypeptide (PACAP) and the excitatory amino acid glutamate which results in increased SCN neuronal activity during day-time (Hannibal and Fahrenkrug, 2004; Hannibal et al., 2002). The signaling from SCN ends up in the pineal gland where melatonin is secreted, through synthesis from serotonin. Signals also reach SCN by projections from the intergeniculate leaflet and midbrain with the inhibitory neurotransmitter GABA but also neuropeptide Y (NPY) and serotonin (Reghunandanan et al., 1993). GABA also projects from the SCN and inhibits the activity of the PVN and the superior cervical ganglion cells which further project to the pineal gland. To summarize, light inhibits while darkness promotes the secretion of melatonin (Gachon et al., 2004; Reppert and Weaver, 2002; Shirani and St Louis, 2009). Melatonin in turn exhibits an negative feedback loop effect on the SCN, where a high density of serotonin receptor can be found and is important in the function of the SCN (Reghunandanan et al., 1993; Reppert and Weaver, 2002; Shirani and St Louis, 2009).

2.4.2 The molecular biology of the circadian clock

The majority of identified clock components are transcriptional activators or repressors. They modulate protein stability and nuclear translocation creating two feedback loops that take 24 hours to complete (Ko and Takahashi, 2006). In the primary feedback loop, the positive elements include members of the two PAS domain basic helix-loop-helix transcription factors, the Circadian Locomotors Output Cycles Kaput (CLOCK) gene and the Brain and Muscle ARNT-like protein 1 gene (ARNTL). CLOCK and ARNTL are the key components of the circadian clock and heterodimerize and activate transcription of genes containing E-box cis-regulatory
enhancer sequences in their promoter. Among those genes is the Period (PER 1, PER2 and PER3) genes and the Cryptochrome (CRY1 and CRY2) genes. Negative feedback is achieved by PER and CRY, that heterodimerize and translocates back to the nucleus to repress their own transcription, by acting on the CLOCK:ARNTL complex (Dardente and Cermakian, 2007; Gachon et al., 2004; Ko and Takahashi, 2006; Reppert and Weaver, 2002). Here, PER2 is a highly connected node of a key transcriptional network, i.e. E/C′-box regulation, that has a crucial role in mammalian circadian systems and is thus inherently vulnerable (Ueda et al., 2005). Other feedback loops, involve the transcription factors orphan nuclear receptors, REV-ER Ba and RORa who’s transcriptions are activated by CLOCK:ARNTL. In turn REV-ER Ba and RORa have the ability to bind to the ARNTL promoter and either repress or activate the transcription of ARNTL. Post-translational modification and degradation of circadian clock proteins are also crucial for maintaining the circadian periodicity of the clock. The two kinases Casein kinase 1ε and 1δ (CK1ε and CK1δ) phosphorylate the PER and CRY proteins, allowing them to enter the nucleus (Gachon et al., 2004; Takahashi et al., 2008). Other genes involved in the circadian regulations are TIMELESS, DECI and DEC2, however their role is not yet fully understood (Takahashi et al., 2008). Both CLOCK and ARNTL have homologues, NPAS2 and ARNTL2 respectively (Dardente and Cermakian, 2007). Moreover, a genome-wide screen and subsequent protein interaction network analysis found recently that dozens of gene products directly or indirectly associate with the known circadian clock components (Zhang et al., 2009).

2.4.3 The involvement of the circadian clock in depressive disorders

The circadian clock controls numerous biological processes and it is therefore not unreasonable to assume that disturbances in this system could lead to various consequences (McClung, 2007a). Disruptions of biological rhythms have been shown to associate with a variety of medical conditions and physiological processes such as
cancer, cellular metabolism and growth, DNA-damage control, xenobiotic response, modulation of behavioral response to drugs and alcohol and behavioral and metabolic disturbances (Barnard and Nolan, 2008; Takahashi et al., 2008). Furthermore, alterations in the circadian clock have also been clearly associated with sleep-disorders such delayed sleep phase syndrome (DSPS), familial advanced sleep-phase syndrome (FASPS) and insomnias (Barnard and Nolan, 2008; McClung, 2007a; Takahashi et al., 2008).

There is considerable support for that circadian dysregulation may also contribute to depressive disorders. Disrupted rhythms are observed in persons with depression and Bipolar Disorder (McClung et al., 2007; Germain et al., 2008; Barnard et al., 2008; McClung et al., 2007). Disrupted rhythms in depressed individuals have been reported for the sleep-wake cycle, body temperature, blood pressure but also several hormonal levels (Barnard and Nolan, 2008; McClung, 2007a; Monteleone and Maj, 2008; Takahashi et al., 2008). Perhaps the most crucial role of the circadian clock is to regulate the sleep-wake cycle. Nearly all individuals suffering from depressive disorders have problems with sleeping, primarily with falling asleep (McClung, 2007a; Monteleone and Maj, 2008). The circadian involvement is especially apparent in SAD, where the depressive episodes follow a seasonal pattern. Most commonly, the depressive episodes occur during the winter (Barnard and Nolan, 2008; Germain and Kupfer, 2008). Depressed individuals tend to display a regular diurnal pattern of symptoms, usually with more severe symptoms in the morning (Germain and Kupfer, 2008; McClung, 2007a). Moreover, SAD patients often display phase delays, specifically in winter due to shorter days and later dawns, whereas Bipolar Disorder patients often are characterized by a diurnal rhythm that is abnormally faster (phase advance), indicating a shortened period (McClung, 2007c). Furthermore, individuals with a behavioral preference for “eveningness” have a greater tendency to develop depression (Barnard and Nolan, 2008). The circadian hormone melatonin has also been postulated to have a central role in seasonal and non-seasonal depression and Bipolar Disorder pathology (McClung, 2007a; Wehr et al., 2001). Due to the wide variety of disrupted rhythms seen in depressed individuals, speculations have led to believe that it is disruptions in the activity of the master pacemaker in the SCN that might be behind these symptoms (Monteleone and Maj, 2008).

There are different theories of how the circadian clock and disrupted rhythms may be involved in depressive disorders and whether the circadian clock exerts an indirect or a direct role in depressive disorders (Barnard and Nolan, 2008; Takahashi et al., 2008). Is it so, that disturbances of the normal sleep-wake cycle (phase-shift hypothesis) could lead to sleep abnormalities such as insomnia that could induce or exacerbate the depressed state (Germain and Kupfer, 2008; Monteleone and Maj, 2008; Turek, 2007). Could those phase-shifts be induced by depression? Or, is it disorganization within the circadian system, at the molecular level, that leads to neurobiological dysfunction which may generate a depressed state (Turek, 2007). However, it is very likely that genetic variations in the circadian clock do result in significant rhythm alterations which in turn may lead to physiological changes such as mood disorders (Monteleone and Maj, 2008). External factors such as alcohol and stressful life events have also been shown to desynchronize the circadian clock and exacerbate mood changes in depressed individuals. This underlies the social zeitgeber theory, which proposes that vulnerable individuals, those with suboptimal circadian gene function,
exposed to life stressors or other environmental factor, such as the shortage of or inappropriate light exposure, can develop depressive or manic episodes through disruption of normal social routines (McClung, 2007c). Desynchronization of the circadian rhythms, that can be induced also by the critical light pulses, underlies a decrease in rhythm amplitude in the SCN (Ukai et al., 2007), and is hypothesized to contribute to depression (Czeisler et al., 1987).

The involvement of circadian disturbances in depressive disorders is further strengthened by the success of chronobiological treatments, such as rhythm shifting performed through light therapy, melatonin pharmacotherapy and total sleep deprivation (Barnard and Nolan, 2008; McClung, 2007). Also, lithium, commonly prescribed in Bipolar Disorder, is reported to lengthen the circadian period in Bipolar Disorder patients. Lithium is thought to have a direct action on the SCN and to modulate the expression of core circadian genes (McClung, 2007c; Yin et al., 2006). Studies have also shown that treatment with antidepressant drugs may alter circadian clock gene expression (McClung, 2007a).

Genetic variants in circadian genes in humans have been linked to various disorders and conditions. For example PER2, PER3, CLOCK, CK1δ and CK1ε have been associated with sleep-disorders and the genes PER1, PER2, PER3 and CLOCK have been linked to diurnal preferences (Barnard and Nolan, 2008; McClung, 2007a). Polymorphisms in several circadian genes have also been found to associate with MD CRY1 and NPAS2 (Soria et al.) and the polymorphism S471L in NPAS2 (Johansson et al., 2003) has been associated with both Bipolar Disorder and SAD (Takahashi et al., 2008). McGrath and colleagues found association of the gene RORB and Bipolar Disorder (McGrath et al., 2009). This is of interest since CRY2 and RORB are the only core circadian genes whose knockout in mice lengthening the circadian period (Ko and Takahashi, 2006). These findings suggest a connection between a normal functioning circadian clock and proper mood regulation. Moreover, Lithium is a potent inhibitor of GSK3 and is used to treat Bipolar Disorder. Yin and colleagues could in cell cultures show that GSK3β phosphorylates and stabilizes the orphan nuclear receptor Rev-erbα. However, treating the cells with lithium lead to rapid proteasomal degradation of Rev-erbα and activation of clock gene Bmal1, demonstrating that the protein stability of Rev-erbα is a critical component of the peripheral clock (Yin et al., 2006). The importance of the circadian system has also been investigated in animal models, were mutations in circadian genes in mice disturb both molecular oscillations and measurable output rhythms (Barnard and Nolan, 2008; Monteleone and Maj, 2008). Also, lesions in the SCN in animals and pituitary tumors in humans have been associated with disruption of circadian rhythms (Hastings, 1998; Shirani and St Louis, 2009).
3 THESIS OBJECTIVES

The overall aim with this thesis has been to identify genetic variants that are associated with depressive disorders in relation with environmental factors, with a focus on the circadian clock system.

