Study of genomic variability in the genetic susceptibility to psychiatric disorders: SNPs, CNVs and miRNAs

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A totes les persones
que pateixen
trastorns psiquiàtrics

A tu, Jordi,
per cada segon que has estat
dia rere dia al meu costat

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Abstract

In this thesis we have studied genetic elements potentially contributing to the pathophysiology of psychiatric disorders, focusing on different sources of human genome variability, including SNPs and CNVs, which can affect not only coding genes but also RNA regulatory elements, such as miRNAs. First, we have interrogated different candidate genes for psychiatric disorders overlapping with known CNVs, finding 14 different genes variable in copy number in psychiatric disorders but not in control individuals. Then, narrowing the analysis on mood disorders, we explored *GSK3β* gene considering both SNPs and a partially overlapping CNV. The *GSK3β* promoter and intron 1 region was found significantly associated with an earlier onset of the major depressive disorder. Finally, we have found evidence possibly pointing to a precise post-transcriptional regulation of circadian rhythms by miRNAs in mood disorder patients. Concretely, a variant in the precursor form of miR-182 could play an important role in fine-tuning its target sites involved in the control of sleep/wake cycles. Overall, we have provided evidence of different types of genome variation on neuronal genes or miRNA regulatory regions that can potentially contribute to the development of psychiatric disorders.

Resum

En aquesta tesi hem estudiat elements genètics que podrien contribuir potencialment en la fisiopatologia dels trastorns psiquiàtrics, centrant-nos en diferents fonts de variabilitat genòmica humana, incloent els SNPs i els CNVs, els quals poden afectar no només a gens codificants sinó també a elements reguladors, com els miRNAs. Primer, vam interrogar diferents gens candidats per trastorns psiquiàtrics solapats amb CNVs coneguts, trobant que 14 gens eren variables en el número de còpia en pacients però no en individus controls. Després, restringint l'anàlisi a trastorns afectius, vam explorar el gen *GSK3β* considerant SNPs així com també un CNV que se solapa parcialment amb el gen. Vam trobar la regió del promotor i de l'intró 1 del gen *GSK3β* associada de manera significativa amb una inferior edat d'inici del trastorn de depressió major. Finalment, hem trobat evidències que possiblement indiquen una precisa regulació post-transcriptional dels ritmes circadians per miRNAs en pacients amb trastorns afectius. Concretament, una variant en la forma precursora del miR-182 podria jugar un paper important en la fina regulació dels seus gens diana implicats en el control dels cicles de son i vigília. En general, hem aportat evidències de què diferents tipus de variació genòmica en gens neuronals o regions reguladores com els miRNAs podrien contribuir potencialment en el desenvolupament de trastorns psiquiàtrics.

Preface

Psychiatric disorders are very prevalent diseases which represent a major public health problem affecting hundreds of millions of people worldwide. As all complex diseases, psychiatric disorders have a strong genetic contribution in their etiopathology, in combination with environmental factors influencing their development. Psychiatric genetics arose in the last century as a new discipline with the aim of deciphering the genetic elements underlying the susceptibility to mental illnesses. Throughout the years, the interrogation of different types of DNA polymorphisms, especially SNPs, by conventional genetic approaches, such as association studies, have led to the discovery of several potential candidate genes for psychiatric disorders. However, very few findings have been strongly replicated and corroborated and, still, the genetic map underlying psychiatric disorders is largely unknown, with an important fraction of the heritability predicted for psychiatric disorders yet to be elucidated. During the last decade, the field of human genetics has evolved dramatically in regards to the study of complex diseases, since there have been tremendous technological advancements that have completely changed the way in which to face the genetic study of a given psychiatric disorder. First, in 2001 the first haploid human genome sequences were released and, few years later, an extensive catalogue of SNPs along the human genome was available. More recently, high–throughput genotyping and sequencing technologies have produced an explosion of whole genome association studies and the sequencing of complete genomes, representing a step further in the unraveling of the genetic basis of psychiatric disorders. This has also brought to light the existence of other important sources of genome variability, such as structural variants, and the relevance of considering not only common variants but also rare ones. Furthermore, it has become apparent the major role of gene regulatory networks, such as those leaded by miRNAs, in the control of a very ample range of physiological and pathological functions, with an evident need to take them into account as contributing factors in the genetic susceptibility to psychiatric disorders. Consequently, in this thesis, we aimed to go a step further in the understanding of the genetic basis of psychiatric disorders taking advantage of different genetic molecular approaches. We have focused on the study of copy number variants and SNPs in candidate genes and regulatory elements such as miRNAs, stressing the importance of using well-defined and homogenous samples to facilitate the identification of susceptibility factors in diseases such as psychiatric disorders.

Contents

Introduction

1.1. Psychiatric disorders

Psychiatric disorders are defined as any illness with a psychological origin, manifested either in symptoms of emotional distress or in abnormal behavior. However, the American Psychiatric Association recognizes that there is no definition that adequately specifies precise boundaries for the concept of mental disorder, and that it lacks a consistent operational definition that covers all situations[1]. At present, psychiatric disorders are commonly occurring and often seriously impairing in many countries throughout the world. For example, estimates made by the World Health Organization (WHO) in 2002[2] showed that 154 million people globally suffer from depression and 25 million people from schizophrenia; 91 million people are affected by alcohol use disorders and 15 million by drug use disorders. Moreover, the World Mental Health (WMH) surveys developed by WHO[3] estimated an inter-quartile range of lifetime psychiatric disorder prevalence of 18.1-36.1%. Consequently, psychiatric disorders have become an important area of study and research, not only from a clinical point of view but also taking into account different aspects of their pathophysiology, such as neurobiological studies and psychiatric genetics, which at the same time can represent a step forward to the diagnosis, classification, prognosis, or treatment of mental illnesses.

1.1.1. Diagnosis and classification

Since the classification of mental illnesses in ancient Greece and Rome in five categories defined by their phenomenology (phrenitis, mania, melancholia, hysteria, and epilepsy) and the four temperaments described by Hippocrates (choleric, sanguine, melancholic, and phlegmatic)[4], the recognition and understanding of mental health conditions has changed over the time across

cultures and the definition, assessment and classification of mental disorders is still nowadays an ongoing debate[5-8].

The ideal diagnostic system labels diseases according to etiology, but as for most mental disorders it is unknown, in psychiatry the diagnosis is normally based upon clinical features, shared natural history, common treatment response, or a combination of all three. Moreover, this psychiatric diagnosis is commonly based on clinical interview and, to a lesser extent, the later course of the patient's illness, contrary to most branches of clinical medicine where diagnoses are made according to the patient's history, with physical examination and investigation playing an important but subordinate role[9].

There are two main systems currently in use worldwide to diagnose and classify mental disorders: the International Classification of Disease (ICD-10)[10], produced by the WHO, and the Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition, Text Revised (DSM-IV-TR)[1], produced by the American Psychiatric Association (APA). While ICD-10 is a wider general medical classification, DSM-IV describes only mental disorders, although the two classifications are broadly similar, having undergone a degree of convergence with subsequent revisions (see box 1 for detailed information of DSM).

Box 1. Diagnostic and Statistical Manual of Mental Disorders (DSM)

The DSM-IV-TR, published by the American Psychiatric Association, is used by clinicians and psychiatrists to diagnose psychiatric illnesses, as well as for research purposes. It covers all categories of mental health disorders for both adults and children, focusing mostly on describing symptoms as well as statistics concerning which gender is most affected by the illness, the typical age of onset, the effects of treatment, and common treatment approaches. Clinicians also use the DMS-IV to classify patients for billing purposes, since the government and many insurance carriers require a specific diagnosis in order to approve payment for treatment. The DSM-IV-TR is a multiaxial approach based on five different dimensions, which allows clinicians and psychiatrists to make a more comprehensive evaluation of a patient's level of functioning, because mental illnesses often impact many different life areas:

Axis I Clinical Disorders: describing clinical symptoms that cause significant impairment, including major mental disorders and learning disorders.
Axis II Personality Disorders and Mental Retardation: Personality disorders have more long lasting symptoms and encompass the individual's way of interacting with the world. Mental retardation is characterized by intellectual impairment and deficits in other areas such as self-care and interpersonal skills.
Axis III General Medical Conditions: including physical and medical conditions that may influence or worsen Axis I and Axis II disorders.
Axis IV Psychosocial and Environmental Factors: Any social or environmental factors that may contribute to Axis I or Axis II disorders.
Axis V Global Assessment of Functioning: allowing the clinician to rate the patient's overall level of functioning. Based on this assessment, clinicians can better understand how the other four axes are interacting and the effect on the individual's life.

In the DSM-IV-TR[1] the psychiatric disorders are classified in the following categories: adjustment disorders; anxiety disorders; delirium, dementia, and amnestic and other cognitive disorders; disorders usually first diagnosed in infancy, childhood, or adolescence; dissociative disorders; eating disorders; factitious disorders; impulse-control disorders; mental disorders due to a general medical condition; mood disorders; schizophrenia and other psychotic disorders; sexual and gender identity disorders; sleep disorders; somatoform disorders; substance-related disorders; personality disorders; mental retardation; and other conditions that may be a focus of clinical attention. Anxiety disorders, eating disorders, schizophrenia and specially mood disorders will be next explained in more detail, as they are the psychiatric disorders studied in this thesis.

In spite of the clear classification of psychiatric disorders, high rates of comorbidity or multiple diagnoses are often present within the same person, with frequent overlap in symptoms and treatments in different psychiatric categories[11]. This is an evident limitation of the non-etiological approach of the DSM classification, which may point to question whether these diagnostic criteria always categorize different psychopathological conditions[6] (Figure 1). Thus, the present DSM classification can be highly reproducible and useful from a clinical point of view but, on the other hand, it may sometimes confuse the basic research of the underlying biological factors conferring susceptibility to psychiatric disorders.

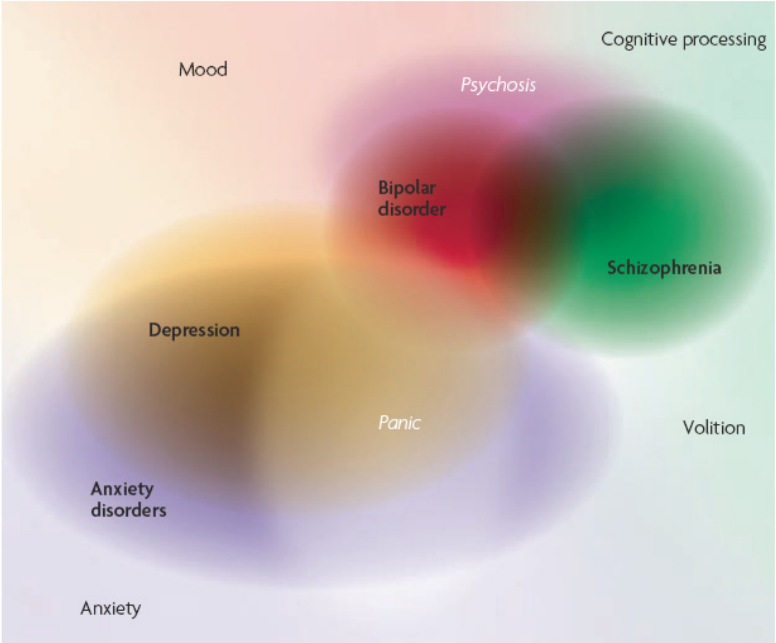


Figure 1. Overlap of psychiatric disorders, possible extremes of personality traits. Psychiatric disorders understood as extreme ends of normal population variations of personality. Genetic and other susceptibility factors affecting levels of these underlying traits can be unique or common for different psychiatric disorders (from Burmeister *et al*.[12]).

1.1.1.1. Anxiety disorders

Anxiety disorders embrace a broad category of heterogeneous disorders which are mainly characterized by abnormal and pathological fear and anxiety. The psychological components of anxiety disorders (as worry and fear) are often accompanied by physiological symptoms such as sweating, rapid heartbeat, or dizziness. Each anxiety disorder has its own distinct features, but they all have in common a heightened sense of arousal or fear that can be episodic or continuous and may be related to exposure to a specific trigger. Anxiety disorders are found to be the most prevalent class of mental disorders in the general population, with estimated life-time prevalence of any anxiety disorder averaging approximately 16% when considering developed and developing countries[3]. The prevalence of anxiety disorders, however, is generally higher in Western developed countries,

being around 29% in United States[13]. The age at onset of anxiety disorders is very variable, some of them (phobias) presenting an early onset in childhood[14]. Regarding gender differences in anxiety disorders, in both childhood and adulthood, girls/women are at greater risk than boys/men for most anxiety disorders[15]. Anxiety disorders have been reported to be frequently comorbid with each other as well as with other mental disorders, for example with mood disorders, eating disorders and substance-abuse disorders among others[16-21]. As outlined in the DSM-IV-TR[1], anxiety disorders include: acute stress disorder, agoraphobia (with or without history of panic disorder), anxiety disorder due to general medical condition, generalized anxiety disorder, obsessive-compulsive disorder, panic disorder (with or without agoraphobia), posttraumatic stress disorder, specific phobia, social phobia, and substance-induced anxiety disorder.

1.1.1.2. Eating disorders

Eating disorders are characterized by an aberrant pattern of eating and weight-control behavior, as well as disturbances in the perception of body image, resulting in a clinically significant impairment of physical health and/or psychosocial functioning[1]. In the DSM-IV-TR, eating disorders are classified as: anorexia nervosa (AN), bulimia nervosa (BN), and eating disorders not otherwise specified (EDNOS), this last category including binge eating disorders[1]. Both AN and BN have in common the underlying psychopathology, where patients judge their-selves worth mainly by their shape and weight and their ability to control them, with most of the other clinical features of these disorders being consequences of this psychopathology[22]. Eating disorders are predominant in Western societies, with a lifetime prevalence estimated from 0.7% to 2% in AN and BN, respectively, and principally affecting females[23]. Most eating disorders commonly develop during adolescence or young adulthood[24]. Regarding the

development and clinical course of eating disorders, migration of patients between the diagnostic categories of AN, BN and EDNOS is frequently observed[25-27]. Furthermore, apart from this comorbidity within different eating disorders' subcategories, a wide range of other psychiatric diagnoses are frequent in patients with eating disorders, such as mood disorders or anxiety disorders[28, 29].

1.1.1.3. Schizophrenia and other psychotic disorders

The category of schizophrenia and other psychotic disorders in the DSM-IV-TR[1] encompasses: schizophrenia (including catatonic, disorganized, paranoid, residual and undifferentiated types), schizophreniform disorder, schizoaffective disorder, delusional disorder, brief psychotic disorder, shared psychotic disorder, psychotic disorder due to a general medical condition (with delusions or hallucinations), substance-induced psychotic disorder and psychotic disorders not otherwise specified. In spite of their heterogeneity, all previously mentioned disorders have in common the presence of psychosis, which can be described as a disintegration of the thinking process and impairment in reality testing, involving the inability to distinguish external reality from internal fantasy. Psychotic symptoms account for presence of delusions (fixed, false beliefs that are not shared by an individual's cultural/religion group) and hallucinations (perceptual experiences that are not shared by others and may actually affect any of the five senses), which can also occur in mood disorders and may be associated with substance abuse, medication side effects or also with a general medical condition[1]. The criteria used to classify psychoses into different categories are based on duration, dysfunction, associated substance use, bizarreness of delusion, and presence of depression or mania[30]. The lifetime prevalence of psychotic disorders is around 2.3%[31], showing similar rates in men and women[32, 33]. Schizophrenia and other psychotic disorders share high levels of

comorbidity with other major mental illnesses, principally with mood, anxiety and substance-abuse disorders[34-36].

1.1.1.4. Mood disorders

The common feature of mood disorders (MD), which include a wide category of different disorders, is a pathological disturbance of mood ranging from extreme elation or mania to severe depression. MD are also characterized by other symptoms such as disturbances in thinking and behavior, which may include psychotic symptoms (for example, hallucinations and delusions). The DSM-IV-TR[1] describes different mood disorders episodes and classifies mood disorders into different categories, as well as it considers different specifiers which provide additional details to a diagnosis, such as the severity of the current episode and how the person is cycling. Mood disorder episodes account for major depressive episode, hypomanic episode, manic episode, and mixed episode. Then, MD are categorized as: depressive disorders, including dysthymic disorder and major depressive disorder (with a single episode or recurrent), bipolar disorders involving bipolar I disorder, bipolar II disorder, cyclothymic disorder, and bipolar disorder not otherwise specified, mood disorder due to a general medical condition (with depressive features, maniac features, or mixed features), substance-induced mood disorder, and mood disorder not otherwise specified. The two major mood disorders categories are major depressive disorder (MDD), also named unipolar major depression, and bipolar disorder (BD), which are distinct in the fact that to be diagnosed of BD is requiered to experience one or more episodes of mania or hypomania, usually accompained by depressed episodes during the course of the illness (Figure 2).

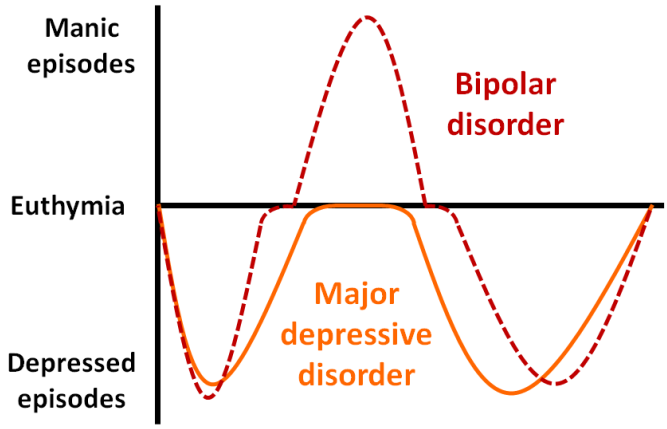


Figure 2. Evolution of MDD and BD. MDD patients present one or more episodes of major depression, while BD patients deviate from euthymia (normal mood) in both directions, combining manic or hypomanic episodes with depressed episodes (adapted from wellesley.edu).

Mood disorders are generally found to be the second most prevalent class of mental disorders worldwide, with lifetime prevalence estimates of any mood disorder averaging approximately 12%, being higher in Western developed countries[3]. The median age at onset of mood disorders is around 30 years old, with a risk of developing a mood disorder being significantly higher in women than in men[13]. Mood disorders show high comorbidity with other psychiatric disorders, most notably with anxiety, substance-abuse, and schizophrenia and other psychotic disorders[17-19, 36, 37].

Major depressive disorder (MDD)

According to DSM-IV-TR[1], MDD is characterized by the presence of major depressive episodes (single MDD or recurrent MDD if at least two major depressive episodes occur), which are mainly defined as a period of at least two weeks with either depressed mood most of the day, nearly every day, or with a markedly diminished interest or pleasure in all, or almost all, activities most of the day, nearly every day, or both. These symptoms are recognized basing on either subjective report or observation made by others, and

moreover they have to be accompanied by other symptoms (in total five or more): significant weight loss when not dieting or weight gain, or decrease or increase in appetite nearly every day; insomnia or hypersomnia nearly every day; psychomotor agitation or retardation nearly every day; fatigue or loss of energy nearly every day; feelings of worthlessness or excessive or inappropriate guilt nearly every day; diminished ability to think or concentrate, or indecisiveness, nearly every day; and/or recurrent thoughts of death, recurrent suicidal ideation without a specific plan, or a suicide attempt or a specific plan for committing suicide. The symptoms cause clinically significant distress or impairment in social, occupational, or other important areas of functioning[1]. MDD represent a current prevalence of 5% to 10% of the general population according to largescale studies, and up to 20% to 25% for the lifetime period[38, 39], with comparable figures obtained worldwide despite being more prevalent in Western developed societies[40, 41], and it affects women twice as often as men[40, 42]. In most countries, the median age at onset of MDD ranges from 20 to 35 years old[40].

Bipolar disorder (BD)

BD category can be subdivided into bipolar I disorder (one or more manic or mixed episodes, or both manic and mixed episodes and at least one major depressive episode), bipolar II disorder (one or more episodes of major depression and at least one hypomanic episode), cyclothymc disorder and bipolar disorder not otherwise specified. According to DSM-IV-TR[1], a manic episode is defined as a distinct period of abnormally and persistently elevated, expansive, or irritable mood, lasting at least one week, and accompanied by three (or more) of the following symptoms: inflated self-esteem or grandiosity, decreased need for sleep, more talkative than usual or pressure to keep talking, flight of ideas or subjective experience that thoughts are racing, distractibility, increase in goal-directed activity or

psychomotor agitation, and excessive involvement in pleasurable activities that have a high potential for painful consequences. In a manic episode, the mood disturbance is sufficiently severe to cause marked impairment in occupational functioning or in usual social activities or relationships with others, or to necessitate hospitalization to prevent harm to self or others, or there are psychotic features. Conversely, hypomanic episodes are not sufficiently severe to cause pronounced impairment in social or occupational functioning and there are no psychotic features, although having many similar symptoms to manic episodes (lasting at least four days) and being associated with an unequivocal change in functioning that is uncharacteristic of the person when not symptomatic and is observable by others. A mixed episode is characterized by a period of at least one week in which the criteria are met nearly every day for both manic and major depressive episodes. BD has an estimated lifetime prevalence of around 1-4% in Western developed countries[13, 43], accounting approximately for 1% in bipolar I disorder and for 1.1% in bipolar II disorder[44]. BD occurs nearly equally in women and men, having the peak age of onset between age 15 and 24 years[44].

Neurobiology of mood disorders

The idea that emotion and emotional behavior is particularly related with a system of brain structures known as limbic system appeared for the first time in the 30's and 40's[45]. The limbic system includes regions such as the hippocampus and amygdala, the anterior and medial thalamus, the cingulated gyrus, and related control areas in the hypothalamus and brainstem. These limbic areas and their connections with other cortical areas (including the ventromedial prefrontal cortex, the lateral orbital prefrontal cortex, the dorsolateral prefrontal cortex and the anterior cingulated cortex among others) constitute highly complex and interconnected networks

implicated not only in mood regulation but also in learning and contextual memory processes[46] (Figure 3).

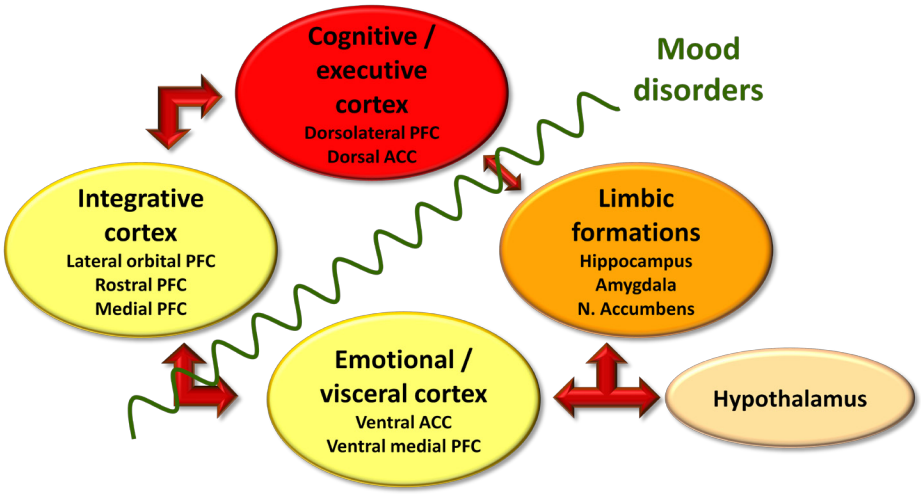


Figure 3. Neuroanatomic circuits involved in the regulation of mood and stress response. In mood disorder patients, disrupted connectivity between limbic and para-limbic areas and rostral integrative prefrontal formations, could result in compromised feedback regulation of limbic activity, causing a hypoactive dorsal cognitive/executive network but active limbic areas which stimulate hypothalamus leading to neuroendocrine dysregulation and sympathetic hyperactivity (adapted from Maletic *et al.*[47]). PFC = prefrontal cortex; ACC = anterior cingulated cortex; N. Accumbens = Nucleus accumbens.

With the use of neuroimaging, neurophatological and neurophysiological techniques, different abnormalities at areas localized in the above-mentioned networks have been identified in mood disorder patients. For example, different studies have found a consistently and significantly reduction in hippocampal volume in MDD and BD patients[48-51], a reduction in grey matter in some areas of the anterior cingulated cortex (ACC) in MDD and BD patients[52-56], a smaller volume of amygdala in unmedicated BD patients[57], and a smaller putamen and caudate nucleus in stratium in MDD patients[58-61], although some of these volume anomalies were only present in some subgroups of mood disorder patients with concrete phenotypes such as the duration of the illness, early-onset or the presence of psychotic

symptoms. Moreover, other studies failed to replicate some of the mentioned findings[62].

Regarding neurochemistry, the increase of extracellular concentrations of different monoamine neurotransmitters in the brain due to effects of antidepressant drugs led to the monoamine hypothesis of depression, which proposes that MD are caused by a deficiency in serotonin and noradrenaline at functionally important receptor sites in the brain[63-66]. Then, as this monoamine hypothesis does not explain all the effects of antidepressants[67], the chemical hypothesis of depression came out, suggesting that MD are produced by a chemical imbalance in the brain caused by structural or functional changes in particular molecules, and that antidepressants function by counteracting these molecular changes[68, 69]. Different studies have found strong evidence of not only disturbances in monoamines but also alternations in other molecules involved in the pathophysiology of MD, such as altered hypothalamic-pituitary-adrenal (HPA) axis and dysfunctions of the extrahypothalamic corticotropine-releasing hormone (CRH) among others[70, 71]. More recently, different studies have pointed to a network hypothesis, which proposes that in MD information processing in particular neural networks does not function properly and that antidepressants drugs and other treatments could function in MD by gradually improving information processing within these networks[67]. This hypothesis is based on the idea that, although chemical neurotransmitters are crucial for the transfer of information between neurons, information in the brain is not stored in a chemical form but processed by the complex interactions of neurons in neural networks, which are constantly being refined through activity-dependent synaptic plasticity to optimally process and store relevant information[72-74]. Thus, both the chemical and network hypotheses are not mutually exclusive, but are complementary. Then, although antidepressants

produce their first effects in monoamines metabolism, the following adaptive changes in the concentrations of those signaling molecules are highly related to the structure of the neural networks, and might be a consequence of the altered information processing rather than its cause. In this scenario, antidepressants can enhance the plasticity of neuronal connections in the hippocampus and cerebral cortex via activation of neurotrophin signaling, where neurotrophic factors such as brain-derived neurotrophic factor (BDNF) could play crucial roles in the selection and stabilization of active synaptic contacts[75-77].

Circadian rhythms and mood disorders

Circadian rhythms are endogenous biological rhythms with a period or cycle length of approximately 24 hours that persist in constant conditions in the absence of environmental input, and their circadian phase reflects where the peak and the trough of the circadian rhythm occur[78]. In mammals, circadian rhythms are controlled by an endogenous biological pacemaker (also called master clock or endogenous oscillator) located in the suprachiasmatic nucleus (SCN) of the anterior hypothalamus[79]. The master clock, in turn, synchronizes circadian oscillators in peripheral tissues and adjusts the rhythmic fluctuation of a broad range of cellular and physiological functions such as body temperature, hormone release, metabolic rate or the sleep/wake cycle[80] (Figure 4).