The specific aims of the studies included in the thesis are:

**Paper I** To test the applicability of two developmental models including environmental factors for depression on our population based Swedish material PART and to determine the power of the model to predict depression in PART.

**Paper II** To investigate association of a proposed functional candidate gene variation for depressive disorder, NPY Leu7Pro to depression in PART. To consider environmental risk factors reported in paper I in these analyses.

**Paper III** To investigate the association between depression and genetic variants within circadian clock genes in PART. To consider potential sleep disturbing stressors in these analyses.

**Paper IV** To investigate the involvement of circadian clock genes in depression and alcohol abuse or dependence comorbidity in the Finnish population based cohort Health2000.

**Paper V** To investigate whether CRY2 mRNA levels differ between healthy controls and patients with depression, before and after one-night sleep deprivation. To identify variations in CRY2 that are associated with depression in Swedish and Finnish samples.

**Paper VI** To further investigate CRY2’s role in affective disorders, by investigating CRY2 genetic association to bipolar disorder.
4 MATERIALS AND METHODS

4.1 SUBJECTS

The majority of subjects used in this thesis (paper I, paper II, paper III and paper IV) belongs to one of two big population based studies, either the Swedish PART study or the Finnish Health2000 study. Paper V and paper VI use clinical case-control material.

4.1.1 The PART study

The PART (Psykisk hälsa, Arbete och RelaTIONer) study is a longitudinal population based study conducted in Stockholm County, with the aim to identify risk and protective factors for mental illness. Epidemiological data have so far been collected two times, 1998 and then in 2001. During fall 2006 genetic material was being collected and beginning of 2010 a third wave was initiated, were questionnaires were sent out and DNA being collected. For more detailed information see (Hällström T, 2003) and www.folkhalsoguiden.se.

The initial study population constituted of randomly selected individuals, aged 20-64, from the Stockholm city council registers, in total 19 742 individuals. A comprehensive questionnaire was sent out with questions regarding risk and protective factors for mental illness as well as several psychiatric symptom scales. The questionnaire included for instance questions regarding childhood conditions, demographic characteristics, social network, financial status, negative life events, use of drugs and somatic health. Psychiatric screening instruments used were Sheehan Patient-Rated (Panic) Anxiety Scale, symptoms of social phobia and agoraphobia according to Marks and Mathews, the Yale-Brown Obsessive-Compulsive Scale, eating disorders according to Beglin and Fairburn, the World Health Organization Short Disability Assessment Schedule (WHO-DAS-S), the Major Depression Inventory (MDI) and harmful alcohol use according to Alcohol Use Disorder Identification Test (AUDIT). For detailed information see (Hällström T, 2003). A random sample of individuals who screened positive, from the questionnaire, with suspected mental illness (n=884) and persons screened negative, with few or no symptoms (n=209) were interviewed by psychiatrists (Hällström T, 2003). The interviews were performed using Schedules for Clinical Assessment in Neuropsychiatry (SCAN) by clinical experienced psychiatrists and one psychologist and diagnosis were set according to DSM-IV criteria (APA, 1994). Of the 1 093 interviewees, 81 persons were diagnosed with MD. The majority of those had at least one previous episode of MD and a majority had been depressed 1-6 month, ~30% of the individuals also had other comorbid psychiatric disorders mostly anxiety syndromes and 74% were untreated. Hundred and thirty-two individuals were diagnosed with other types of depressive disorders, two with Bipolar Disorder, 30 with Dysthymia, 29 with Mixed Anxiety Depression and 71 with Minor Depression. Of these individuals 23% also had other comorbid diagnosis, mostly anxiety syndromes. Other types of psychiatric disorders were diagnosed in 136 individuals, 65 were diagnosed with anxiety syndromes, and others had alcohol dependence/abuse or various types of sleeping disorders. The majority of individuals (n=744) did not fulfill the criteria for any psychiatric disorder (Hällström T, 2003).
At the second wave, all individuals who participated in wave one (n=10,441) were sent a questionnaire with almost the same set of questions as in wave one and 8,613 responded. Those individuals who were diagnosed with depression or alcohol dependence/abuse in wave one were re-interviewed (Hällström T, 2003).

The DNA collection (both saliva and blood) started in 2006 and involved all the 1,050 individuals with a depression diagnosis (MD, Dysthymia and Mixed Anxiety Depression) and the 334 individuals with an anxiety diagnosis (obsessive-compulsive disorder, social phobia, general anxiety disorder, agoraphobia or panic syndrome). To be placed in the depression or anxiety group, the individual should have been diagnosed with either depression or anxiety respectively in at least one of the waves. A random number of individuals (n=3,326) who participated in both waves and had no psychiatric diagnosis or psychopathological symptoms in any of the two waves were also asked to contribute with DNA. No psychopathological symptoms were defined as having no pathological symptoms of panic attacks, agoraphobia, social phobias, eating disorder, obsessive-compulsive disorder, use of illicit drug, depression or social disability due to psychological problem. Also, the controls stated they had never received health care for any psychiatric disorder or nervous discomfort (Hällström T, 2003). Of the individual qualifying as controls, a sub-group was identified and referred to as “mental resilience”. In addition to the controls, the “mental resilience” individuals had a wellbeing score ≥ the average in controls and “mental resilient” group combined, being ≥21 out of a maximum 30. This criterion should have been fulfilled in both waves, despite the occurrence of one or more potentially traumatic negative life events within 12 months before the second wave (Forsell, Lavebratt manuscript). The saliva DNA collection yield samples from 484 depressed individuals, 175 with anxiety, 1,877 controls and 481 “mental resilience” subjects, all with questionnaire date from both waves (Sjoholm et al., 2009). The blood collection yielded blood samples from 17 depressed individuals and 71 controls. The ethical committee of Karolinska Institute approved the study and specified written consent was obtained from all the participants, both for the questionnaire and DNA collection.

Paper I is based on all the sub-set of individuals interviewed in wave one. Paper II and paper III are based on the individuals participating in the DNA collection. More specifically, in Paper II the individuals with depression, anxiety or individuals fulfilling the criteria as controls were used. In Paper III, individuals with depression were compared to the controls and the mental resilience groups. These controls were also used in Paper V.

4.1.2 Health2000 study

The Health2000 study is a nationwide population based health examination and interview survey conducted in Finland between the fall of 2000 and spring of 2001. The Health2000 study aimed at investigating the general health of the Finnish population by identifying health problems, their cause and possible treatment. A random selection of almost 10,000 individuals over the age of 18 were recruited and examined using interviews, questionnaires and clinical examinations. Blood samples were also collected. The National Public Health Institute (KTL) in Finland was responsible for the study (Health2000).
Selected groups of Health2000 were studied in paper IV, and healthy controls from Health2000 were used in paper V and in a study (Sjöholm et al., manuscript) referred to in paper III. The individuals used in paper IV were selected based on 1) having both and depression and AUD (n=76), 2) having AUD diagnosis and no other mental disorders (n=446) and 3) being healthy sex and age-matched controls with no psychiatric symptoms (n=517). The diagnoses (depression and AUD) were based on CIDI and set according to the DSM-IV criteria (APA, 1994). In the depression group MD and dysthymia are included. The local ethic committee at National Public Health Institute, approved the study protocol, and all the participants signed an informed consent (Sjöholm et al.).

4.1.3 Swedish SAD material

The patients, 118 cases (13% men), met the DSM-IV diagnostic criteria for major depressive disorder with the seasonal (winter) pattern (SAD) (APA, 1994). They were recruited from outpatient services by senior specialists in psychiatry. The consensus diagnosis of two independent psychiatrists was required for inclusion of each patient. Controls, 1 011 adults (29% men) had no current or past psychotic or mood disorder as assessed with interviews or self-rated questionnaires. The Swedish cases and controls lived in Västerbotten and Stockholm areas. Most of the controls came from the PART study. The local ethic committees, Karolinska Institutet, and University of Umeå, approved the study-protocol, and all the participants signed an informed consent after the protocol had been fully explained (Lavebratt et al.).

This material was used in paper V and in a study (Sjöholm et al., manuscript) referred to in paper III.

4.1.4 Finnish SAD material

All patients, 86 cases (29.1 % men), met the DSM-IV diagnostic criteria for major depressive disorder with the seasonal (winter) pattern, SAD (APA, 1994). The patients were recruited from outpatient services by senior specialists in psychiatry. For each patient the consensus diagnosis of two independent psychiatrists was required for inclusion. The patients lived in the Helsinki area with inhabitants originating from all the regions in Finland. As controls, 1 096 healthy screened individuals (29% men) from Health2000 were used and they had no present or past psychotic or mood disorder. The cases and controls were matched for ethnicity. The local ethic committee at National Public Health Institute, approved the study protocol, and all the participants signed an informed consent after the protocol had been fully explained (Lavebratt et al.).

This material was used in paper V and in a study (Sjöholm et al., manuscript) referred to in paper III.

4.1.5 The Swedish bipolar material

The bipolar patients used in paper VI were recruited from outpatient clinics in the whole of Sweden. All were clinically diagnosed with Bipolar Disorder after being
interviewed by a psychiatrist or a trained psychiatric nurse over the telephone. The diagnoses were set with SCAN according to DSM-IV criteria (APA, 1994). Individuals were excluded from the study if mania was the result of alcohol or drug abuse, medication or somatic disease, as well as individuals that were known to be relatives. Of the 577 bipolar individuals used in the study, 497 individuals (43% men) were diagnosed with Bipolar Disorder 1 and 60 individuals with Bipolar Disorder 2 (36% men) and 20 cases had the diagnosis BP-NOS. Information regarding rapid cycling, mixed episodes and the age of onset of mania, as well as depression were registered. As controls, 1,044 population controls (59% men) were used, being anonymous blood donors (ABD), collected at the Karolinska University Hospital, Stockholm, Sweden. The analyses were carried out using both a case-control design and a case-by-case design. In particular, patients with bipolar disorder with rapid cycling (n=155, 35% men) were studied and compared with bipolar disorder patients without rapid cycling (n=422, 43% men). Written informed consent was obtained from the patients after complete description of the study. The study was approved by the Regional Ethical Review Board in Stockholm (Sjöholm et al. manuscript).