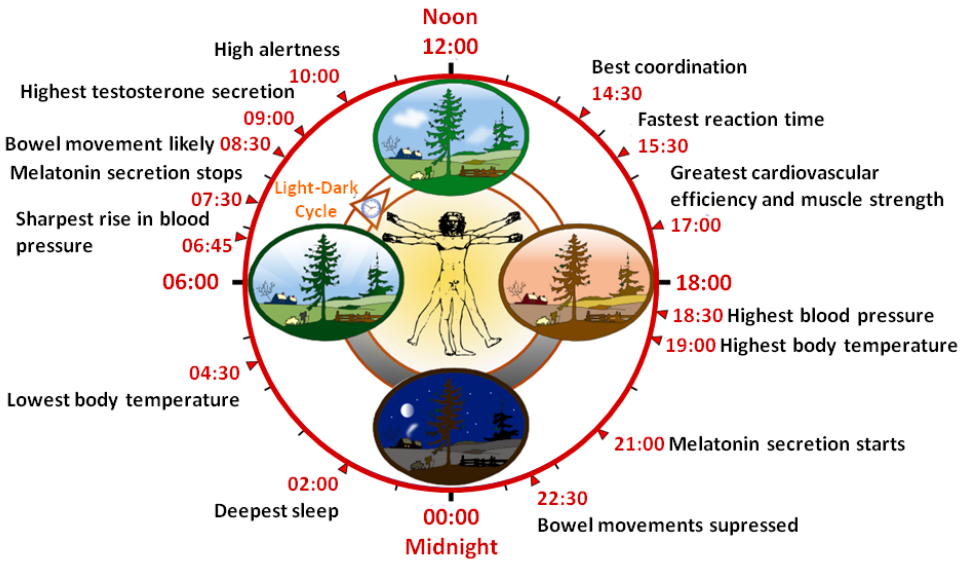


Figure 4. Circadian rhythms in humans. The biological circadian clock affects the daily rhythms of many physiological processes. This figure exemplifies circadian patterns of someone who rises early in the morning, eats lunch about noon, and sleeps at night. Although circadian rhythms tend to be synchronized with cycles of light and dark, other factors such as ambient temperature, meal times, stress, and exercise can influence the timing as well (adapted from Smolensky *et al.*[81]).

Each SCN neuron can independently generate self-sustained circadian rhythms when dissociated form SCN tissue demonstrating its role as the circadian biological pacemaker[82-85]. Nevertheless, circadian clocks are normally set or entrained by periodic environmental cues (called zeitgebers), with the daily light-dark cycle being the most universal and potent entrainment stimulus in mammals, ensuring that expressed rhythms in physiology and behavior are coordinated to the 24 hour length[86]. In mammals, circadian oscillators contain positive and negative elements that form autoregulatory feedback loops, regulating in this way the circadian rhythms of hundreds of clock-controlled genes which allow organisms to anticipate daily changes in the environment[78, 87] (Figure 5).

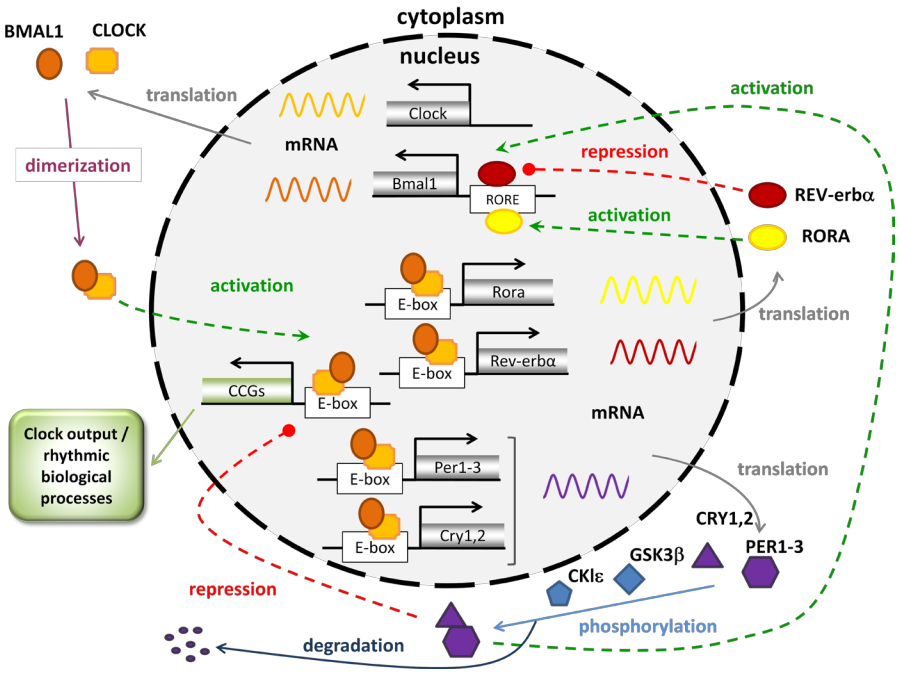


Figure 5. Mammalian circadian-feedback loops regulation in endogenous oscillator. *Clock* is constitutively expressed in the SCN, while peak transcription of *Bmal1* occurs in the middle of the circadian night. CLOCK and BMAL1 heterodimerize in the cytoplasm to form a complex that, following translocation to the nucleus, activates transcription from target genes containing E-box *cis*-regulatory enhancer sequences, such as Per, Cry, Rev-erbα, Rora, and clock-controlled genes. After transcription, translation, and post-translational modifications of PERs and CRYs, they form a heteromultimeric complex that translocates to the nucleus and directly abrogates the transcriptional activity of CLOCK:BMAL1 complex (lowering RNA levels of PER and CRY among others), as well as PER2 positively regulates Bmal1 transcription. REV-ERBα accumulates quickly and inhibits Bmal1 transcription, then RORA, which accumulates more slowly, activates Bmal1 transcription, both acting through RORE. The core clock proteins are post-translationally modified by phosphorylation and ubiquitination to alter their stability, sub-cellular localization or protein-protein interactions. Thus, the mammalian oscillator is composed of interconnected feedback loops that regulate the abundance and activity of transcription factors which, in turn, control the expression of clock-controlled genes in the output pathways from the oscillator, resulting in behavioral and physiological rhythms[78, 80, 86, 88] (adapted from Beckett *et al.*[89]). Abbreviations: BMAL1= ARNTL, aryl hydrocarbon receptor nuclear translocator-like; CLOCK= circadian locomotor output cycles KAPUT; PER1-3= period1-3; CRY1,2= cryptochrome1,2; REV-ERBα= NR1D1, nuclear receptor subfamily 1, group D, member 1; RORA= RAR-related orphan receptor A; CK1ε = CSNK1ε, casein kinase 1ε; GSK3β = glycogen synthase kinase 3β; RORE= retinoic acid-related orphan receptor response elements; CCG= clock-controlled genes.

Mood disorder patients commonly show biological rhythm-related symptoms, such as abnormal sleep/wake, appetite, and social rhythms[90-93]. Indeed, the prevalence of mood disorders such as MDD and BD may be higher in individuals born with an abnormally shifted or arrhythmic clock, leading to hypothesize that abnormalities in the molecular clock could underlie the development of these disorders[88]. There is strong evidence supporting a role of biological clocks in mood disorders, such as: marked diurnal mood variation (mood usually worse in the morning) in patients undergoing major depressive episodes[94, 95], an abnormal elevated nocturnal body temperature with a phase advance in the overall 24-hour pattern in depressed patients[96-98], an overall increased of cortisol secretion with a phase advance of the cortisol circadian rhythm occurring in depressed patients[99], lower blood melatonin concentration and a phase advance or a trend toward a phase advance of the melatonin circadian rhythm in some MDD patients[100, 101], and subjective reports of sleep-wake cycle alterations by mood disorder patients, as well as the presence of sleep architecture abnormalities[102] (some of these circadian rhythms-related phenotypes often present in mood disorder patients are further described in Box 2). In addition, some of the major neurotransmitters from monoaminergic pathways implicated in mood regulation, including serotonin, norepinephrine and dopamine, have a circadian rhythm in their levels, release, and synthesis-related enzymes[103-108]. Interestingly, the previously mentioned manifestations of abnormal circadian function return to normality with pharmacological antidepressant or mood stabilizer treatments and patient recovery[88]. Accordingly, different drugs used in the treatment of MD act on circadian rhythms regulation, as for example: lithium, a mood stabilizer used in the treatment of BD and as an augmentation agent of antidepressant treatment in MDD, which is known to alter circadian period possibly through inhibiton of GSK3β (a modifier of

multiple members of the molecular clock)[109-113], fluoxetine, a broadly used selective serotonin reuptake inhibitor antidepressant, which produces a phase advance in the firing of SCN neurons in rat slice culture[114], or the antidepressant agomelatine, a potent agonist of the melatonin receptors and an antagonist of some serotonin receptors, which resynchronizes circadian rhythms in body temperature, cortisol, and other hormones in animal models and in humans[115, 116]. Moreover, other non-pharmacological treatments acting by shifting or resetting the circadian clock, including sleep deprivation, light therapy, and interpersonal and social rhythm therapy, have shown to be effective in the treatment of MD[117-119]. Finally, one study showed that mice carrying an inactivating mutation of the *Clock* gene display an overall behavioral profile resembling human mania, and that chronic administration of lithium returns many of the behavioral responses to the wild-type levels[120].

Box 2. Circadian rhtythms-related phenotypes in mood disorders.

Seasonal Affective Disorder (SAD)

SAD affects approximately 1-3%[121] of the population in temperate climates, being a specifier of a seasonal pattern that can be applied to major depressive episodes (MDE) in BD-I, BD-II, or recurrent MDD, and it is described and diagnose regarding the following DSM-IV-TR criteria[1]:

A. There has been a regular temporal relationship between the onset of MDE and a particular moment of the year.

B. Full remissions (or a change from depression to mania or hypomania) also occur at characteristic time of the year.

C. In the last 2 years, two MDE have occurred that demonstrate the temporal seasonal relationships defined in Criteria A and B, and no non-seasonal MDE have occurred during that same period.

D. Seasonal MDE (as described above) substantially outnumber the non-seasonal MDE that may have occurred over the individual's lifetime.

The presence of SAD can be assessed with the Seasonal Pattern Assessment Questionnaire (SPAQ)[122].

Insomnia

MD patients exhibit higher rates of sleep disturbance than the general population, even during periods of remission, with 50-90% of MD patients complaining about impairment of sleep quality[123]. Insomnia can be assessed in MD patients with items 4, 5, and 6 of the HAM-D scale (Hamilton rating scale for depression)[124, 125], being possible to differentiate three different types of insomnia:

- **Early insomnia:** difficulty in falling asleep at the beginning of the night.
- **Middle insomnia:** frequent awakenings during the middle of the night, difficulty maintaining sleep.
- **Late insomnia:** early morning awakening.

Chronotype

In circadian rhythms, an entrained phase is described by chronotype, a term that reflects the preferred timing of activity and rest during the day. Chronotype can be assessed with Horne-Östberg Morningness-Eveningness Questionnaire (MEQ)[126, 127]. Individuals with a relatively early circadian phase are morning types (morningness chronotype), *versus* those with a relatively late circadian phase being evening types (eveningness chronotype). Both morningness and eveningness indivudals are more likely to develop a MD[128].

1.1.2. Genetic basis of psychiatric disorders

Psychiatric disorders are a wide range of complex diseases, also termed multifactorial diseases, which are defined as any illness that does not exhibit classic Mendelian recessive or dominant inheritance attributable to a single gene *locus*, but are caused by multiple genes interacting with each other and with environmental factors to create a gradient of genetic susceptibility to disease[129, 130]. Some examples of complex diseases, a part from psychiatric disorders, include such high prevalent illnesses as hypertension, obesity, type 1 and 2 diabetes, heart disease, multiple sclerosis, arthritis, asthma, cancer and many more metabolic disorders, autoimmune diseases and complex degenerative processes, among others. As it has been previously mentioned, among the scientific community there is a great interest in the study of psychiatric disorders, as well as of complex diseases in general, as they are very common worldwide and represent a global public health problem in developed and developing countries.

The genetic component of psychiatric disorders was already recognized in the beginning of the twentieth century by Emil Kraepelin, who is considered the founder of psychiatric genetics. Since then, the genetic basis of psychiatric disorders has been mainly established by means of family, twin and adoption studies[131], confirming work that started in the 1930s[132]. The basic assumption of these familial aggregation studies is that if a disorder is caused by genetic factors, individuals who are genetically related should share similar risks for the disorder. Family studies aim to determine whether a disorder of interest aggregates in families, comparing the prevalence of the disorder among first-degree relatives of affected probands (cases) to the prevalence in the population or among relatives of unaffected probands (controls). When there is a higher risk among relatives of affected proband indicates the existence of familial aggregation of the disorder, but this can be

due to genetics or environment, as family members usually share both types of influences. Twin and adoption studies are used to separate the effect of genetic background from shared environmental factors. Twin studies compare the concordance rates between monozygotic (MZ) twins (who are genetically identical) and dizygotic (DZ) twins (who share on average half of their genes). Shared environmental influences on both MZ and DZ twins are assumed to be the same, thus, significantly higher concordance rates in MZ twins reflect the genetic influence. Adoption studies examine the shared environment of non-genetically related family members and the shared genetics of family members who have different family environments. If genes influence the risk of a disorder, genetically related family members should resemble each other more than do adoptive (environmentally related) family members. By means of adoption and twin studies it can be estimated the heritability, which indicates the proportion of phenotypic variation in a population that is attributable to genetic variation among individuals[133]. In this way, during the last century heritability rates have been estimated to range from 33-45% in major depressive disorder to 70-85% in schizophrenia[134, 135]. Table 1 summarizes estimations of heritability, morbidity risk in first-degree relatives of patients with psychiatric disorders, and concordances in twin studies found in the main psychiatric disorders studied in this thesis.

Table 1. Psychiatric disorders: heritability estimations, family and twin studies.

Psychiatric disorders classification		Heritability (%)	Morbidity risk in first-degree relatives (%)		Twin concordance (%)		Ref
			Cases	Controls	MZ	DZ	
Anxiety Disorders	OCD	60-70	2.6-23	2.4-5.2	50-80	20-40	136-143
	PD	30-40	8-18	0.7-4.2	30-70	0-17	139, 144-155
Mood Disorders	MDD	33-45	15-25	5-10	30-50	15-25	156-164
	BD	65-85	3-15	0.5-1.8	44-62	4-10	156, 157, 163, 165-170
Schizophrenia		70-85	2-9	0-1	40-50	5-14	170-178
Eating Disorders	AN	55-70	2.2-6.1	0-0.3	25-55	0-13	179-186
	BN	55-60	4-9.6	0.9-3.5	23-33	0-11	179, 180, 183, 187-189

Abbreviations: OCD, obsessive-compulsive disorder; PD, panic disorder; MDD, major depressive disorder; BD, bipolar disorder; AN, anorexia nervosa; BN, bulimia nervosa; MZ, monozygotic; DZ, dizygotic.

Although the genetic contribution to psychiatric disorders has been broadly demonstrated by means of familial aggregation studies, the genetic basis of psychiatric disorders remains largely unknown. Throughout the years great efforts have been made to comprehensively understand the origin of psychiatric disorders and, more concretely, to unravel the genetic factors playing a role in their etiopathology, pursuing potential targets for effective treatment, screening and prevention. Nevertheless, some of the main characteristics given their nature of complex diseases complicate the study of their genetic basis.

First, complex diseases follow a polygenic inheritance, in which a number of genotypes or mutations at different loci may be required to develop the pathological condition. Consequently, low penetrance is also one of their principal characteristics, meaning that the genotype at a given *locus* may

influence the probability of developing a disease, but not fully determine the outcome. Accordingly, two different phenomena can be present in complex diseases: incomplete penetrance (one can inherit a predisposition allele but may not manifest the disease) and phenocopy (one carrying no predisposing allele might get the disease as a result of environment or random causes)[190]. Hence, a specific genotype at a concrete *locus* is not necessary neither sufficient, albeit having a possible contribution, to develop a complex disease.

Second, genetic heterogeneity also makes harder the gene mapping of these diseases, as mutations in any of a number of genes or *loci* may result in identical phenotypes, conferring disease susceptibility independently of each other[191]. This complexity is indeed enhanced by epistasis or gene interaction, in which the possession of a certain mutation or genotype interferes with the phenotypic influence of other ones, in a way that their combined effect on phenotype could not have been predicted as the sum of their separate effects[192].

In addition, other non-genetic factors play a major role in the liability to complex diseases, such as influence on gene products and specific phenotypes by environmental and epigenetic factors, which may include infection, diet, environmental insult, level of exercise, stress and, moreover, developmental and time-dependent expression of genes. In this context, interactions between genes and environment may exist, causing deleterious effects of genes only in the presence of a particular environmental stimulus[191].

Finally, the genetic study of complex traits is further complicated by additional models of non-classical Mendelian genetic inheritance, namely:

mitochondrial inheritance, in which mitochondrial genome is maternally inherited and each meiotic transmission may involve selection from a potentially mixed population of mutant and normal organelles, imprinting, accounting for differential expression of the paternal and maternal copies of a gene, uniparental disomy, occurring when inheriting two copies or a part of a chromosome from one parent and no copies from the other one, mosaicism, when two or more genetically different cell lines deriving from a single zygote coexist in one individual, and genetic anticipation, an earlier age at onset and increased severity in successive generations of a pedigree due to the expansion of trinucleotide repeats[190].

Currently, there are three general models proposed to explain the genetic basis of a complex disease. The one that has been more widely accepted for a lot of years is the common disease-common variant model[193, 194], which postulates that genetic factors underlying common diseases are common polymorphisms each of which with a moderate contribution, and generally explaining no more than 5% of disease susceptibility. In this scenario, single nucleotide polymorphisms (SNPs) were thought to be the most prevalent form of interindividual genetic variation and also the most explored by means of association studies in order to find common variants underlying this genetic susceptibility in complex disorders[195]. Although some common variants have been identified and replicated in different studies for some complex diseases (such as type 2 diabetes)[196-200], recent whole genome association studies have shown that is not always possible to find common variants associated with all common disorders and, moreover, some of the results found cannot be confirmed by different studies (this would be the case for some psychiatric disorders, including bipolar disorder and schizophrenia)[196, 201-207]. Hence, alternative models have been proposed and discussed, as the rare alleles of major effects[208, 209], which supports that

common diseases are highly heterogeneous with respect to their etiology, and that rare variants with frequencies lower than 1% can promote disease. The recent finding of rare copy number variants enriched in schizophrenic and autistic patients compared to control individuals is one example giving strong evidence for this model[210-212]. Finally, the infinitesimal model holds that hundreds or thousands of genetic variants with a relative risk lower than 1.2 are likely to contribute to common disease, explaining only a fraction of a percent of susceptibility. Overall, these three models are neither mutually exclusive and possibly nor sufficiently each of them to individually explain the genetic etiology of complex diseases, which is likely to be based on a combination of multiple rare and common susceptibility[213] (Figure 6).

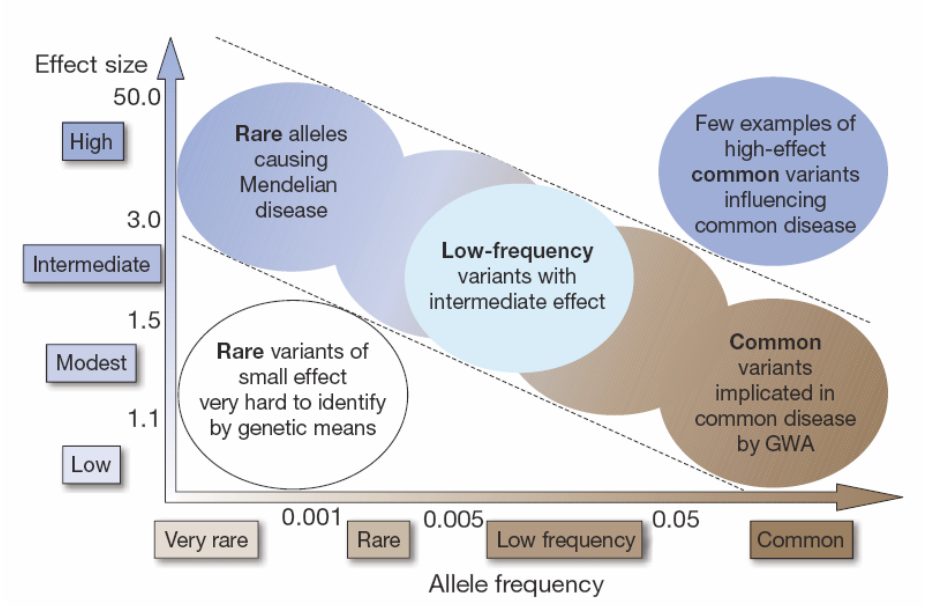


Figure 6. Identification of genetic variants in disease susceptibility depending on risk allele frequency and genetic effect (odds ratio). Nowadays, most genetic studies of complex diseases focus on identification of risk variants with characteristics shown within diagonal dotted lines (from Manolio *et al.*[214]).

1.1.3. Conventional genetic approaches

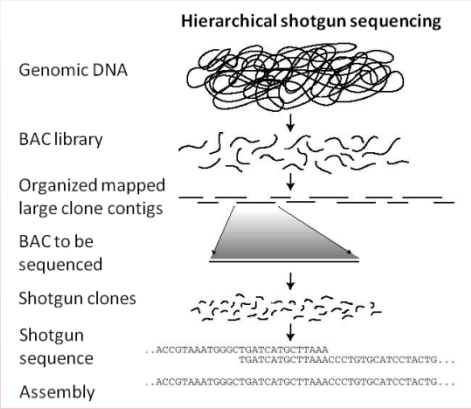
Throughout the years, different methodological approaches have been used to unravel the genetic components known to play a role in the susceptibility to psychiatric disorders and, in general, to complex diseases. Most of these genetic approaches take great advantage of the availability of different genetic markers, which account for genomic variability present in the human genome. In the late 70's, single nucleotide variants were first reported in the *Hpa*I restriction site that lies downstream of the β-globin gene[215]. Since then, other DNA markers have been described and used in the genetic study of complex diseases to assess individual genetic profiles and perform human genome mapping, including: restriction fragment length polymorphism (RFLP)[216, 217], minisatellites[218, 219], variable number of tandem repeats (VNTRs)[220], and microsatellites, also named short sequence repeat (SSR) variants or short tandem repeats (STR)[221-228]. The completion of the haploid human genome sequence, simultaneously by two different projects[229, 230], represented a remarkable step further in the knowledge of human genome variation and in the genetic research of human diseases (see Box 3 for further detailed information regarding the two projects leading to the first drafts of the human genome sequence). Some years later, the first diploid human genome sequence was released[231], and since then, several individual genomes have been sequenced by means of new and improved high-throughput DNA sequencing technologies[232-243]. In fact, in less than ten years time since the release of the first draft of the human genome, the sequencing strategies have evolved dramatically, and nowadays the so-called "next-generation" sequencing instruments have the ability to produce fast and inexpensive enormous volume of data, thus, largely facilitating the resequencing of human genomes to enhance a better understanding of how genetic differences affect health and disease[244]. These technological advances have enabled such ambitious projects as the 1000 Genomes

Project, an international collaboration for resequencing the genomes of approximately 2,000 people from different worldwide populations to describe most of human variation and diversity[245, 246].

Box 3. Human Genome Sequence

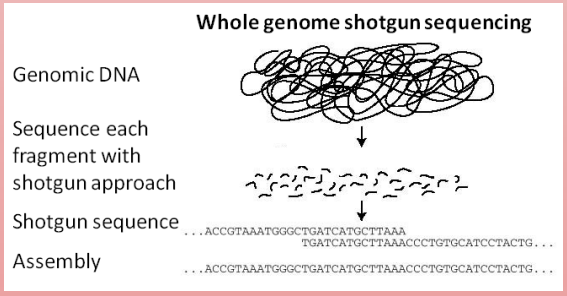
In February 2001, two draft versions of human genome sequences were published the same week in Science and Nature: one was a private initiative from Celera Genomics leaded by Craig Venter, and the other was from the Human Genome Project supported by public funds under the direction of Francis Collins. Both publications consisted in a haploid version of the human genome sequence without annotations of genetic variants.

Human Genome Project (HGP)[229]



The HGP was a publicly funded project started on 1990, being an international collaboration between a number of sequencing centers in the United States, Europe, and Asia. The reference genome was deduced from a collection of DNAs from anonymous individuals, using the strategy of hierarchical shotgun sequencing. In this approach, genomic DNA is cut into pieces of about 100-200 kb and cloned into bacterial or yeast artificial chromosomes (BACs and YACs, respectively). The genomic DNA fragments represented in the library are then organized into a physical map and fragmented into smaller pieces (500 bp), with their posterior selection and sequencing by random shotgun strategy. Finally, the clone sequences are assembled to reconstruct the sequence of the genome.

Celera Discovery System[230]



In 1998, Craig Venter started a private project at his firm Celera Genomics, causing an evident competition between both the public and private projects. DNA from five different individuals were used for the whole genome shotgun sequencing method, which consists in randomly shearing genomic DNA into small pieces (~2 Kb) which are cloned into plasmids, sequenced, and then aligned and assembled into human reference genome sequence. The Celera assembly, however, benefited from data produced in the HGP in its human genome assembly.

Hence, apart from the more conventional genetic markers previously mentioned, the development of new computational and experimental strategies has allowed higher resolution analyses of structural genetic variation known since 1980[247, 248], accounting for deletions, insertions, duplications and complex rearrangements of genomic regions in 1 Kb or larger, called copy number variants (CNVs). Nowadays, CNVs are under the scope of several genetic studies of human diseases, including psychiatric disorders[249], thus, they will be further discussed in more detailed in their own section of the introduction.

Nevertheless, although all the advancements in the knowledge of human genome variation, by now, single nucleotide polymorphisms (SNPs) have been the most widely used DNA markers in the genetic study of complex disorders, constituting the major source of inter-individual genetic and phenotypic variation. SNPs are DNA sequence variation occurring in a single nucleotide, and can be classified as synonymous (the same polypeptide sequence is produced) and nonsynonymous (the SNP modify the polypeptide sequence of the coding protein). The use of SNPs in these genetic studies has been largely facilitated with the outcome of the HapMap Project, which in 2005 genotyped one million SNPs in its first phase[250] (see Box 4 for more detailed information regarding the International HapMap Project).

Overall, the study of genetic and genomic variation of the human genome is essential in the genetic research of human diseases. In psychiatric genetics, however, the phenotypic classification of the disorder is an additional difficulty in this research. As it has been previously mentioned, the nosology of psychiatric disorders is mainly based on clinical symptoms, and it does not necessarily reflect the underlying genetic substrates and pathological pathways[251]. Moreover, psychiatric diseases have a high etiological

heterogeneity and, consequently, patients displaying the same clinical symptoms might in fact belong to different etiological subgroups (for example, psychosis can be part of the diagnosis of schizophrenia, bipolar disorder and psychotic depression), as well as, comorbidities, found in high percentages in psychiatric patients, further complicate the diagnostic classification[12]. The use of endophenotypes, or intermediate phenotypes, in complex diseases, and concretely in psychiatric disorders, arose as an effort to close the gap between neurobiological pathways and the observed clinical symptoms. An endophenotype is a biological marker that is associated with the illness in the relevant population, state-independent (namely, present both during periods of illness and wellness), heritable, present in unaffected family members more frequently than in general population, and that it cosegregates with the illness within families[252, 253]. Initially, endophenotypes were only applied to psychological processes, but this was soon extended to biological mechanisms, and now they include cognitive, neuropsychological, biochemical, endocrinological, neurophysiological, neuroanatomical, and neurofunctional measures[254]. Furthermore, subphenotypes are also been used in psychiatric disorders with the aim to reduce the heterogeneity inherent in sampling based on a diagnostic category, thus, identifying more homogenous subgroups of complex syndromes which will improve chances of identifying susceptibility genes (for example, early onset of major depression or bulimia nervosa with self-induced vomiting)[255, 256].

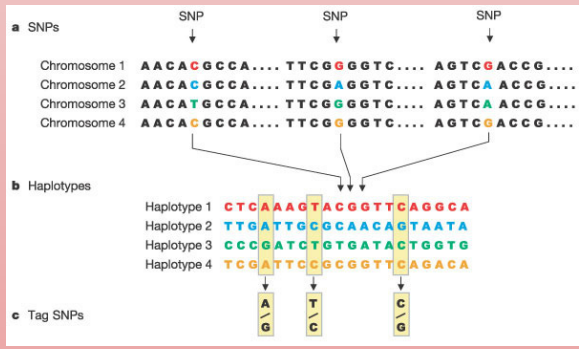
Box 4. The International HapMap Project.