4.1.6 The American bipolar material

In paper V 13 Bipolar Disorder patients and eight healthy volunteers were included in a sleep deprivation study performed by the University of California Irvine (UCI) and San Diego (UCSD). Of the patients there were 10 men and three women, 11 had a European decent, one was African and one was Asian. The mean age was 40.2 years (range 18-57 years). All patients had a Bipolar Disorder 1 diagnosis according to the DSM-IV criteria, and were depressed at the time of the study (APA, 1994). The patients had a mean score of 17.3 (SD = 6.4) on the 21-item Hamilton Depression Rating Scale (Hamilton, 1960). The eight controls all had a European decent, four were women and four were men with a mean age of 23.6 years (range 19-34 years). The participants were hospitalized at the sleep research center at University of California at Irvine Medical Center (UCIMC) for 48 hours and deprived of sleep for 21 hours after an overnight stay. The study was conducted during summer for 10 of the patients and all the controls and during winter for three of the patients. The patients were all on an SSRI and on lithium (n=8), valproate (n=1) or lamotrigine (n=4). None of the controls was on medication. Informed consent was obtained from all participants using an approved protocol from University of California Institutional Review Board (Lavebratt et al.).

4.2 METHODS

4.2.1 Path analysis

To investigate environmental risk factors involved and predisposing for depression (paper I) path analysis were used. Path analysis is a special form of structural equation modeling (SEM), a statistical technique to test and estimate causal relationships. In a path analysis one can simultaneously model several related regression relationships. Also, a variable can be modeled as an independent variable in one relationship and as a dependent in another, and is then referred to as a mediating variable. The path analyses were performed with probit regression, since the dependent outcome variable was binominal, and as estimator a robust weighted least square estimator (WLSE) were used. The outcome variable, MD as well as the predictors, except the
Childhood variables were treated as binary dependent. The childhood variables were treated as binary independent. The two-headed arrows indicate correlation relationships and the one-headed arrows indicate regression relationships for risk factors 1-5 (see figure below). The proportion of the variance, $R^2$, explained by the model was calculated for the dependent variables. Also, the overall $R^2$, telling us the proportion of variability (depressed or not depressed) in the data set that could be accounted for by our model was calculated. To evaluate the model's ability to fit the data, several goodness of fit indices were calculated. Generally, fit indices measure goodness of fit by evaluating the discrepancy between observed and expected data. Tucker–Lewis index (TLI), comparative fit index (CFI) and the root mean square of approximation (RMSEA) were used (Kendler et al., 2002; Smith T.D, 2001). The statistical program Mplus 4.2 was used to perform the analyses (Muthén, 1998-2006).
4.2.2 Gene candidate selection approach

The genes studied in this thesis were selected through a candidate gene approach. A focus of this thesis has been to investigate the genes constituting the circadian clock. These genes are responsible for creating and maintaining the body’s internal rhythms, which are often disturbed in persons with depressive disorders. The genes investigated in paper II – paper VI are given in the Table below.

- In paper II, our aim was to investigate the NPY gene due to previous reported association for NPY to depression and anxiety. At the time of the study two nonsynonymous SNPs were reported in the NCBI’s SNP database, rs16139 and rs5571. rs16139 was reported to be functional, and had been associated so several clinical conditions, but had not been repeatedly associated to depression.

- In paper III the aim was to investigate the involvement of circadian clock genes in depression. SNPs in 18 known circadian clock genes were selected using the Tagger software and HapMap data being in total 115 SNPs. Cut-off value for $r^2$ was 0.8 and minor allele frequency (MAF) was ≥0.1. The tag-SNP tool in Haploview was also used to further verify and if possible decrease the number of SNPs to genotype. Non-synonymous SNPs were also selected to cover potentially functional variants. Overall haplotype tagging SNP coverage was 72% and 66% (the lower value when including RORA and NPAS2).

- Also in paper IV the aim was to select SNPs focusing on the circadian system to identify gene variants associated to depression and AUD comorbidity. SNPs in 20 clock genes were selected. Up- and down-streams genes of the circadian pathway were also picked. In total were 39 SNPs selected. Primarily SNPs with a possible functionality, like amino acid change or published data on functional alteration were selected. However, for core clock genes, the Tagger program and HapMap data were used to increase the variation coverage applying the same cut off values as in paper III.

- In paper V CRY2’s role in depressive disorders was investigated. The same SNP selection process as stated for paper III was performed.

- As paper VI was a replication study of paper V the same four SNPs in CRY2 were genotyped.
<table>
<thead>
<tr>
<th>Gene</th>
<th>Gene name</th>
<th>Paper</th>
</tr>
</thead>
<tbody>
<tr>
<td>Core clock</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ARNTL</td>
<td>Aryl hydrocarbon receptor nuclear translocator-like</td>
<td>III,IV</td>
</tr>
<tr>
<td>ARNTL2</td>
<td>Aryl hydrocarbon receptor nuclear translocator-like 2</td>
<td>III,IV</td>
</tr>
<tr>
<td>BHLHB2</td>
<td>Basic helix-loop-helix domain containing, class B, 2*</td>
<td>III</td>
</tr>
<tr>
<td>BHLHB3</td>
<td>Basic helix-loop-helix domain containing, class B, 3*</td>
<td>III</td>
</tr>
<tr>
<td>CLOCK</td>
<td>Clock homolog (mouse)</td>
<td>III,IV</td>
</tr>
<tr>
<td>CRY1</td>
<td>Cryptochrome (photolyase-like)</td>
<td>III</td>
</tr>
<tr>
<td>CRY2</td>
<td>Cryptochrome 2 (photolyase-like)</td>
<td>II, V, IV</td>
</tr>
<tr>
<td>CSNK1E</td>
<td>Casein kinase 1, epsilon</td>
<td>III</td>
</tr>
<tr>
<td>NPAS2</td>
<td>Neuronal PAS domain protein 2</td>
<td>III, IV</td>
</tr>
<tr>
<td>PER1</td>
<td>Period homolog 1 (Drosophila)</td>
<td>III</td>
</tr>
<tr>
<td>NR1D1</td>
<td>Nuclear receptor subfamily 1, group D, member 1</td>
<td>III</td>
</tr>
<tr>
<td>PER2</td>
<td>Period homolog 2 (Drosophila)</td>
<td>IV</td>
</tr>
<tr>
<td>PER3</td>
<td>Period homolog 3 (Drosophila)</td>
<td>III</td>
</tr>
<tr>
<td>RORA</td>
<td>RAR-related orphan receptor A</td>
<td>III</td>
</tr>
<tr>
<td>TIMELESS</td>
<td>Timeless homolog (Drosophila)</td>
<td>III, IV</td>
</tr>
<tr>
<td>TIPIN</td>
<td>TIMELESS interacting protein</td>
<td>III</td>
</tr>
<tr>
<td>Circadian related genes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ACADS</td>
<td>Acyl-Coenzyme A dehydrogenase, C-2 to C-3 short chain</td>
<td>IV</td>
</tr>
<tr>
<td>ADA</td>
<td>Adenosine deaminase</td>
<td>IV</td>
</tr>
<tr>
<td>ADCYAP1</td>
<td>Adenylate cyclase activating polypeptide 1 (pituitary)</td>
<td>IV</td>
</tr>
<tr>
<td>ANKK1</td>
<td>Ankyrin repeat and kinase domain containing 1</td>
<td>IV</td>
</tr>
<tr>
<td>DBP</td>
<td>D site of albumin promoter (albumin D-box) binding protein</td>
<td>III</td>
</tr>
<tr>
<td>DRD2</td>
<td>Dopamine receptor D2</td>
<td>IV</td>
</tr>
<tr>
<td>FDFT1</td>
<td>Farnesyl-diphosphate farnesyltransferase 1</td>
<td>IV</td>
</tr>
<tr>
<td>GLO1</td>
<td>Glyoxalase I</td>
<td>IV</td>
</tr>
<tr>
<td>NCOA3</td>
<td>Nuclear receptor coactivator 3</td>
<td>IV</td>
</tr>
<tr>
<td>NFIL3</td>
<td>Nuclear factor, interleukin 3 regulated</td>
<td>III</td>
</tr>
<tr>
<td>NPY</td>
<td>Neuropeptide Y</td>
<td>II, IV</td>
</tr>
<tr>
<td>OPN4</td>
<td>LIM domain binding 3;opsin 4 (melanopsin)</td>
<td>IV</td>
</tr>
<tr>
<td>PLCB4</td>
<td>Phospholipase C, beta 4</td>
<td>IV</td>
</tr>
<tr>
<td>VIP</td>
<td>Vasoactive intestinal peptide</td>
<td>IV</td>
</tr>
<tr>
<td>VIPR2</td>
<td>Vasoactive intestinal peptide receptor 2</td>
<td>IV</td>
</tr>
</tbody>
</table>

*BHLHB2 and BHLHB3 are nowadays named BHLHE40 and BHLHE41 respectively.
4.2.3 Genotyping methods

The genotyping methods used in this thesis are Pyrosequencing® in paper II, TaqMan® in paper IV and paper VI, and iPLEX Gold in paper III and paper V.