The International HapMap Project (www.hapmap.org)[257] started in October 2002 with the aim to identify and catalog genetic similarities and differences in human beings through obtaining a complete map of SNPs in the whole genome in different populations around the world. The project is a collaboration among scientists and funding agencies from Japan, the United Kingdom, Canada, China, Nigeria, and the United States, and all the information generated is released into public domain through the database dbSNP (http://www.ncbi.nlm.nih.gov/projects/SNP/).

HapMap phases and populations

In 2005, more than one million SNPs were genotyped in the Phase I HapMap[250] in 270 samples coming from four populations: 30 trios (two parents and an adult child) from Yoruba people of Ibadan, Nigeria (YRI); 30 trios of U.S. residents of northern and western European ancestry (CEU); 45 unrelated individuals from Tokyo, Japan (JPT); and 45 unrelated Han Chinese individuals from Beijing, China (CHB). In 2007, the Phase II dataset was published[258], adding over 2.1 million SNPs to the original map in the same individuals. Finally, the Phase III dataset was released in 2009, genotyping and sequencing additional samples from the HapMap populations and samples from seven additional populations: Maasai in Kinyawa, Kenya; Luhya in Webuye, Kenya; Chinese in metropolitan Denver, CO, USA; Gujarati Indians in Houston, TX, USA; Tuscans in Italy; African ancestry in the Southwest USA; and Mexican ancestry in Los Angeles, CA, USA.

TagSNPs



The construction of the HapMap occurs in three steps:

a) SNPs are identified in DNA samples from multiple individuals.

b) Adjacent SNPs that are inherited together are compiled into haplotypes (a haplotype is a combination of consecutive alleles at multiple *loci* that are transmitted together on the same chromosome).

c) TagSNPs (representative SNPs in a region of the genome with high linkage disequilibrium, highly correlated with the nearby SNPs) within haplotypes are identified that uniquely identify those haplotypes. By genotyping the three tag SNPs shown in this figure, researchers can identify which of the four haplotypes shown here are present in each individual *(from www.hapmap.org).*

Following, the main methodological approaches used to find susceptibility genes for psychiatric disorders, and for complex diseases in general, are described.

Genetic linkage studies

Linkage occurs when gene loci are close, together on the same chromosome and fail to follow Mendel's law of independent assortment, resulting in their being inherited together: a DNA marker close to an allele on the same chromosome will tend to be inherited with that allele within a family. If a gene influences a given characteristic, relatives who share the DNA marker will be more similar for the characteristic than relatives who do not. Based on these phenomena, genetic linkage analyses are a hypothesis-neutral search that tests cosegragation of genetic markers and a phenotype of interest, being used to identify regions of the genome that contain genes that predispose to disease. Linkage analysis methods can be applied to both major gene disorders (parametric linkage), analyzing the cosegragation of genetic loci in pedigress through estimating the recombination fraction (genetic model for the disease must be specified), and multifactorial diseases (model-free or non-parametric linkage), testing the excess sharing of alleles between affected relatives, irrespective of the mode of inheritance[259, 260].

Although in the 80's and 90's there was a great expectation regarding the possible identification of single genes involved in psychiatric disorders through linkage studies, the linkage era for psychiatric disorders finished at 2005 with no single locus unequivocally replicated across multiple independent samples[12]. Further meta-analyses of linkage studies in psychiatric samples to gain power neither found any consistent result, with only one genome-wide significant linkage peak in schizophrenia[261], two for bipolar disorder on 6q and 8q[262], an a peak on chromosome 9 on obsessive-

compulsive disorder[263, 264], being not confirmed by a later and much larger study[265]. However, the linkage results for psychiatric disorders have been useful as a start point in positional candidate gene association studies and pathways analyses.

Positional cloning

Positional cloning aims to locate the responsible gene of a trait solely on the basis of map position, assuming no functional information, correlating in this way a sequence that is significantly different in cases and controls with a phenotype. Preliminary localization can be defined using techniques such as cytogenetic variation or linkage analysis, and positional cloning is then used to narrow the region until the gene and its mutations are found. There is strong evidence of the success of positional cloning in isolating the genetic effects in single gene disorders, with such clear examples as the identification of disease genes for Duchenne muscular dystrophy, Huntington's disease and cystic fibrosis, among others[266, 267]. However, it is less powerful for complex disorders and, actually, it is not widely used in common psychiatric disorders, as it can only lead to gene identification in rare families with unique, essentially Mendialian forms of mental illness[12].

Genetic association studies

Genetic association studies test for a possible association between one or more common genetic polymorphism (with a minor allele frequency, MAF, higher than 5%) and a trait, which can be a discrete or quantitative phenotype, for example, testing if an allele or genotype frequency differs significantly between a sample of cases compared with a control sample. Since 1996, genetic association studies are known to be more powerful than linkage studies when testing genetic variants having only small individual effect on risk, as it happens with complex diseases[268]. In association studies

one or more markers can be tested, accounting for a direct association when the tested polymorphisms are themselves putative causal variants, or indirect association, when the polymorphism is a surrogate for the causal locus[269]. The latter would be the case of association studies interrogating for TagSNPs. Both direct and indirect association studies have benefit from the large catalogue of SNPs described in different worldwide populations in the HapMap project[250, 258]. Nonetheless, when testing several markers in the same association study, it is important to bear in mind that this multiple testing introduces statistical artefacts leading to possible false-positive results, thus, the raw p-value need to be corrected for this[259]. Another potential problem related with association studies is population stratification, which may account for the lack of replication in different studies for psychiatric disorders, as well as for other complex diseases. Population stratification occurs when the sample studied contains several genetically distinct subsets, and has different frequencies of the disease as well as different frequencies of the marker allele, which can lead to confounding associations, generating false findings or obscuring true causal associations. There are different ways to avoid this difficulty, as for example matching cases and controls for ethnicity and geographical region, matching them by family, using genetic markers (such as ancestry informative markers) for testing population substructure, or applying the genomic control to results, which controls the false positive rate by increasing the threshold required for statistical significance[269].

Genetic association analyses can be family-based tests or population-based analyses. The former avoid the problem of confounding by population structure but they are not always very powerful and difficult to undertake on a sufficiently large scale to detect genetic association reliably, while the latter are normally more powerful due to the possibility of selecting large

sample size from whole population. Moreover, association studies can also be classified into candidate gene approach or genome-wide association studies (GWAS), depending on the existence or not of a previous hypothesis and the number of polymorphisms tested.

Population-based analyses

Population-based analyses can present a case-control or cohort design, among others. In a cohort design, a subset of individuals from a population are selected, genotyped and followed for disease incidence during a specified period of time. As cohort studies are expensive to follow-up and entail some difficulties regarding issues with drop-out, the normally used population-based strategy are the case-control analyses, in which the genotype of the tested variants are determined in a number of affected (cases) and unaffected (controls) individuals. Then, in this design, the allele or genotype frequencies at the site of interest are compared in samples of cases and controls, and a higher frequency in cases is taken as evidence that allele or genotype is associated with increased risk of disease or the studied phenotype, with the usual conclusion that the associated polymorphism affects the studied trait directly or is a marker for some nearby genetic variant which is the causal one[195, 269].

Family-based tests

To circumvent the problems with unmatched control groups that can arise in population-based analyses, there are family-based methods (including case-parent triad, case-parent-grandparent and pedigrees designs) with internal controls to detect allelic association, which can be analyzed with different statistical methods, such as the transmission disequilibrium test (TDT). It depends on at least one parent of each affected subject being heterozygous at the marker allele, and compares the frequency of affected offspring to

whom a particular allele is transmitted with the frequency of those not receiving that allele. There is evidence of association with the tested trait when the alternative genotypes are unequally transmitted to the probands[259].

Candidate gene approach

The genetic association studies based on candidate genes approach account for *a priori* knowledge of the possible role of the selected genes in the etiology of the studied phenotypes. They include biological candidate gene approach, where candidate genes are selected on the basis of knowledge of the pathophysiology underlying the studied disease or in the effects of treatment drugs on protein targets, or positional candidate approach, which rely on previously identified linkage peak to select the candidate genes[270].

Several genes have been found to be associated with psychiatric disorders or related phenotypes by means of candidate gene association studies. In the case of the biological gene approach, for example, a functional null allele of aldehyde dehydrogenase 2 (ALDH2) has been demonstrated to protect from risk of alcoholism[271], and a promoter variant in the serotonin transporter (*SLC6A4*), which is the target of a major class of antidepressants, is an established risk factor for depression[272, 273]. Moreover, association studies based on positional candidate gene approach have also reported strong evidence for different genes associated with other conditions, including the gamma-aminobutyric acid receptor alpha 2 subunit (*GABRA2*) gene as one risk factor for alcoholism[274-276], and the involvement of D-amino acid oxidase activator (*DAOA*) in the susceptibility to bipolar disorder[277, 278], among others.

Genome wide association studies (GWAS)

GWAS consist in exploring genetic variation across the whole genome designed to identify genetic association with the disease or trait of interest. Samples genotyped can account for case-control individuals or family-based populations, and they are normally limited to a single ancestry to avoid confounding effects (as some DNA markers have markedly different frequencies across populations). P value correction for multiple testing is an important consideration to take into account in the statistical analyses of GWAS[279]. This approach allows the detection of new susceptibility variants as it uses markers throughout the genome, in comparison to other methodologies testing only candidate genes[270]. Since few years ago, GWAS have become a real possibility due to the advancements in the human genome sequencing, the development of the HapMap Project, and the improvement of the high-throughput genotyping technologies[250, 258, 280-282].

GWAS findings are recently emerging for psychiatric disorders. In schizophrenia, for example, two large studies have found rare deletions significantly associated with the disorder on chromosome 1q21.1 and 15q13.3, as well as the more established 22q11 deletion[211, 283], and other studies reported a significant increase in schizorphrenia case subjects in their total genomewide count of rare long copy number variants (CNVs)[212, 283, 284]. In relation to CNVs and autism, different studies have also found association with a deletion on chromosome 16p11.2[285-287] and, in addition, other studies have shown an excess of rare CNVs in autistic patients compared to control individuals[210, 285, 288]. Regarding SNPs genotypes, none of the GWAS performed in schizophrenia patients reported genomewide significant results[201, 203, 289], finding the same scenario of no evidence of strong and replicable associations when performing GWAS interrogating other psychiatric disorders, such as major depressive disorder[290-293] and bipolar

disorder[196, 202, 204-207]. Nevertheless, when combining data of three GWAS for bipolar disorder, two genes were found to be significantly associated with the disorder: *CACNA1C* (encoding for the alpha 1C subunit of the L-type voltage-gated calcium channel) and ankyrin-G (*ANK3*), both being downregulated in mouse brain in response to lithium[196, 202, 205, 294, 295]. Thus, in summary, by the moment GWAS have not clearly elucidated the genetic basis of psychiatric disorders, although the study of larger sample might bring new light in the near future[12].

1.1.3.1. Candidate genes for mood disorders

Several candidate genes have been found in psychiatric disorders taking advantage of the previously reviewed genetic methodologies and other biological and more functional strategies, as well as from considering drug efficacy in their treatment. The most significant and abundant genes involved in psychiatric disorders pertained to the neurotransmitter system in the central nervous system (CNS, including mainly monoamines, acetylcholine, aminoacids, peptides, gases and single ions), are related to neurodevelopmental and plasticity processes of the CNS (such as neurotrophic factors and hormones), or are regulatory elements involved in the complex networks of gene regulation in the CNS at transcriptional or translational levels, such as microRNAS[296-302].

Regarding candidate genes in mood disorders, no genes of major effect have been found with the use of linkage studies, as previously mentioned. Nevertheless, individual studies have repeatedly implicated some regions in MDD or BD, although usually not consistent enough to be highlighted by meta-analyses[12]. The strongest signals derived from linkage studies in MD are summarized in Table 2.

Table 2. Summary of candidate regions from linkage studies in mood disorders.

Region	Disorder	Subphenotype	Comments	Ref
12q22-24	MDD	---	Modest linkage signal	303
	MDD	males	Significant linkage peak	304
	BD	---	Genome-wide significance	305, 306
15q25-q26	MDD	Early onset	Suggestive evidence of linkage	307, 308
8p22-p21.3, 17p12,	MDD	Early onset	Suggestive linkage signals (including *SLC6A4* at the 17p12)	308
2p14, 8p23.3, 17p12	MDD	---	Suggestive linkage signals (including *SLC6A4* at the 17p12)	309
2q	MDD	Early onset females	Signal peak close to *CREB1* gene	310
13q, 22q	BD	---	First meta-analyses of BD genome scans, genome-wide significance	311
9p22.3-22.1, 10q11.21-22.1, 14q24.1-32.12, regions of 18	BD	---	Meta-analysis with only modest singnificant results	312
6q21-q25	BD	---	Genome-wide significant or suggestive evidence for linkage	305, 313-315
6q21-q25, 8q	BD	---	Meta-analysis, genome-wide significance	262

Abbreviations: MD, mood disorder; MDD, major depressive disorder; BD, bipolar disorder; *CREB1,* cAMP responsive element binding protein 1; *SLC6A4,* solute carrier family 6 (neurotransmitter transporter, serotonin), member 4.

Moreover, several association studies have been performed interrogating different candidate genes in MD, such as monoamingergic neurotransmitters. For example, serotoninergic pathways are thought to play an important role in the etiology of MD, based on strong evidence of the known role of serotonin (5-HT) in many physiological and behavioral processes (including mood, appetite, sleep, activity, suicide, sexual behavior and cognition, all of them affected in depression), the diminished serotonergic function involved in the onset and course of depression (decreased plasma tryptophan levels, decreased levels of 5-HT metabolites in cerebrospinal fluid -CSF-, reduction in pre- and post-synaptically 5-HT

receptors), the mood-lowering effect of tryptophan depletion, and the efficacy of serotonin-modulating antidepressants[316]. Consequently, several association studies on MD have focused on genes involved in synthesis, release, reuptake or metabolism of 5-HT. Apart from 5-HT, other monoaminergic neurotransmitters are suggested to play a role in the pathophsyiology of MD, like catecholamines, including dopamine (DA) and norepinephrine or noradrenaline (NA). The study of DA has been mainly focused on BD, as psychostimulants increasing dopamine activity in the brain produce effects similar to mania, and the effects of these can be attenuated by drugs that are effective in mania, such as lithium and antipsychothics. In accordance, the theoretical bases that support dopamine excess in mania, apply equally to a dopamine deficiency in depression, being mainly supported by pharmacological manipulations[317]. Moreover, the CSF levels of the homovanillic acid (HVA), a metabolite of DA, are usually elevated during mania[318-321]. The strongest evidence of NA involvement in MD is based on the efficacy of serotonin–norepinephrine reuptake inhibitors (SNRIs) as antidepressant medication[322, 323]. Thus, different molecules form the catecholamines metabolism have been targeted in genetic studies of MD, such as different dopamine receptors (DRD1, DRD2, DRD3, DRD4, and DRD5), tyrosine hydroxylase (TH), the rate-limiting enzyme of catecholamines synthesis, catechol O-methyl transferase (COMT), involved in the breakdown pathways of catecholamines, monoamine oxidase (MAO), which catalyzes the oxidation of monoamines, and dopamine transporter (SLC6A3), which reuptake DA in the synapses.

During the last years, it has become also apparent that the pathophysiology of MD could be related with impairment of neuronal plasticity, and that depressed patients could display an inability to adapt to environment and may be more vulnerable to challenging experiences[324-326]. Indeed, the brain

of depressed subjects show structural abnormalities and reduced expression of several markers of neuronal function and viability, among which neurotrophic factors, in particular brain-derived neurotrophic factor (*BDNF*), seem to be playing a pivotal role[327]. In addition, *BDNF* provides trophic support to different monoamines widely implicated in the development and evolution of MD[328-331]. Different studies have shown decreased plasma levels of *BDNF* in BD, maniac and depressed patients[332-334], which can be reversed by antidepressant treatment[335-337], an increase in serum BDNF levels in resistant depressed patients after electroconvulsive shock therapy (ECT)[338, 339], reduced BDNF protein levels in the hippocampus of postmortem brains of suicide victims[340-342], and BDNF upregulation in the hippocampus and prefrontal cortex of patients treated with antidepressants[343].

Table 3 briefly summarizes some of the most interesting and widely studied candidate genes interrogated by means of association studies in MD.

Table 3. Summary of candidate genes from association studies in mood disorders.

Gene	Disorder	Comments	Ref
SLC6A4	MD	Some studies found statistical significant association with MD, while others reported negative results.	344, 345
	MDD	Associated with family history of depression and MDD in different studies, including a meta-analysis. Nevertheless, negative results have also been reported. Different studies also point to an interaction of *SLC6A4* gene and environment in the development of MDD, although other studies do not support this hypothesis.	272, 346-355
	BD	Statistical significant association in a meta-analysis.	350
HTR1A	MDD	Significant association with a genotype which may reduce serotonergic neurotransmission, predisposing to depression and suicide.	356
TPH2	MDD	One study reported SNP and haplotype associations, and another found a loss-of-function polymorphism associated to MDD, although no subsequent studies could identify the same mutation in a large number of MDD patients.	357-363
DRD1	BD	A SNP and a haplotype have been associated to BD, but also negative results have been reported.	364-368
DRD2	BD	Association between BD and *DRD2* in a large European study, and also in Chinese population but not in Caucasians. Negative results of association in a clear majority of genetic studies.	365, 369-384
DRD3	BD	Suggestive evidence of association in two different studies, and negative findings in others.	373, 376, 378, 385-392
DRD4	BD	Nominal association with low harm avoidance in BD. Negative findings in BD also reported.	379, 393-395
	MDD	Significant associations with MDD in individual studies and in a meta-analysis.	355, 374
DRD5	MD	No significant results found in different studies.	378, 396, 397
COMT	BD	Meta-analysis: modest effect size; associated with the occurrence of rapid cycling and ultra-rapid cycling in BD patients, but negative findings have also been reported.	398-401
	MDD	Significant association with early onset of the disorder.	402
TH	BD	Significant association in two individual studies, but no association in a meta-analysis.	403, 404
SLC6A3	BD	Statistical significant associations in two independent studies.	405, 406
MAO-A	MD	Significant associations with MDD and BP, being a common candidate gene in MD.	407
BDNF	MDD	Significantly associated with reduced volume and abnormal activation of hippocampus, as well as with increased susceptibility to geriatric depression.	408, 409
	MD	Associated with childhood onset and with antidepressant treatment outcome in MD patients. However, other studies including meta-analyses found no association with MD.	355, 410-418
	BD	Different alleles from the same polymorphism have been found to be a a risk and a protective factor for BD.	419-422

Gene	Disorder	Comments	Ref
NTRK3	MDD	Nominal associations not statistically significant after multiple testing correction.	423
DAOA	BD	Statistical significant associations in different studies, but no association in a meta-analysis. Also significant association with decreased grey matter density in temporal lobe and amygdale.	278, 424-430
	MDD	Suggestive evidence of association.	431
APOE, GNB3, MTHFR	MDD	Strongest significant association signals found in a meta-analysis.	355

Abbreviations: MD, mood disorder; MDD, major depressive disorder; BD, bipolar disorder; *SLC6A4,* solute carrier family 6 (neurotransmitter transporter, serotonin), member 4; *HTR1A*, 5-hydroxytryptamine (serotonin) receptor 1A; *TPH2,* tryptophan hydroxylase 2; *DRD1-5,* dopamine receptor D1-D5; *COMT*, catechol-O-methyltransferase; *TH*, tyrosine hydroxylase; *SLC6A3,* solute carrier family 6 (neurotransmitter transporter, dopamine), member 3; *MAO-A,* monoamine oxidase A; *BDNF*, brain-derived neurotrophic factor; *NTRK3*, neurotrophic tyrosine kinase, receptor, type 3; *DAOA*, D-amino acid oxidase activator; *APOE,* apolipoprotein E; *GNB3*, guanine nucleotide-binding protein beta 3; and *MTHFR*, methylene tetrahydrafolate reductase.

Another group of candidate genes of particular interest in MD are those involved in the circadian clock regulation or modulation since, as previously mentioned, there is ample evidence pointing to abnormalities of circadian rhythms underlying some aspects of the pathophysiology of MD[88]. Thus, circadian-related genes have been tested in association analysis as possible candidates for MD, and several of them (including *CLOCK*, *ARNTL*, *NPAS2*, *BHLHB2*, *CSNK1*ε, *PER2*, *PER3*, *VIP*, *GSK3*β and *CRY1)* have been implicated in the susceptibility to MDD, BD, disease recurrence, age at onset, treatment response and circadian subphenotypes typically observed in MD, such as insomnia and diurnal preference[432-450]. Table 4 summarizes the strong positive findings of association studies interrogating genes involved in regulation and modulation of circadian clock in relation to different MD phenotypes and subphenotypes. However, some findings have not been confirmed in further studies. For example, different studies found no relation

with *ARNTL*, *TIMELESS*, *PER3*, *CLOCK*, *CRY*, and *GSK3β* genes when interrogating for MDD, BD or MD[451-459], and the reported association between a SNP in *CLOCK* gene and early, middle, and late insomnia in BD patients[434] has not been confirmed in a very recent association study testing the same polymorphism in an untreated MDD sample[460].

Focusing on *GSK3β*, it has been widely studied in MD patients, since it is inhibited by lithium (a mood stabilizer in BD patients and an augmentation antidepressant therapy in MDD) and other mood stabilizers (including valproic acid and electroconvulsive seizures)[461, 462]. Moreover, *in vivo* inhibition of GSK3β causes antidepressant-like activity in mice[463] and, in accordance, enhanced serotonergic activity or antidepressants acting on serotonergic systems cause inhibition of GSK3β[464]. Regarding genetic studies, a SNP present in the promoter region of *GSK3β* has been associated to bipolar II disorder female patients[439], while no link between *GSK3β* and BD was found in two other studies[456, 465]. Besides, the same SNP has been associated to different subphentopes in BD, such as age at onset, severity of depression, effects of total sleep deprivation[435], and lithium response[437, 441], although this last finding has not been replicated in two further studies when testing the same or different polymorphisms[466, 467]. In MDD patients, a four-marker haplotype was associated to antidepressant drug response[445]. Finally, another study reported BD patients to present increased number of gains in one CNV overlapping with *GSK3β* gene compared to controls individuals[443].

Table 4. Potential susceptibility circadian-related genes in mood disorders.

Gene	Polymorphisms	Disorder	Subphenotype	Ref.
NPAS2	471 Leu/Ser	SAD	---	432
PER3	647 Val/Gly	SAD	Diurnal preference	432
	rs10462020, rs57875989, rs2640909	MD	Age at onset, response to selective serotonin reuptake inhibitors (SSRIs) treatment, and circadian mood oscillation	440
	rs57875989	BD	Age at onset	447
PER2, *ARNTL (BMAL1)*, *NPAS2*	haplotype	SAD	---	442
BHLHB2, CSNK1ε, CLOCK	rs6442925, rs1534891, rs534654	BD	---	448
CRY1, NPAS2	rs2287161, rs11123857	MDD	---	450
CLOCK, VIP	rs10462028, rs17083008	BD	---	450
CLOCK	3111 C>T (rs1801260)	BD	Higher recurrence rate	433
	3111 C>T (rs1801260)	BD	Greater insomnia , decreased need for sleep	434
	3111 C>T (rs1801260)	MD	Insomnia improvement during antidepressant treatment	438
	3111 C>T (rs1801260)	BD	Diurnal activity pattern	444
	3111 C>T (rs1801260)	MD	Information processing	446
	rs3736544	MDD	Fluvoxamine treatment response	449
GSK3β	- 50 C>T (rs334558)	BD	Age at onset	436
	- 50 C>T (rs334558)	BD	Response to total sleep deprivation	435, 436
	- 50 C>T (rs334558)	BD	Severity of deperession	436
	- 50 C>T (rs334558)	BD	Lithium treatment response	437, 441
	- 50 C>T (rs334558)	BD	Bipolar II disorder in females	439
	- 50 C>T (rs334558), rs2319398, rs6808874	MDD	Therapeutic response to antidepressants	445
	CNV	BD	---	443

Abbreviations: MD, mood disorder; MDD, major depressive disorder; BD, bipolar disorder; SAD, seasonal affective disorder; *NPAS2*= neuronal PAS domain protein 2; *PER2-3*= period2-3; *BMAL1*= *ARNTL*, aryl hydrocarbon receptor nuclear translocator-like; *BHLHB2*= basic helix-loop-helix domain containing, class B, 2; *CSNK1ε*= casein kinase 1ε; *CLOCK*= circadian locomotor output cycles KAPUT; *CRY1*= cryptochrome1; *VIP*= vasoactive intestinal peptide; *GSK3β* = glycogen synthase kinase 3β.

1.1.4. Unexplored human genome variation

Throughout the years, the genetic study of complex disorders has focused mainly on the research of common variants, in particular SNPs, relying on different strategies such as the previously reviewed genetic approaches. This research has undoubtedly brought to light several biological pathways and a number of candidate genes possibly involved in the pathophysiology of complex disorders and, more concretely, psychiatric diseases[195]. Indeed, the recent publication of a large amount of GWAS represent a step further in this research, assaying several hundred thousand to more than a million SNPs in thousands of individuals[468]. Nevertheless, these studies only target common gene variants, with the associated SNPs usually having very small effect sizes, and the proportion of heritability explained is at best modest for most traits[469]. Hence, at present, the scientific community is still far from the complete understanding of the human genome variation underlying the susceptibility to psychiatric disorders and complex diseases in general. This missing heritability can be partially due to the uncommon (MAF between 1% and 5%) and rare (MAF <1%) variants. In consequence, during the last years it has become clear the need of exploring these uncommon and rare variants through novel resequencing technologies, which allow a further evaluation of their contribution to disease risk[470].

Moreover, besides rare variants progressively gaining importance in the study of genetic contribution to mental illness[471], it is likely that they do not reflect the complete spectrum of variability in the genome. Thus, other type of unexplored human genome variation has emerged as an important source of genomic diversity with putative regulatory consequences worth to take into account in the study of complex disorders. These could include epigenetic changes, editing modifications, non-coding RNAs, mobile genetic elements, and structural variants, among others[472].

From all the above-mentioned sources of genomic diversity possibly contributing to susceptibility to psychiatric disorders, CNVs and microRNAs are under the scope of this thesis, being further discussed in the next sections.

1.2. Structural variations

A structural variation is a change of genomic DNA greater than 1 kilobase in size that distinguishes two genomes in one species. Structural variation can be either unbalanced (CNVs, including insertions and deletions) or balanced (for example, genomic inversions, and reciprocal translocations) (Figure 7). Both types of structural variation require a break in the DNA phosphodiester backbone.

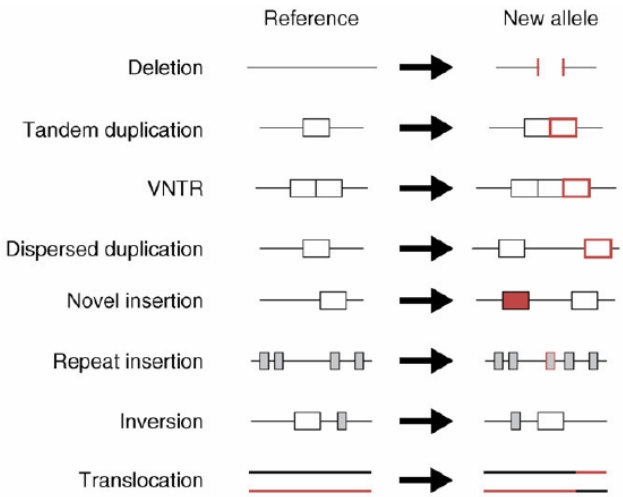


Figure 7. Types of structural variants. Eight different types of structural variants are shown, defined in relation to the reference genome sequence (from Hurles *et al.*[473]). Abbreviation: VNTR: variable number tandem repeat.