Pyrosequencing

Pyrosequencing® (Qiagene/Biotage AB, Uppsala, Sweden) is a quantitative mini-sequencing method and was used to genotype the rs16139 polymorphism in NPY (paper I). First the area of interest is amplified in a PCR where one of the primers is labeled with biotin. The biotin enables purification and separation of the strands of the PCR fragment. A sequencing primer is then hybridized to the single stranded DNA and the fragment is ready to be quantitatively sequenced on the Pyrosequencer. A mix of four enzymes DNA polymerase, ATP sulfurylase, luciferase and apyrase as well as the substrates adenosine 5’ phosphosulfate, and luciferin are added to the reaction. Thereafter dNTPs are added one at a time. If the dNTP is complementary to the base next to the sequencing primer on the template strand the dNTP is incorporated and pyrophosphate (PPi) is released in a quantitative manner relative the amount of nucleotides incorporated. Next, the PPI is converted to ATP by ATP sulfurylase, a reaction also in need of adenosine 5’phosphosulfate (APS). The generated ATP catalyzes the step of converting luciferin to oxyluciferin, a conversion that generates visible light proportional to the amount of ATP produced. The light produced is then detected by a charge coupled device camera and converted to a peak seen in the Pyrogram. The height of the peak is proportional to the number of dNTPs incorporated. Before the next nucleotide is added, unincorporated nucleotides are degraded by apyrase. The process continues and a complementary DNA strand is built up (Lavebratt and Sengul, 2006; Ronaghi et al., 1998). The PSQ™ reactions were performed on a PSQ96™ instrument using the PSQ96™ SNP reagent kit (Biotage AB).

![Figure 5](image-url)

On each 96-well plate at least one negative and one positive control were included, and gel electrophoresis was run on a number of samples to ensure the right size of the amplified fragment (Sjoholm et al., 2009).
Pyrosequencing is typically preferred when the number of SNPs and individuals to analyze are few. Also this technique enables the study of the actual sequence, approximately 50-100 base pairs.

TaqMan allelic discrimination
AB\'S TaqMan allelic discrimination technique (Applied Biosystems, Foster City, CA, USA) is based on probe technology and was used in paper IV and paper VI. A short description of the technique will now follow. A PCR reaction is performed where locus-specific forward and reverse primers, as well as two allele-specific probes, anneal to the template. Each probe is labeled with a 5\' reporter dye, in our case VIC on one probe and FAM fluorophore on the other probe, and a 3\' quencher dye. When the PCR is carried out the AmpliTaq Gold enzyme, which has 5\'-3\' activity, cleaves off the probe and releases the corresponding fluorophore. It\'s emitted fluorescence (VIC or FAM) is no longer quenched. The fluorescence is measured after the PCR on the ABI PRISM 7900HT SDS and analyzed with the SDS 2.2.1 software. Having both FAM and VIC signal is synonymous with being heterozygote at that particular locus. Furthermore, having either a VIC or a FAM signal means the individual is homozygous at that locus. Both positive and negative controls were used (Biosystems, 2008a).

![Figure 6](image)

The TaqMan technique can be used both in a 96- and 384-well format. It is also possible to multiplex several SNPs simultaneously, since the allele specific probes can be labeled with different fluorophores. Another advantage with TaqMan is that all assays are run under the same PCR-conditions, which makes it user friendly.

MALDI-TOFF MS with iPLEX Gold
The SNP genotyping method used in paper III and paper V was Sequenom\'s MassARRAY technology with the iPLEX Gold chemistry (Sequenom,Inc., San Diego, CA, USA) and MALDI-TOFF (matrix-assisted laser desorption-ionization-time of flight) mass spectrometry detection. The spectroDESIGNER software was used to design the
Multiplexes of 24-34 SNPs were run. iPLEX Gold assay is based on a multiplex PCR (with universal PCR conditions) followed by a phosphatase treatment with SAP (Shrimp Alkaline Phosphatase) which inactivates unincorporated nucleotides. An allele-specific primer-extension reaction is then performed, with the result that the allele-specific extension product will differ in their mass. The PCR product is then transferred to a spectroCHIP array and detected by the massARRAY analyzer (based on the MALDI-TOF MS) (Sauer et al., 2006). The spectra produced were analyzed using the spectroTYPER software and the analyses were manually read by two persons. Also negative and positive controls were used. As quality controls, 2.5% of the samples were genotyped in duplicates (Lavebratt et al.; Lavebratt et al.,).

The advantage with the Sequenom method is that one can multiplex up to 40 SNPs in one run for 384 individuals. Also, the same standardized PCR conditions are used for all reactions.

**Figure 7**
4.2.4 qPCR

Quantitative PCR, also called real-time PCR was performed to measure CRY2 mRNA levels in paper V. The technique uses the PCR principle to in real time amplify and quantify the gene product of interest. To be able to measure the mRNA levels reverse transcription is performed, where single stranded DNA is produced from the mRNA template. TaqMan Reverse Transcription Reagents and Oligo d(T)16 primer were used. The DNA was then amplified with CRY2 specific DNA probes labeled with a fluorescent reporter. When the DNA is then amplified the reporter is cleaved off from the probe and emits light that can be detected, thus the same principles in TaqMan allelic discrimination. The CRY2 primer sequences spanned exon 6–7 junction within target sequence. The analyses were done on an ABI 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA, USA). All samples were run in triplicates and GAPDH, C1ORF82 and TFG were used as endogenous control genes (Biosystems, 2008b).

4.2.5 Statistical analyses of the genetic data

Data management and summary statistics

PLINK was used in paper III, paper IV, paper V and paper VI. The settings for the inclusion thresholds for the MAF check was >0.01, maximum per-SNP missing frequency check was >0.1, maximum per-person missing frequency check was >0.2 and Hardy-Weinberg equilibrium (HWE) test was p-value cut-off >0.05 for the control group.

The Hardy-Weinberg law states that both the allele and genotype frequencies should remain constant in a population generation after generation and are then in equilibrium. The allele and genotypes frequencies can thus be estimated by these two equations:

\[ p + q = 1 \]
\[ p^2 + 2pq + q^2 = 1 \]

where \( p \) is the frequency of the minor allele, \( q \) the frequency of the major allele, \( p^2 \) is the frequency for the rare homozygotes, \( 2pq \) the frequency for the heterozygotes and \( q^2 \) the frequency for the common homozygotes. Deviations from this assumption can be tested with a \( \chi^2 \) test, comparing expected versus obtained frequencies. Violations of the HWE-principle could indicate inbreeding, mutations, assortative mating, genetic drift and genotyping error. HWE-test was done separately in the case and the control groups for all SNPs. If the control group was not in HWE the SNP raw data were further checked and then excluded from further analyses. However, deviations from the assumption for a SNP in the case group could indicate a possible disease association, thus those SNPs were not excluded (Balding, 2006).

The power of a statistical test is the probability that the test will reject a false null hypothesis. Power analysis can be used to calculate the minimum sample size required to accept the outcome of a statistical test with a particular level of confidence. It can also be used to calculate the minimum effect size that is likely to be detected in a study using a given sample size. Power analyses (post-hoc) were performed in paper II, paper III, paper IV, paper V and paper VI (Cardon and Bell, 2001).
Haplotypes and linkage disequilibrium
A haplotype is a combination of alleles on different loci but on the same chromosome. Alleles at nearby sites can co-occur on the same haplotype more than is expected by chance, the SNPs are then said to be in linkage disequilibrium (LD) (Wall and Pritchard, 2003). Different measurements for assessing the strength of LD have been proposed, we have used the D’ measurement. Haplotype and LD analysis were performed in paper III, paper IV, paper V and paper IV using PLINK (Purcell et al., 2007) and Haplovie (Barrett et al., 2005).

Association testing
Testing for genetic association in case-control studies is the testing for difference in allele, genotype or haplotype frequency between cases and controls. The testing of significance of an association can be performed in a number of ways depending on the data types. If having a dichotomous outcome variable (e.g. depressed versus controls) and a categorical predictor (e.g. genotype) a chi2 test can be used to test for significance of an association between the predictor and the outcome. Logistic regression can also be used, and is needed when a covariate is being added. A covariate is an independent variable affecting the outcome variable. This effect might be of direct interest or it may be a confounding factor. In genetic association analysis gender is often modeled as a covariate (Newton-Cheh and Hirschhorn, 2005). This is the general equation of a regression relationship:

\[ y = b_0 + b_1x_1 + b_2x_2 + \ldots \quad (b_i \text{ is the regressions coefficients for } x_i, \ b_0 \text{ is the intercept}) \]

Genotype frequency differences can be tested assuming different inheritance models:
- Genotype model = AA vs AB vs BB
- Dominant model = AA + AB vs BB
- Recessive model = AA vs AB + BB

Testing for trend can also be informative, where a linear relationship between genotypes is being tested.

In paper II, Kruskal-Wallis test were performed to test for difference in age between affected states and between genotype groups. A 2-sided Person chi-square test was used to assess possible difference in gender between affected states and between genotype groups. No difference in age or gender between genotype groups was found. Therefore, analyses of genotype frequency differences between cases and controls were performed controlling for age and gender using either a 2-sided Person chi-square test or 2-sided Fisher’s exact test. These tests were also used checking the association between environmental risk factors and outcome. Verifications with logistic regression were performed (PLINK) (Purcell et al., 2007). Programs used in this study were STATA (Stata-Corp) and SPSS (SPSS).

In paper III, the nonparametric Kruskal-Wallis test and Fisher’s exact test were used to assess difference in age and gender between cases and controls. Since gender distribution differed between cases and controls allele frequency difference was tested using logistic regression with gender as covariate. The difference in effect sizes between controlling and not controlling for gender did not differ. Therefore, in genotype and haplotype analysis gender was not controlled for. STATA (Stata-Corp),
SPSS (SPSS), PLINK (Purcell et al., 2007) and Haploview were used (Barrett et al., 2005).

In paper IV, paper V and paper VI, logistic regression with gender as covariate was used to test for allele and genotype frequency differences, as well as testing for trend. Calculations were performed with PLINK (Purcell et al., 2007) as well as R in paper IV (Team R, 2007).