A copy number variation (CNV) is defined as a DNA segment ranging from 1 kilobase to several megabases in size that represents an imbalance between two genomes from one species, that is, present at a variable copy number in comparison with a reference genome[474]. Since some time ago, structural variants are known to be present in the human genome and, in fact, different genomic rearrangements have been described to cause neurodevelopmental and neurodegenerative disorders, for example: a microdeletion of chromosome 15q11-q12 causing Prader-Willi syndrome[475], a microduplication involving copy number change of the dosage-sensitive gene *PMP22* (peripheral myelin protein 22) in Charcot-Marie-Tooth disease type 1A[476], a microdeletion at 22q11.2 region found in patients with DiGeorge and

velocardiofacial syndromes[477], and a microdeletion at 7q11.23 in Williams-Beuren syndrome[478]. Besides, it was not until 2004 when two independent groups first reported the widespread presence of CNVs in healthy human individuals[479, 480] and, since then, a bulk of studies have confirmed these findings[481-491]. The first map of CNVs in the human genome was published in 2006[488] and, ever since, the study of control individuals by means of different technologies has yielded a catalog of 57,829 CNVs and 14,478 CNV loci in the human genome according to the last version of Database of Genomic Variants (http://projects.tcag.ca/variation/) in March 2010 (Figure 8).

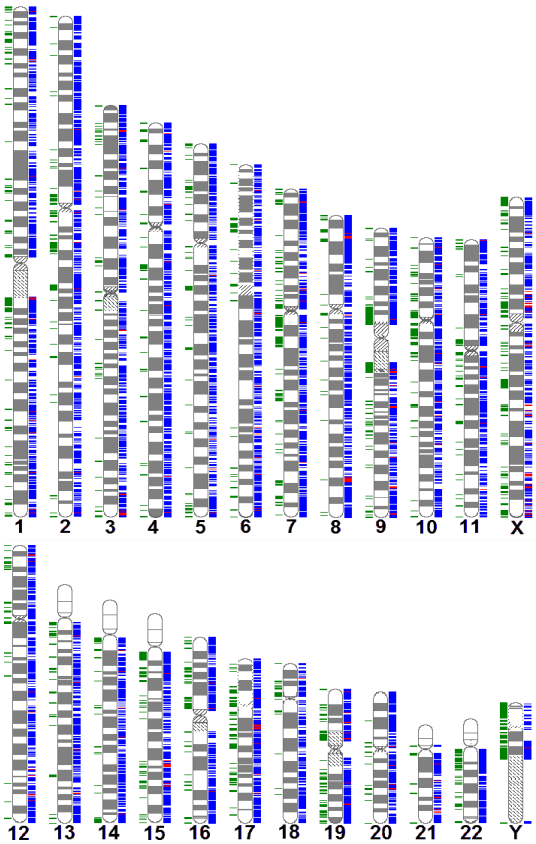


Figure 8. Genomic distribution of CNVs in the human genome. Blue bars indicate reported CNVs; red bars indicate reported inversion breakpoints; and green bars to the left indicate segmental duplications (from Database of Genomic Variants, March 2010, http://projects.tcag.ca/variation/).

Methodologies for the detection of structural variants

Initially, microscopic structural variation larger than 3 Mb could be detected by conventional cytogenetic methods, allowing the analysis of visible chromosomal heteromorphisms, reciprocal translocations, deletions, duplications, insertions and inversions. Then, the development of FISH[492], and subsequently stretched-fiber FISH[493] allowed the mapping of specific DNA sequences at high resolution for the first time. By using fiber FISH, the resolution has improved from the whole chromosomes in metaphase spreads (at a resolution of 5 Mb), or interphase nuclei (50 Kb – 2 Mb) to the level of chromatin strands (5 – 500 Kb)[494].

In the past decade, the progress in high-throughput technologies and the completion of the human genome DNA sequencing increased the resolution of the genetic variation detection, with two main categories of methodologies: whole genome analysis and interrogation of targeted genes.

Microarrays are within the technologies that provide a genome-wide screening of variations, although being unable to detect copy number neutral variants (such as balanced rearrangements) and cannot precisely delineate the breakpoints and other fine structure details for the genomic rearrangements. Array-based comparative genomic hybridization (aCGH), for example, can be used to detect gene gain or loss in an accurate and rapid manner, with thousands of locus specific probes immobilized onto microarrays, where the tested and reference genomic DNA are compared by hybridization[495]. Microarray probes can account for genomic clones (such as BACs, BAC-array), cDNAs, polymerase chain reaction (PCR) products and oligonucleotides, being BAC and oligonucleotide arrays the ones most widely used for whole genome screening. BAC arrays have a resolution limited to about 50 Kb due to its large insert size of the probes but, nevertheless, it is

still widely used to detect large-size variations[496]. Genome-wide genotyping arrays have been also applied in CNV detection, as for example Affymetrix SNP Array 6.0, which features 1.8 million genetic markers, representing more than 906,600 SNPs and 946,000 probes for CNVs[497], and Illumina Human 1M BeadChip, which includes a total of over 1.07 million SNP markers for CNV analysis covering 14,000 total CNV regions[498]. More recently, alternative microarrays have been developed for higher resolution, such as exon arrays and representational oligonucleotide microarrays (ROMA). The exon array can detect CNVs at the single exon level[499], and ROMA has a high resolution of 30 Kb throughout the genome, where digested DNA is ligated with adapters, amplified by PCR, labeled with different fluorophores and co-hybridized to a microarray with probes specific to locations across the entire human genome[500, 501].

Other methodologies targeting concrete regions account for multiple PCRs, allowing the verification of the accurate seize of variation regions, such as quantitative real-time PCR, MLPA (multiplex ligation-dependent probe amplification) or MAPH (multiplex amplifiable probe hybridization). The advantages of these technologies are the low cost, the low input of genomic DNA (0.5 – 1 μg) without previous manipulation or amplification, and the possibility to detect the specific changes at 50 – 100 genomic loci in a single experiment[502]. MAPH mainly consists in fix the genomic DNA onto a membrane, hybridized it with a set of probes in different sizes flanked by the same primers, removed unbound probes, stripped the remain specifically bound probes and amplified them by using the universal primer pair, finally separating the PCR products by gel electrophoresis where the relative band intensities and peak heights indicate the changes of copy numbers[503]. Besides, MLPA can be used to detect the gain or loss of one copy of single exons in human genomic DNA, designing and hybridizing two probes flanked

by universal primers for each target, a step of ligation between the two probes, and a further amplification by PCR using the universal primers[504]. Still another approach has been recently developed, called multiplex ligation-dependent genome amplification (MLGA), with decreased probe amplification background and the total assay time[505].

Finally, computational methods, based on the optimization of algorithms and the access to large numbers of public sequence data, provide another approach to detect structural variations. First, Tunzun *et al*.[482] aligned over 1.1 million paired-end sequences from a high-density fosmid library against the human genome referece assembly, describing as putative sites of insertion, deletion and inversion those discordant regions which showed discrepancy by length and/or orientation. In 2007, Korbel *et al*.[491] described the pair-end mapping (PEM), a new large-scale and high-throughput method, where the paired-ends of 3 Kb fragments generated by hydrodynamical shearing are sequenced and compared to the human reference genome. Significant differences between the paired-end reads and the corresponding reference genomic regions revealed the presence of structural variants, including deletions, inversions, mated and unmated insertions larger than 3 Kb, and simple insertions of 2-3 Kb, with the fine-mapping of more than 1000 structural variations. Finally, DNA sequence alignment[506] is the simplest way for identifying all kinds of variations if the whole genome sequence is available, with no limitation to its resolution and the possible mapping of all type of variants at the nucleotide level. With the development of sequencing technology, such as Roche/454, Solexa (Illumina) and SOLid (ABI), which are all high-throughput and time-saving, variants between individuals can be easily mapped through alignment and the analysis of the human genome has been strongly accelerated.

1.2.1. Copy number variants and complex diseases

Structural variants can have an important biological impact, either by gene dosage alternation, disruption of genes, positional effects, uncovering deleterious alleles or modulating the action of other sequences[507]. The effect of structural variants on gene expression can be due to alteration of gene dosage, as well as alteration of gene structure or regulation (Figure 9). Indeed, there is some evidence that correlates CNVs with gene expression levels, and it has been estimated that about 20% of the measurable genetic impact on gene expression is driven by CNVs, although it may be an underestimation due to CNV maps are biased toward large CNVs[485, 486, 508, 509]. It is important to consider that the effects of structural variants on gene expression are complex, since any kind of functional element, including enhancers, promoters, total o partial genes, and microRNAs, can be located within these types of polymorphisms, thus influencing expression at different levels of the regulatory network[510]. Moreover, CNVs can also alter expression of genes that are located in *cis*, far away, or even in other chromosomes[509].

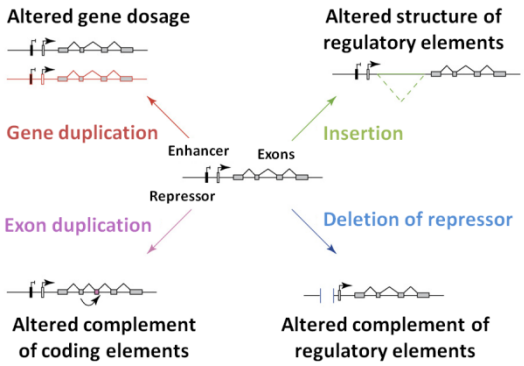


Figure 9. Influence of structural variation on gene regulation. Exons: grey boxes; enchancer: white box; repressor: black box. Four general mechanisms by which structural variants can impact on gene expression are depicted. For each mechanism, an exemplar structural variant (in colour) is shown relative to the central reference gene structure (from Hurles *et al.*[473]).

In summary, CNVs represent a layer of genetic complexity in the regulatory network of expression phenotypes important to take into account, having effects not only in Mendelian and genomic disorders[511] but also possibly determining the susceptibility to complex disorders through alteration on

gene expression. Since 2005, 18 new genomic disorders have been described, more than doubling the number of disorders described in the previous 20 years[512]. Clinically relevant CNVs can be found in DECIPHER (DatabasE of Chromosomal Imbalance and Phenotype in Human using Ensembl Resources, https://decipher.sanger.ac.uk)[513] and ECARUCA (European Cytogeneticists Association Register of Unbalanced Chromosome Aberrations, http://agserver01.azn.nl:8080/ecaruca/ecaruca.jsp). Furthermore, in the literature there are different examples showing association between CNVs and common complex disorders, such as inflammatory and autoimmune common disorders, neurological disorders, and cancer types in which CNVs have been identified in tumour samples[510].

1.2.1.1. Copy number variants and psychiatric disorders

As previously mentioned, CNVs have been demonstrated to be involved in complex diseases and, accordingly, their possible contribution to neuropsychiatric conditions has recently emerged as a new field to explore[249]. First, Wilson *et al.*[514] reported copy number aberrations at four loci in patients with schizophrenia and BD, but not in control individuals. Nevertheless, a following study did not replicate these results[515]. Another study identified 35 aberrant chromosomal regions, accounting for CNVs gains and losses, in patients with schizophrenia[516]. After these initial attempts to find CNVs related to psychiatric disordes, Lachman *et al.*[443] described a significant association between a CNV overlapping with the *GSK3β* gene and BD, finding an increased number of gains in the *GSK3β* in BD patients compared to control individuals.

More recently, genome-wide surveys have demonstrated and overall burden of rare CNVs in psychiatric disorders such as autism spectrum disorders[210] and schizophrenia[211, 212, 283, 284, 517].

Regarding autism spectrum disorder, in a genome-wide CNV association study, *de novo* rare CNVs were more frequent in patients with autism spectrum disorder than in unaffected individuals, with an approximate 3-fold increased in the *de novo* mutation rate, and with all CNVs found in autism spectrum disorder patients harboring at least one gene, some of which have been previously implicated in these disorders[210]. In an independent study, 370 and 254 CNVs were detected in unaffected individuals and patients in autism, respectively, with about 5% of the CNVs found in autistic patients being *de novo* and some of them previously implicated in autism[518]. Still another study identified a recurrent microdeletion and its reciprocal microduplication located on 16p11.2 in four children with autism spectrum disorder, being 100 times more frequent than in control participants of this study[287]. This deletion was also observed in individuals with BD, attention-deficit hyperactivity disorder, schizophrenia and dyslexia.

In the case of schizophrenia, Walsh *et al.*[212] reported that novel rare microduplications and microdeletions were present in 15% of schizophrenia cases, a frequency three times higher than in controls. Other studies have reported *de novo* or rare CNVs to be specific or more frequent among schizophrenic patients compared to unaffected individuals[211, 283, 284, 517].

Finally, several other studies exploring CNVs in psychiatric disorders are being performed with some noticeably finding, such as different studies supporting the role of CNVs in autism spectrum disorder[285-288, 519-523] and schizophrenia[524-532], an association of rare and recurrent exonic CNVs with a subset of patients with Tourette Syndrome[533], and one study from Zhang *et al.*[534] reporting the presence of singleton deletions in 16.2% of BD patients in contrast to 12.3% of control individuals, being significant this increased frequency of deletions in cases.

1.3. Non-coding RNAs: miRNAs

It is well-known that the biological complexity of organisms is not directly related with the number of protein-coding genes. In fact, with the sequence of the human genome it was estimated that humans have only approximately 25,000 genes (representing less than 2% of the total genomic sequence)[280], being very similar with the number of protein-coding genes from other less complex eukaryotes such as mouse, chicken, pufferfish, or even the nematode worm *Caenorhabditis elegans (C. elegans)*[535]. Thus, over the past decade it has become clear that differences in complexity of organisms cannot be explained entirely by the number of protein-coding genes. Indeed, it is widely recognized that complex organisms utilize a wide range of regulation steps in the control of gene expression, at the epigenetic, transcriptional and post-transcriptional levels, including DNA methylation, chromatin modification, availability, localization, quantity or activity of transcription factors, mRNA splicing, polyadenylation and localization, and mechanisms of proteins localization, modification, and degradation, among others. In this context, it is worth noting that the vast majority of the human genome is transcribed and that the biological complexity generally correlates with the proportion of the genome that is non-protein-coding[536, 537]. In accordance, one layer of gene expression regulation accounts for non-coding RNAs (ncRNAs), which do not code for proteins and directly function as RNAs. Different classes of non-coding RNAs have been described in mammals (Table 5).

Table 5. Classes of non-coding RNAs in mammals (adapted from Taft *et al.*[538]).