Correction for multiple testing
The rational behind correcting for multiple testing is that when performing many tests, one is by chance more likely to get significant association even if the hypothesis is false (Balding, 2006). There are several different methods to correct for multiple testing. A classical method is the Bonferroni correction, where the new corrected significance threshold is the alpha (usually 0.05) divided by the number of independent statistical tests being performed. However, Bonferroni correction is considered very strict. Therefore, in paper III and paper IV we used another approach to correct for multiple testing. We used a significance threshold being alpha (0.05) divided by the number of SNP groups, each defined by D'>0.80 between any two included SNPs (Lavebratt et al.). The rational behind this correction method is that SNPs in LD are not independent from each other and each SNP should therefore not be accounted for. Permutation tests to obtain empirical significance were also performed in paper III, paper IV, paper V and paper VI. A permutation test is a form of resampling method and involves the shuffling of alleles between the two exposures groups to determine how likely the observed outcome is.
5 RESULTS

5.1 PAPER I – ENVIRONMENTAL FACTORS IMPORTANT FOR DEPRESSION

Kendler and colleagues proposed in 2002 and 2006 two gender-specific developmental models for MD where known vulnerability factors were structured according to when they occur in life (Kendler et al., 2002, 2006). Our aims were to test these models on our Swedish population based material PART including both females and males, and to test the specificity of the model to predict MD and other depressive disorders versus mental illness in general. Because of the latter aim, the material used was overrepresented for other psychiatric diagnoses (see paper I for more detailed description of the material used). Path and correlation analyses were performed on 16 risk factors (see picture below). The strongest unique influence on MD was found for early adolescence neuroticism, low self-esteem and anxiety, and for history of depression. The path analysis revealed that the models predicted two-thirds of the variance in liability to MD. The models predicted depression to a similar degree when cases were defined by both MD and other depressive disorders. When comparing MD with other psychiatric diagnoses the prediction of depression was only slightly weakened. The prediction values of MD were also similar between the female and the male model. Therefore, and because women were overrepresented in our material, primarily the female model data were reported in paper I. Our results support similar etiology, the sharing of risk factors, between MD and other depressive disorders. Moreover, our results also show the model’s ability to specifically predict depression and not mental illness in general. Two etiological pathways seem to be of importance, an adversity pathway and an internalizing pathway both originating in disturbed family environment.
5.2 PAPER II – PREPRONPY PROTECTS AGAINST DEPRESSION

There is extensive evidence for that reduced levels of NPY are involved in the pathophysiology of anxiety and depression. The Pro7 allele in the exon 2 encoding the signal peptide of preproNPY has been associated with higher processing rate into mature NPY (Kallio et al., 2001), and with high alcohol consumption and overweight/obesity-related disorders. In a material of 51 MD patients and 140 presumably healthy volunteers from Sweden the Pro7 frequency was lower among patients compared to controls (Heilig, 2004). In PART, the Pro7 allele was less common among depressed cases compared to controls (frequency=1.7% among cases; OR=2.6; P=0.0004; 95% CI=1.6–4.4). The dominant model showed a protective effect of Pro7 (OR=2.7, P=0.0004, 95% CI=1.6–4.6). Combining the depression and anxiety cases in a dominant model further strengthened our results (OR=2.6, 95% CI=1.6–4.1, P=0.0001). Furthermore, we tested if the protective property of Pro7 allele was present among those exposed to environmental risk factors. Risk factors shown to be of importance for depression development in paper I were considered. Pro7 heterozygotes were compared with homozygotes for the Leu7 allele. Pro7 heterozygosity had a protective role despite exposure to environmental risk for three of the seven investigated risk factors. These risk factors were family problems during childhood (OR=2.1, 95% CI=1.1–4.2), one or more last year independent stressful life events (OR=2.3, 95% CI=1.1–5.0) and last year dependent stressful life events (OR=2.5, 95% CI=1.3–5.0). Thus, the Pro7 allele protected against depression despite exposure to environmental vulnerability factors in the PART cohort.

5.3 PAPER III – PER2 VARIATION ASSOCIATES WITH DEPRESSION

Eighteen circadian or circadian related genes were studied for genetic association to depression in PART. The strategy used was to first identify SNPs suggestively associated to depression using a mental resilience sample as control set. SNPs with an indication of allelic association where then further tested for genotype and haplotype frequency differences and also investigated in a second sample set, depressed individuals versus ordinary healthy control individuals. Genetic variations suggestively associated with winter depression (Sjöholm et al, manuscript) were also further analyzed in this second sample set. Association to depression was found for haplotype variants in PER2, ARNTL and RORA supported by at least two of the three sample sets used. Further, we investigated whether the genetic risk variants found were independent from environmental risk factors that possibly could contribute to sleep disturbances and hence possibly rhythm disturbances. The results showed that the genetic risks were not dependent on an exposure to financial strain or last year negative life events.
5.4 PAPER IV – GENETIC INVESTIGATION BEHIND DEPRESSION AND ALCOHOL ABUSE OR DEPENDENCE COMORBIDITY

To investigate if circadian clock or circadian related genes are associated with the comorbidity of depression and AUD three sample sets were used.
1. Depression or Dysthymia + AUD versus controls
2. AUD versus controls
3. Depression or Dysthymia + AUD versus controls and AUD

Analyses were first carried out in sample set 1 and if SNPs showed suggestive allele or genotype association (p<0.05) they were further investigated in sample set 2 and sample set 3. Genes from the core circadian clock as well as genes involved in both up and down-stream pathways were investigated. Three SNPs in CLOCK, ARNTL and ACADS showed either allelic or genotypic association in sample set 1 and were hence further analyzed in sample set 2 and sample set 3. The CLOCK gene showed the strongest association of the three genes regardless of what sample set was used. The minor G allele of rs11240 in CLOCK was suggestively associated with an increased risk for comorbid depression and AUD (OR of 1.65 and p-value 0.0077). Using additional genotype models further strengthened the results. The rs11240 SNP was not associated with AUD using sample set 2, thus with sample set 3 weak association was found. LD analysis was performed in CLOCK with sample set 1 and one block was formed including all four SNPs analyzed (rs3805151, rs2412648, rs11240, rs2412646). Of the haplotypes formed, one (TTGC included the rs11240 risk allele) showed association in both the comorbidity sample set 1 (OR=1.6 and p-value=0.0077) and in sample set 3 (OR=1.5 and p-value=0.0084). Our results suggest an association between the CLOCK gene and the comorbidity between depression and AUD.

5.5 PAPER V – CRY2 AND DEPRESSION

CRY2 Expression Analysis

The CRY2 mRNA levels were assessed in depressed individuals with a Bipolar Disorder 1 diagnosis and controls before, during and after total sleep deprivation. The CRY2 expression showed a diurnal pattern in healthy controls and sleep deprivation induced a 2.0-fold (p=0.02) increase in CRY2 mRNA expression. In depressed patients the CRY2 mRNA expression was decreased as compared to controls during sleep deprivation (p=0.03). Furthermore sleep deprivation did not induce any increased mRNA levels for CRY2 in patients.
**Genetic Association Analysis**

Two different SAD materials, a Swedish and a Finnish material, were used to investigate possible involvement of CRY2 sequence variation in depression. In the Swedish material three out of the four SNPs analyzed were significantly associated with SAD and in the Finnish material two out of four SNPs. rs10838524 minor allele was overrepresented among depression cases in both the Swedish and the Finnish material (OR=1.6, p-value 0.0017 and OR=1.7, p-value 0.0020, respectively), although the minor allele for the SNP in the two materials was not the same. Genotype and trend test analyses in both populations were in line with the allelic data. For both materials three of the four SNPs, rs7123390, rs10838527 and rs3824872, was associated with an increased risk for SAD in the Swedish material (OR =1.7, P=0.012). Adding rs10838524 to the block (LD measure to rs7123390: D'=0.85) further lowered the p-value (OR = 1.8, P=0.0059). The GGAC haplotype was protective in the Swedish material (OR=0.75, P=0.048) and conferred a risk for depression in the Finnish material (OR=1.8, p=0.0004). Haplotype analyses in both materials show how the risk haplotypes span from CRY2 intron 1 to downstream the 3’UTR. If we assume that the same functional variant in both materials contributes to depression, we can use the two materials to narrow down the interval containing the functional variant. This may point to a location of a potential functional variation somewhat upstream exon 12 in 3’UTR. However, different vulnerability variations may be more likely.

**5.6 PAPER VI – CRY2 ASSOCIATED WITH RAPID CYCLING**

The aim of this study was to investigate whether the CRY2 variants associated to SAD were associated also to Bipolar Disorder. Such an association would also be in agreement with the CRY2 mRNA level association found to depression in Bipolar Disorder 1. Therefore, the four SNPs previously analyzed were genotyped in a Swedish Bipolar Disorder material. Allelic association testing in a case-control manner was performed, where Bipolar Disorder 1, Bipolar Disorder 2 and Bipolar Disorder patients with rapid cycling (BP-RC) were compared to population controls being ABD. Analysis was also performed in a case-by-case design, where rapid cycling was compared with Bipolar Disorder patients without rapid cycling (nonRC-BP). No association was found for the case-control analyses with Bipolar Disorder 1 and Bipolar Disorder 2. However, comparing the allele frequencies between BP-RC and the ABD showed that the A allele of rs10838524 conferred an increased risk (OR=1.4, CI=1.1-1.8, P=0.0076) for BP-RC. Further, genotype and trend test analyses with the BP-RC patients supported the importance of the A allele. The case-by-case analysis performed in BP-RC versus nonRC-BP showed a trend (P=0.0041) and association with a dominant model (OR=1.80, P=0.0098) for rs10838524 allele A. Due to strong LDs between all four SNPs, all SNPs were included in the haplotype. The haplotype with the strongest association was GGAC, including the protective G allele from rs10838524, and showed a protective role against rapid cycling compared to non-RC-BP and ABD (OR=0.7, P=0.0055 and P=0.024, respectively). AAAC and AGGA were found to be suggestive risk haplotypes for rapid cycling including the risk allele rs10838524 A (OR=1.34, P=0.043 and OR=1.55, P=0.048, respectively). This study is the first to report association between the circadian gene CRY2 and rapid cycling, a severe form of...
human bipolar disorder. Also, this is one of few findings showing a molecular discrimination between rapid cycling and other subtypes of bipolar disorder.
6 DISCUSSION

6.1 ENVIRONMENTAL STRESSORS IN DEPRESSIVE DISORDERS

We applied a developmental model for MD proposed by Kendler et al. (Kendler et al., 2002, 2006) to test whether it could predict development of MD and other depression diagnoses, and thereby explain the etiology of these disorders, in unrelated Swedish adult men and women. We demonstrate in paper 1 that the model with five life stages (tiers), composed of 16 previously reported risk factors for MD, predicted approximately two-thirds of the variance of liability to have a depression episode at the time of the interview.

![Diagram of the developmental model](image)

**Figure 10**

The predictability of depression by the model was only slightly lowered by including persons with other psychiatric diagnosis (15%) among the controls. The correlations between risk factors and depression, as well as the predictability of depression and path estimates between the risk factors were quite similar when including only MD diagnosis to when including also other depression diagnoses (62%). This suggests that depression as a whole has a common etiology. This is supported by studies showing that the affected persons move in and out of different subtypes of depression over time (Chen et al., 2000; Kessler et al., 1997). However, neuroticism and low education were estimated to be stronger risk factors for MD than for other depression diagnoses. The high predictability of depression despite overrepresentation of non-depressed persons with psychiatric symptoms in the sample suggests a potential of the model to identify specifically those with depression. The latter is notable given the
fact that environmental risk factors for mental disorders individually are known to in part be shared. The risk factors with strongest unique influence on depression episode were early adolescence neuroticism and history of depression. Early adolescence low self esteem and anxiety also showed strong relationship with depression. These four factors built up an internalizing pathway, originating in disturbed family environment, within the model. Disturbed family environment appeared to be the origin of also an adversity pathway for the development of MD.

How similar were the findings from PART to the original findings by Kendler et al on the twin samples from Virginia, USA? They developed two gender-specific models, with the same risk factors but somewhat different sets of inter-connections (Kendler et al., 2002, 2006). The models each explained ~50% of the variance in liability to MD, i.e. somewhat lower predictive power than each of these two models had in the combined set of men and women in PART. The higher predictive power of the model in PART may be explained by a number of differences between the studies such as the material, the scoring of risk factors and the definition of outcome. Kendler et al proposed not only the internalizing and the adversity pathways but also an externalizing pathway. Their externalizing pathway originated in conduct disorder and passed through substance misuse. We could not study this pathway due to unreliable data for conduct disorder. In our Swedish sample the internalizing pathway may to be of higher relative importance compared to in the Virginia twin sample where the adversity pathway appeared to have a higher relative impact on MD development.

6.2 RELATIONS BETWEEN GENES AN ENVIRONMENTAL FACTORS IN DEVELOPMENT OF DEPRESSION

Identified environmental risk factors were considered in the genetic analyses of paper II, paper III and paper IV. In paper II, protective NPY Pro7 allele was found to protect against depression despite exposure to some of the previously known risk factors, severe problem in family during childhood and last year stressful life events. In paper III, PER2, ARNTL, NPAS and RORA risk genotypes remained vulnerability factors in the absence of exposure to the potential sleep disturbance factors last year negative life events and financial strain. In paper IV, CLOCK variation was found to predispose for comorbid depressive and alcohol use disorders. CLOCK was not found however to be associated with AUD only. These results and earlier reports of ethanol’s direct action on the SCN (McElroy et al., 2009) led us to hypothesize that the CLOCK variation found here may be a vulnerability factor for depression given the exposure to alcohol in these AUD individuals.

Consideration of environmental factors in genetic association studies in depression has been limited. Instead a multifactorial focus has been on GWAS and molecular pathway analyses. Ideally would be to enter genetic and epigenetic variation data for pathways into a model like that presented in paper I. However, sample size, and thereby statistical power, limits the feasibility. Also, far from all DNA collections from depressive disorder cohorts have life history data. Furthermore, integrating both genetic and epidemiological data into models will require enormous data sets. A possible solution is to simplify the model, by reducing the number of factors and thereby reducing the complexity. However, some of the risk factors in the
developmental model will likely be found explained by genetic and epigenetic variations.

6.3 DO THE CIRCADIAN CLOCK GENES CONTRIBUTE TO DEPRESSIVE DISORDERS?

In paper II, paper III, paper IV, paper V and paper VI we investigated the role of circadian clock genes and related genes in the etiology of depressive disorders. In the previous section it was discussed whether the associations seen with depression and circadian genes were influenced by certain environmental risk factors (paper II and paper III). The results indicated that the associations found may alone be due to a genetic effect of the clock genes in depression.

Our results in paper II support a protective effect of NPY and is in line with previous reports suggesting anxiolytic-like and antidepressant-like effects of NPY (Mikkelsen et al., 1994; Stogner and Holmes, 2000). The protective effect of Pro7 found in this study is consistent with the findings of higher NPY processing in endothelial cells and higher plasma NPY levels after exercise in Pro7 carriers compared to Leu7 homozygotes (Kallio et al., 2001). NPY signaling is an “alarm-system” with the amygdala and hippocampus as core regions. Its activation appears to counteract the behavioral effects initially triggered by stressful situations in order to cope with the threat (Heilig, 2004). Moreover, NPY also seems to be involved in the circadian regulation, signaling from the intergeniculate leaflet (IGL) to the SCN. However, the mechanisms of IGL NPY neuronal activity and the nature of regulatory NPY signaling in the SCN clock are poorly understood (Glass et al., 1993). Moreover, the reduction of NPY levels in CSF seen in Swedish MD patients (Heilig, 2004) may reflect central NPY-signaling levels, at least if the decrease is genetically predisposed.

In paper III genetic variation in PER2 were associated with depression vulnerability in a Swedish population-based sample. Our results agree with earlier finding of winter depression association with SNP #10870 (Partonen et al., 2007). Also, the risk allele G of rs2304672 was nominally associated with bipolar disorder (Kripke et al., 2009), and with morning preferences in healthy volunteers (Carpen et al., 2005). If the desynchronization of circadian rhythms (Ukai et al., 2007) is linked to the onset of depression in vulnerable individuals, it is highly interesting to note that in mice the Per2 gene is involved in synchronization (Kornmann et al., 2007). If this role of PER2 holds for humans as well, then PER2 gene variants identified herein may be associated with a reduced synchronicity.

CLOCK was found to associate with depression and AUD in paper IV. CLOCK is one of the most central genes in the circadian clock (Dardente and Cermakian, 2007; Takahashi et al., 2008). This CLOCK variation found here may be a vulnerability factor for depression given the alcohol exposure in AUD, but not considerably increasing the risk for depression without AUD. This view is supported by the findings from other studies of the Finnish general population through the Health2000 Study where they could not detect any CLOCK association with MD disorder or dysthymia (Utge et al.) or anxiety disorders (Sipila et al.). The SNPs analyzed in the study of Sipilä et al. are in high LD with rs11240 analyzed in the current study (according to HapMap public release). Neither could we detect any indication that CLOCK variation was
associated with AUD only. Our results are also strengthened by alcohol’s properties to influence and alter the circadian rhythm. Alcohol may even act on the central pacemaker in the SCN (Rosenwasser, 2001). Studies also show that alcohol sensitivity and preference vary along with the circadian oscillation (McClung, 2007b; Wasielewski and Holloway, 2001). Studies show that rats and other rodents have a preference for alcohol during their active phase (dark-phase) (McClung, 2007b; Wasielewski and Holloway, 2001). Drug-induced changes of gene expression have been reported for several clock genes and the CLOCK:ARNTL transcription activity was increased in in vivo experiments when the dopamine D2 receptor was stimulated (McClung, 2007b). The Period (Per) genes in rats have a decreased circadian expression pattern in SCN and various other brain areas after alcohol intake (Chen et al., 2004) The shared overlap of genetic and environmental factors influencing depression and AUD was estimated to be only 9-14% (Prescott et al., 2000).

In paper V, we observed a marked diurnal variation in human CRY2 mRNA levels from peripheral blood mononuclear cells and a significant up-regulation following one-night total sleep deprivation, a known antidepressant. In depressed bipolar patients, levels of CRY2 mRNA were decreased and did not respond to sleep deprivation. Our results support earlier findings showing oscillating properties of CRY2 (Miyazaki et al., 2004) and demonstrate the effect of sleep deprivation, a known antidepressant on the circadian oscillations of CRY2 mRNA in human peripheral blood mononuclear cells. Our data indicated that depression in bipolar disorder is related to lowered levels of CRY2 mRNA. To investigate a possible genetic contribution of CRY2, we also undertook SNP genotyping in paper V using two independent population-based samples from Sweden and Finland. The CRY2 gene was significantly associated with winter depression in both samples. Differences in CRY2 risk haplotypes were observed between the Swedes and Finns. Overall there are genetic differences between these populations, which could explain our results (Lappalainen et al., 2009). Despite this difference, if we assume that the same functional variant in the Swedish and the Finnish samples, the two samples could be used to narrow down the interval of that functional variant. The risk haplotypes spanned from CRY2 intron 1 to downstream 3′UTR, and pointed at a location of a potential functional variation somewhere
upstream exon 12 in 3′UTR (rs10838527) from the Finnish data, with a distance from the intron 1 marker rs10838524 to rs10838527 corresponding to 33 kb (NCBI build 130). The vulnerability locus from the Swedish data overlapped the Finnish locus, but at the intron 7 marker rs7123390, and extended downstream rs10838527.

In paper VI we proposed that the circadian gene CRY2 is associated with rapid cycling in bipolar disorder. This is the first time a clock gene is implicated in rapid cycling, and one of few findings showing a molecular discrimination between rapid cycling and other forms of bipolar disorder. CRY2 is one of the core circadian genes and has role in photoreception. Deletion of Cry2 gene in mice prolonged the circadian period by approximately 48 min (from 23.7 to 24.5 hours) (Thompson and Sancar, 2002). CRY2 participates in regulation of the evening oscillator and is therefore of interest in mood disorders where a lack of switch from evening to morning oscillators has been postulated (Daan et al., 2001). Those results are in line with data that indicates intact morning oscillator in patients with bipolar disorder (Elsass et al., 1979) and in winter depression patients (Koorengevel et al., 2002). SAD has earlier been associated with PER2 variants (Partonen et al., 2007). CRY1 variants have also been shown to associate with MD (Soria et al., 2010). This is interesting because reduced CRY2 levels are presumably due to over-expressed CRY1 binding PER2, making less PER2 available for facilitating CRY2 binding with ARNTL:CLOCK dimers (Chen et al., 2009). In addition, abnormalities in the rest-activity cycles, or the sleep-wake cycle, may cause that support to PER1 (to inhibit PER2) by melatonin secretion fails and therefore PER2 overrules. When the adenylate cyclase sensitization, that is induced by melatonin disappears (Wagner et al., 2008) and the orphan nuclear receptor Nr1d1 gene expression is activated (Hazlerigg et al., 2004) there is insufficient support to have a robust morning peak of the Per1 gene expression. As Per1 guides Per2 (Pando et al., 2002) the reduced Per1 gene expression might permit Per2 to be over-expressed in general and with an earlier schedule than usual, thus generating the advanced phase position, which is often seen in seen in depressed. Moreover, the involvement of circadian genes in Bipolar Disorder is in strengthened by the effect of lithium. Lithium targets the clock gene glycogen synthase kinase 3 (GSK3) which interacts with PER2 and promotes the nuclear translocation of PER2 (Iitaka et al., 2005). We propose that a CRY2 locus is associated with vulnerability for depression, and that mechanisms of action involve dysregulation of CRY2 expression.

To summarize, we can in paper II, paper III, paper IV, paper V and paper VI demonstrate the involvement of core circadian genes in depressive disorders. Moreover, the associations found in paper III could be semi-replicated. The findings in paper V could be further strengthened in paper VI that is the association of CRY2 with depressive disorders. In paper II and paper III we could also demonstrate that the exerted effect of these genetic associations seemed to be independent of exposure to known risk factors for depression (paper I), implicating a substantial impact of the clock genes in affective disorders. Only in paper IV where we found an association with comorbid depression and AUD and CLOCK, could in part possibly be due to the exposure of alcohol. Our results support earlier findings of the involvement of circadian genes in depressive disorders and rhythm disturbances. Hence, one of the symptoms of depression is sleep disturbances implicating that disruption of the sleep-wake cycle and sleep alterations may lead to misalignment and result in depression.
and that mutations in the circadian genes might cause, contribute, increase the risk for depressive symptoms thus depression.

6.4 STRENGTHS AND LIMITATIONS

6.4.1 Paper I, paper II, paper III

PART and environmental risk factors
The Swedish population based material PART is extensively examined at two occasions, so far. The nonparticipation rate was 47% in the PART-study. An attrition analysis was done linking the personal identification number of each participant to several official registries. The result showed that the odds ratios for psychiatric diagnoses in the inpatient registries were quite similar among participants and non-participants when related to gender, age, income, education, country of birth, number of days with sickness allowance and socio-economic group (except for persons affected by psychotic disorders). However, prevalence of psychiatric disorders representative for Stockholm County cannot be calculated from PART since most likely the severe affected individuals did not participate.

No prevalence could be estimated in paper I due to the high non-participation rate and oversampling of persons with psychiatric symptoms to the interviews. The higher predictive power of the model in paper I than in Kendler’s studies might be due to a number of differences between the studies. First, the Swedish population is genetically and culturally homogenous whereas sample in the Kendler et al. study was from Virginia, USA. Kendler et al. studied twins which are more homogeneous than unrelated person with regard to genetic and early environmental risk factors. Second, in paper I the outcome variable was defined as depression episode the last month, whereas in Kendler et al.’s studies, the outcome variable was MD any time during the last year. The depression diagnoses in paper I were based on extensive psychiatric interviews using SCAN which has a high reliability, and repeatedly has bee used as the golden standard (Eaton et al., 2000; Leentjens et al., 2000). Third, the present study used DSM-IV criteria whereas Kendler et al. used DSM-III. DSM-IV but not DSM-III takes into account the impact of the disorder on functioning in daily life. Any of this may have strengthened our predictive power of the model. Conduct disorders, as well as childhood sexual abuse were assessed in the studies by Kendler and colleagues, but not in paper I. Moreover, the weak association between family history of MD and MD may be explained by a high degree of false negatives since the criteria for positive assignment were restrictive. The weak influence of parental loss on depression could be due to that only 7% reported parental loss. Lack of power may also explain the absence of association between major depression and substance abuse. Recall bias of childhood problems exists in paper I and paper II, but is limited since only 14% of those 2 633 persons reporting problems during childhood in the first questionnaire wave did not do so in the second wave 3 years later (Forsell and Lundberg, unpublished). Furthermore, stressful life events were self-reported and can thus be biased by the person’s mental condition. The internalizing risk factors in early adolescence, as well as the adversity factor life time trauma were not studied in paper II since this information was not available.
The genetic analyses
A limitation in paper I is that genetic risk was assessed using reports on heredity since no genetic analyses were performed. In paper II, the individuals with anxiety diagnosis were few. The environmental risk factors in paper II were analyzed one at a time because of the low frequency of the Pro7 allele. A limitation in the genetic analyses in paper III is that only a semi-replication approach was used, since the cases were the same in sample set 1 and sample set 2. Moreover, a genetic finding in sample set 1 not detected in sample set 2, may reflect a low effect size in sample set 2, a genetic association to mental resilience rather than to depression in comparison to controls, or a false positive signal in sample set 1.

6.4.2 Paper IV

The individuals used in paper IV are derived from Health2000 that is nationwide and representative of the general population aged 18 or over. However a limitation in paper IV is thus the small size of the comorbid sample and the lack of a group of patients having depressive disorder only. Moreover, the individuals without psychiatric symptoms as well as the AUD individuals were used in two sample sets.

6.4.3 Paper V and paper VI

A strength of paper V is that our findings of the involvement of the CRY2 gene in depressive disorders are based on data from both expression- and genetic association data. There are however some limitations in paper V. The diagnoses studied the expression and the genetic association analyses were not the same, although depressive state was characterized both groups. In the expression analysis individuals diagnosed with Bipolar Disorder were studied and in the genetic association analysis individuals diagnosed with SAD were studied. A limitation in the expression analysis of CRY2 was that too few time points for the cases were assessed, thus we can not analyze the CRY2 rhythm and possible phase shift among the cases. Moreover, we cannot exclude that the CRY2 mRNA levels in the patients were influenced by medication. All the patients were on an SSRI together with a mood stabilizer. Since all controls and 10 of the patients were studied during the summer, the season of the examinations did not likely explain the CRY2 mRNA level difference between patients and controls. A limitation in the genetic analysis was the number of patients. However, the cases and the controls in both the Swedish materials represented an ethnically quite homogeneous population. For both the Swedish and the Finnish materials the controls were extensively scored to exclude any mental illness, reducing bias due to the ethnic variation and dilution of genetic effects due to disorder heterogeneity among the controls. Moreover, the differences in CRY2 risk haplotypes seen between the Swedish and the Finnish materials could have been more extensively studied by genotyping more SNP in CRY2.

Paper VI was initiated based on the results from paper V. In general the results in paper VI did strengthen the findings in paper V. The risk and protective CRY2 haplotypes AGGA and GGAC, respectively, that were suggested to be associated with rapid cycling, were identical to those recently suggested to be associated with winter depression in Swedish patients, with similar case and control haplotype frequencies, and thereby similar effect sizes, between the two studies. The rapid cycling risk and
protective haplotypes AAAC and GGAC, respectively, were the opposite, that is protective and risk haplotypes, in the Finnish winter depression sample. We could not detect association for the CRY2 SNPs in paper VI to Bipolar Disorder 1 or Bipolar Disorder 2. However we cannot exclude the possibility of a true association between CRY2 and bipolar disorder at low effect sizes. On the other hand, bipolar disorder cases are heterogeneous with regard to clinical symptoms and severity, and to genetic underpinnings. Moreover, the rapid cycling group may reflect a more genetic homogenous group, thus increasing the power.
7 CONCLUDING REMARKS

Disturbed sleep-wake patterns, as well as disruptions of other circadian rhythms have been observed in depressive disorders. Therefore, it is possible that identification of genetic variants in circadian genes will give us a better understanding of the pathology in depressive disorders. The associations between genetic variants and depressive disorders were investigated in relation to environmental factors, using a candidate gene approach focusing on the circadian clock genes. In conclusion, our results support the hypothesis that circadian gene variants are involved in depression, seasonal affective disorder and bipolar disorder. The associations found with NPY and PER2 did not seem to be affected by exposure to known depression risk factors, thus indicating that variants in circadian genes might cause, contribute to, or increase the risk for depressive symptoms. However, there is a need for replication and further investigation of the variants found to be associated with depression disorders in this thesis.

Studies of depressive disorders most certainly face the same problems as the study of other complex disorders in that they are multifactorial. Probably many of the genes involved in psychiatric disorders have not yet been identified due to this complexity (Akil et al.). The “rare variant hypothesis” and the “common variant hypothesis” (Bodmer and Bonilla, 2008) are often mentioned when discussing susceptibility to complex disorders. A single gene might contribute to depression with a small effect, but it is also possible that one gene might contribute to depression in one family only, thus making it difficult to have enough power (Akil et al.; Nestler et al., 2002). The “common and rare variant hypothesis”, one could say, advocate two different approaches to complex genetics. We have tried to reduce complexity by studying Nordic samples that may be ethnically more homogenous than those from other parts of the world. Since we have not had large families to work with we have had low power to detect association to rare gene variants and therefore we have not selected such SNPs to study.

Another difficulty one faces when working with genetics of psychiatric disorders is that the diagnoses of different depressive disorders overlap, and moreover that the symptoms vary within a disorder (Bergen et al.; Burmeister et al., 2008). One way to overcome this problem is to investigate a more well-defined group of individuals with a specific phenotype, thereby being more likely to have a genetically homogenous sample (Camp and Cannon-Albright, 2005). Thus, analyzing subjects stratified based on variation in disease characteristics (endophenotypes), such as rapid cycling in bipolar disease, one enhances the signal to noise ratio by reducing heterogeneity and may thus increase the power to detect an association (Bergen et al.; Camp and Cannon-Albright, 2005). Moreover, it is possible that epigenetic modifications and gene-environment interactions (Cichon et al., 2009) make it more difficult to identify genetic variants that increase the susceptibility to complex diseases.

I believe that the approach used in this thesis, i.e. to focus on one feature of depression, rhythm disturbances, is a good way to address the complexity of depressive disorders. However, depression is most probably due to several genetic mutations as well as several life stressors, epigenetic changes, and other cellular and
morphological lesions (Akil et al.), making it important to complement this approach with many other approaches.
8 FUTURE PERSPECTIVES

There are many questions remaining regarding the genetics behind depressive disorders and to what extent the circadian system may be involved in the disease pathogenesis. Below is a list of ongoing research projects and a number of possible experiments to conduct in the future.

- An ongoing project investigates the relationship between DNA methylation in GR, 5-HTT, COMT and MAOA genes in depressed individuals subjected to stressful life events during the childhood, taking into account genetic variations in these genes. This project also aims to analyze whether the DNA methylation pattern for these genes differ between blood and saliva.

- To be able to test for epigenetic differences in brain tissue we are conducting a study using an animal model of depression (the FSL and FRL rats) looking at the brain regions hippocampus and prefrontal cortex. Certain genes with altered expression in depressive rats are investigated with regard to gene sequence, promoter methylation, histone modification and upstream mechanisms of epigenetic changes.

- The genetic associations presented in this thesis needs to be replicated in other materials, to determine their involvement in depressive disorders.

- It would also be of interest to conduct expression analyses on circadian clock genes in areas such as the SCN using post-mortem brains from healthy and depressed individuals.

- The findings in paper IV of whether alcohol can induce depressive symptoms could be tested in a CLOCK mutant animal model.
9 ACKNOWLEDGEMENTS

During my PhD years I have had the chance to work and got to know many amazing people. I would therefore like to express my sincere gratitude to you all.

First I would like to thank all participants in the PART study for patiently filling in the extensive questionnaires, making it possible for us to conduct our research and hopefully make a contribution back to you.

I would also like to thank the following:

Catharina “Cattis” Lavebratt, my main supervisor. I remember the first day at the lab like yesterday! 😊 Thank you for letting me be a part of the Neurogenetics group but also for being one of the most enthusiastic and optimistic persons I know. These are good qualities when being in the researching “business”.

My co-supervisor Yvonne Forsell for letting me do “non-genetic” research (plus all the non-job related discussions we had had during my four years at KI).

Timo Partonen for giving me the chance to work and live in Finland and being a good host. I really enjoyed living in Finland. You always give me good comments on my articles and fast replies on my emails.

My un-official mentor, Martin Schalling, thanks for letting me be a part of the neurogenetics lab and the scientifically guidance through my four years.

My dear dear friends, I can laugh with you and I can cry with you!
Anna, my mommy (I dubbel bemärkelse nu), who ALWAYS takes care of me + Kennet I might add. My stand-in-twin! 😊 Ida, for being who you are, kind and always listening. Karin, you’re fun, always positive and my-know-it-all, what will I do without you?! Malin, the rock, you always gives great advises and have the energy like no one else. Phille, my baby-brother, what will you do without me! ;) For all the nice time traveling with you to the US and A. It has been nice “fighting” with you, I will miss that.

My dear colleagues but also my friends. It’s because of you I always go to work with a smile on my face! 😊 Selim “ Storebro” for putting a smile on my face every day and helping me out when I was new in the lab. Elin och Pernilla for being such nice colleagues and friends and listening to all my research problems. It has been a pleasure working with you. Jeanette and Sivonne, for being such wonderful persons. Tina, nice to have someone doing almost “the same thing” as me. Alex, AnnaLee, Annika, Björn, Charlotte, Dzana, Katharina, Louise N, Marie, Neman, Santi and Ulrika!

The “new” 00-floor members, the Hillert group, Anna F-H, Anna M, Boel, Elin K, Helga, Iza, Jenny L, Kerstin, Rasmus, Roger, Wangko and Jan Hillert. Thanks for interesting and tasty Friday breakfast-seminars and nice AWs. ;)

46
The Hemathology lab, for contributing to a nice work-atmosphere at the 00-floor, you are always so friendly and helpful.

All former colleagues of the Neurogenetics group and the 00-floor, Alicia, Anna K, Ann-Sophie, Banu, Dalila, Elsebritt, Frida, Lina R, Maria K, Priya, Rifat and Ryan. It has been a pleasure to have worked and known you!

Thanks to all people at CMM L8:02 for nice breakfast seminars and book club. The people at L8:04 for journal club and “all the other stuff”! ;) Good luck, Ame and Melanié!

Thanks for taking care of us!
Lennart “Lelle” och Catharina. IT-department, Daniel, Dagmar, Rudolf. The administration at MMK, Britt-Marie, Ann-Britt, Kerstin, Helena, Kristina, for always helping out and taking such a good care of us PhD-students.

My “other” colleagues, my Finnish colleagues!
I will always remember my time in Finland with a smile. Thanks for making me feel like home. Leena, Pia, Emma, Annu, Ollie and all the other nice people I got to know! Kiitos!

The ”BH-gänget”, Daniel and Ewa-Carin, Sweden goes US and A. Thanks for all the nice re-unions after the course in Bar Harbor, Maine! Vart åker vi härnäst?! ;)

Tord, ännu än en till……tvilling! ☺ Som under min vår på Norrbacka varit den som stått för stöd, smakråd och skratt! Tack för all hjälp!

Mickie, du har skämt bort mig med mer än bara stöd och snälla ord under våren. God mat, mycket goda viner och glass (från den nyinköpta glassmaskinen)! ;)

Familjen Lindholm, familjen som vet hur det är! ;) Kerstin, Jan, Stoffe, Alex och Jocke. Intensivt både i backen, med matlagningen å snacket! Bästa svängen… Vem får stavfelet nästa gång?!?

Universitets-tjejerna: Diana, Erika, Jenny, Jossan, Kim, Stella and Regina. I remember it like yesterday! Now what?! ;)

My dear childhood friends, what have we not done together? ☺ Dandryds-tjejerna, Calin, Johanna, Louise, Lojsan, Malin and Maria. My girls! Vi har känt varandra nästan hela livet och delat både glädje och sorger. Ni är underbara, roliga och vackra!
My dear family
Camilla "Milli". Min kära Myra, det har lixom varit du och jag hela livet. Kärlek vid första ögonkastet?! ;) Vad firar vi i år, 25 år som vänner?! Så olika men ändå så otroligt lika! Min personliga rådgivare i allt, för dig kan man aldrig göra fel! TACK!

Mina kära föräldrar, Pappi (Bengt) och Mamma (Åsa), som har tagit så väl hand om och curlat mig hela livet! Först i rollen som föräldrar "till trillingarna" men nu som otroligt bra vänner! TACK!

Era respektive, Ann-Marie och Olle, tack för att ni tar så väl hand om mina föräldrar! Olle den evige entusiaster som FAKTISKT läser mina "pek" och gör understrykningar som vi sedan diskuterar. 😊

Min älskade "Mojmoj" Alice, du är rolig du, jag är alltid så glad efter våra samtal! Hade man hört oss prata och inte vetat bättre så kunde man lätt tro att det var två vänninor som pratade! ;) Och just det, tack för "dina gener", de har kommit väl till pass! ;)

Mina andra 2, Andreas (nr1) och Tess (nr2), jädrans att man skulle vara sist. Men det gick ju bra trots allt. Mina kära syskon som jag delat allt med i livet ända sedan fostertiden! Otroligt glad att det blev just vi, så kul som vi har tillsammans!

Deras respektive, Erik och Marta, tack (hehe) för att ni "valt" att vara en del av vår familj! Ni gör skridresor, famijemiddagar och fester till ett sant nöje!

This work was supported by KID funding for postgraduate training (KI), the Swedish Research Council, Stockholm County Council, the Söderström-Königska Foundation at the Swedish Society of Medicine, and Karolinska Institutet Foundation, Academy of Finland #201097, #210262 and #203425, The Finnish Medical Foundation and Helsinki University Central Hospital #TYH538.
10 REFERENCES


Koyama, R., and Ikegaya, Y. (2005). To BDNF or not to BDNF: that is the epileptic hippocampus. Neuroscientist 11, 282-287.


52


Meaney, M.J. Epigenetics and the biological definition of gene x environment interactions. Child development 81, 41-79.


SPSS. PASW for Windows ( Chicago, SPSS Inc.).

Stata-Corp, L. STATA (College Station, Texas).


patients with seasonal affective disorder. Archives of general psychiatry 58, 1108-1114.