NcRNA class	Characteristics	Functions	Ref.
Established ncRNA classes			
Long (regulatory) non-coding RNAs (lncRNAs)	Broadest class. Encompass all non-protein-coding RNAs species >~200nt, including mRNA-like ncRNAs.	Epigenetic regulation, sequence-specific tethers for proteins complexes, and specifying subcellular compartments or localization.	539, 540
Small interfering RNAs (siRNAs)	~21-22 nt long, produced by Dicer cleavage of complementary dsRNA duplexes. Form complexes with Argonaute proteins.	Gene regulation, transposon control and viral defence.	541-543
microRNAs (miRNAs)	~22 nt long, produced by Dicer cleavage of imperfect RNA hairpins encoded in long primary transcripts or short introns. They associate with Argonaute proteins.	Primarily involved in post-transcriptional gene regulation.	543-545
PIWI-interacting RNAs (piRNAs)	Dicer independent small RNAs ~26-30 nt long, principally restricted to the germline and somatic cells bordering the germline. They associate with PIWI-clade Argonaute proteins.	Regulate transposon activity and chromatin state.	542, 543
Promoter-associated RNAs (PARs)	A general term encompassing a suite of long and short RNAs, including promoter-associated RNAs (PARs) and transcription initiation RNAs (tiRNAs) that overlap promoters and transcription start sites (TSSs).	May regulate gene expression.	546, 547
Small nucleolar RNAs (snoRNAs)	Traditionally viewed as guides of rRNA methylation and pseudouridylation.	Emerging evidence of gene-regulatory roles.	548
Other recently described classes			
X-inactivation RNAs (xiRNAs)	Dicer-dependent, processed from duplexes of two lncRNAs, Xist and Tsix.	X-chromosomal inactivation in placental mammals.	549
Sno-derived RNAs (sdRNAs)	Some are Dicer-dependent, processed from snoRNAs.	Some function as miRNA-like regulators of translation.	550-552
microRNA-offset RNAs (moRNAs)	~20 nt long, derived from the regions adjacent to pre-miRNAs.	Unknown.	553, 554
tRNA-derived RNAs	tRNAs can be processed into small RNA species by a conserved RNase (angiogenin).	Induce translational repression.	555
MSY2-associated RNAs (MSY-RNAs)	~26-30 nt long, largely restricted to the germline. Associated with the germ cell specific-DNA/RNA binding protein MSY2.	Unknown.	556
Telomere small RNAs (tel-sRNAs)	Dicer-indepenent ~24 nt RNAs principally derived from the G-rich strand of telomeric repeats.	May have a role in telomere maintenance.	557
Centrosome-associated RNAs (crasiRNAs)	~34-42 nt small RNAs, derived from centrosomes.	Evidence of guiding local chromatin modifications.	558

From all the non-coding RNAs, microRNAs (miRNAs) have been well-characterized and also thoroughly investigated in relation to human physiology and disease, thus, during next sections miRNAs main features, functions and their role in human diseases will be further addressed.

1.3.1. Biogenesis and way of action of miRNAs

MicroRNAs (miRNAs) are approximately 21-nucleotide-long RNA regulators of gene expression[559] that are present not only in multicellular organisms but also in unicellular ones[560, 561], indicating that miRNAs are evolutionary old. In 2001, tens of miRNAs were identified in humans, flies, and worms by small RNA cloning and sequencing, thereby establishing miRNAs as a new class of small silencing RNAs[562-564]. Nowadays, the last version of the miRBase (September 2010, Sanger miRBase, release 16.0) recognizes 1,048 known miRNAs in humans. Bioinformatic predictions indicate that mammalian miRNAs can regulate about 30% of all protein-coding genes[565]. Many of the bilaterian animal miRNAs are phylogenetically conserved, which indicates that miRNAS have had important roles throughout animal evolution[566]. Furthermore, most mammalian miRNAs genes have multiple isoforms (paralogues) that are probably the result of gene duplications, and albeit the paralogues are thought to act redundantly in most occasions, members of the same family might have distinct roles *in vivo*[567].

Around 50% of mammalian miRNA loci are found in close proximity to other miRNAs, called clustered miRNAs, which are normally transcribed from a single polycistronic transcription unit (TU), with some exceptions of individual miRNAs derived from separate gene promoters[568]. miRNAs can be generated from non-coding or protein-coding TUs. Approximately 40% and 10% of miRNAs loci are located in the intronic and exonic region of non-

coding transcripts, respectively. Similarly, miRNAs in protein-coding TUs are usually placed in intronic regions, which account for about 40% of all miRNA *loci*. Some miRNA genes can be assigned to either intronic or exonic miRNA group depending on the alternative splicing patterns[569] (Figure 10).

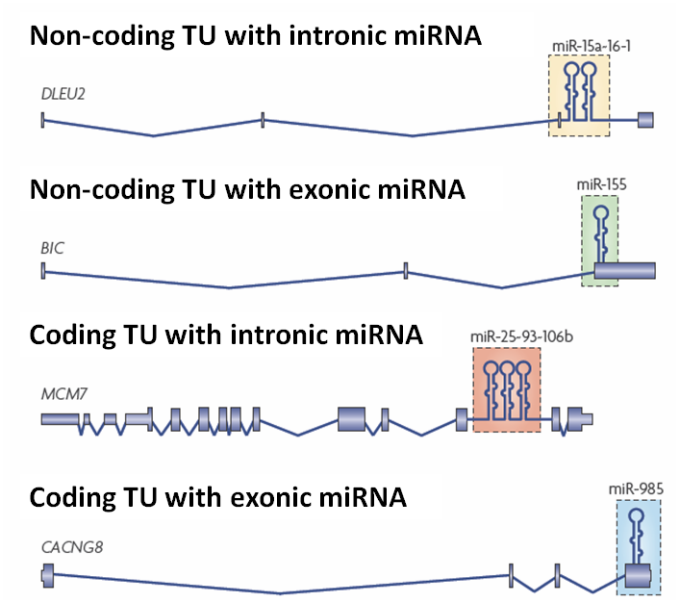


Figure 10. Genomic location and gene structure of miRNAs. MiRNAs can be categorized into four different groups according to their genomic location relative to exon and intron positions. One example of each category is depicted (from Kim *et al*.[569]).

The first step of miRNA biogenesis is the transcription of primary transcripts (pri-miRNAs) generally mediated by RNA polymerase II (Pol II)[570, 571], although a minor group of miRNAs that are associated with Alu repeats can be transcribed by Pol III[572]. Pri-miRNAs are usually several kilobases long, contain local stem-loop structures, often include sequences for several different miRNAs, and are generally polyadenylated and capped. The first step of miRNA maturation is cleavage at the stem of the hairpin structure by means of the nuclear RNase III-type protein Drosha, which releases a small hairpin of about 65 nucleotides with a 3' overhang that is the precussor form

of the miRNA (pre-miRNA)[568]. Drosha requires a cofactor, the DiGeorge syndrome critical region gene 8 (DGCR8) protein in humans, forming the Microprocessor complex[573, 574]. Some intronic miRNAs are processed co-transcriptionally before splicing, with the pre-miRNA entering the miRNA pathway, whereas the rest of the transcript undergoes pre-mRNA splicing and produces mature mRNA for protein synthesis. Other intronic miRNAs (mirtrons) are produced from spliced introns and debranching, forming a hairpin structure that resembles pre-miRNAs, and bypassing the Drosha-processing step[569]. Following nuclear processing, pre-miRNAs are exported to the cytoplasm mediated by exportin-5-Ran-GTP, where exporting 5 recognizes the pre-miRNA with the short 3' overhang[575]. Then, pre-miRNAs are cleaved near the terminal loop by the RNase III Dicer, releasing miRNA duplexes of about 22 nucleotides[576]. Human Dicer interacts with two closely related proteins, TRBP (TAR RNA-binding protein) and PACT. Although neither TRBP nor PACT are required for processing activity itself, they seem to contribute to formation of the RNA-induced silencing complex (RISC)[577, 578]. Thus, following Dicer cleavage, the resulting 22-nt RNA duplex is loaded onto an argonaute protein (AGO) so as to generate the effector complex. One strand of the RNA duplex remains in AGO as a mature miRNA (the guide strand or miRNA), while the other strand (the passenger strand or miRNA*) is degraded, although some hairpins produce miRNAs from both strands[579]. Dicer, TRBP (and/or PACT) and AGO proteins (AGO1-4) contribute to RISC assembly by forming a RISC loading complex (RLC) in humans[580] (Figure 11).

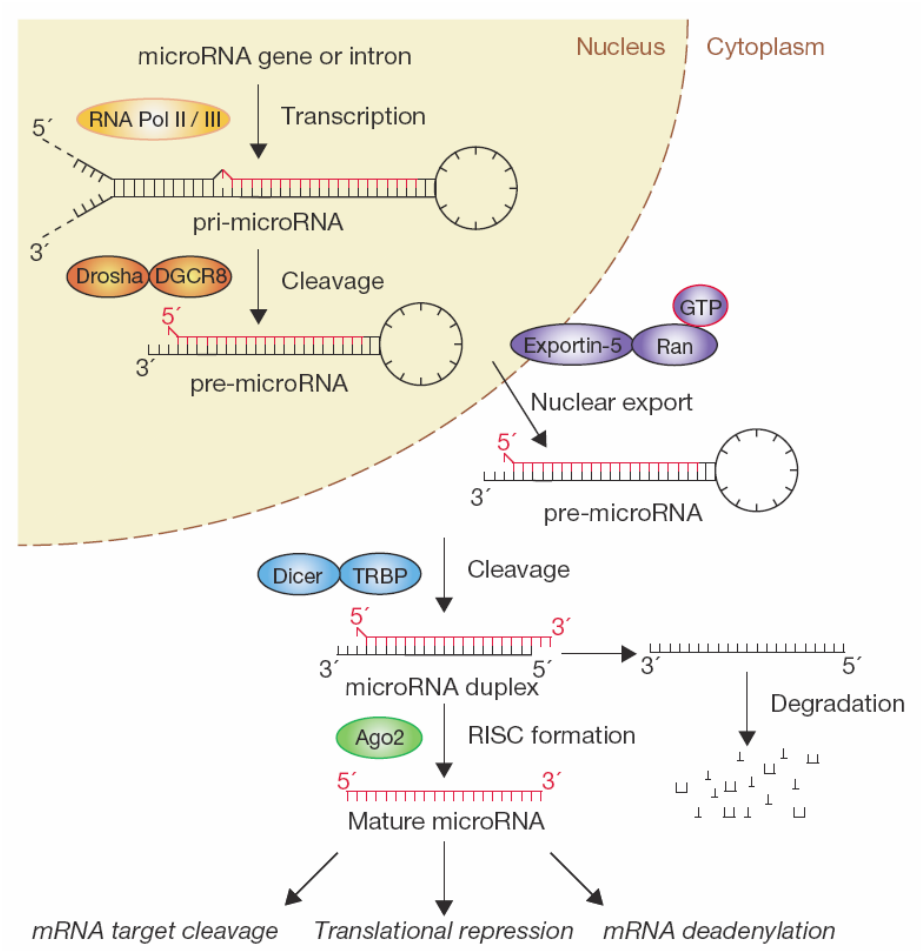


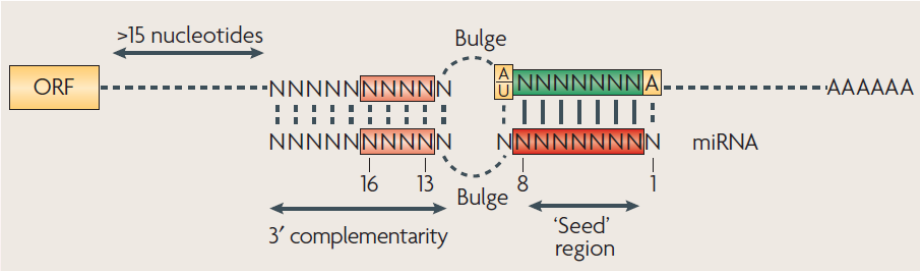
Figure 11. Canonical pathway of miRNAs biogenesis. The figure shows the lineal canonical pathways of miRNAs processing. However, there is evidence of multiple non-canonical steps in the miRNAs biogenesis, some of which are commented in the text (from Winter *et al.*[545]).

Thus, the functional strand of the mature miRNAs is located together with AGO proteins into the RISC complex, where it guides RISC to silence target mRNAs, through translational repression, mRNA decay or deadenylation, or target cleavage, although other types of regulation, such as translational activation[565] and heterochromatin formation[581], have also been described. MiRNAs function as guide molecules in post-transcriptional gene regulation by base pairing with the target mRNAs, usually in the 3' untranslated region (3' UTR, interaction principles between miRNAs and mRNAs are detailed in

Box 5). The degree of miRNA-mRNA complementarity is considered a key determinant of the regulatory mechanism. In this way, perfect complementarity allows AGO-catalyzed cleavage of the mRNA strand, while central mismatches exclude cleavage and promote repression of mRNA translation. The mechanisms by which miRISC regulates translation have still not been clarified, but it seems that repression could occur at all steps of translation: initiation, elongation, and termination. Furthermore, mRNA repression can be associated in some cases with mRNA destabilization, due to deadenylation, decapping, and exonucleolytic digestion of the mRNA[544]. Translationally repressed mRNA is either stored in P-bodies (cytoplasmatic foci that contain translationally repressed mRNA-proteins complexes) or enters the mRNA-decay pathway for destruction. Depending on cellular conditions and stimuli, stored mRNA in P-bodies can re-enter either the translation pathway or the mRNA-decay pathway[559].

There are different computational approaches that predict target sites for miRNAs, using different algorithms mainly based on the following criteria: complementarity of the seed region (although G:U wobbles within the seed region are tolerated) and extensive base pairing with the remainder of the miRNA which may compensate missing complementarity of the seed, the thermodynamics of miRNA-mRNA duplexes, and the conservation of target sites among related genomes[582-585]. Some of the most commonly used miRNA target prediction programs are: PicTar (http://pictar.mdc-berlin.de), TargetScan (http://www.targetscan.org), miRanda (www.microrna.org*),* and microCosm (http://www.microcosm.com, previously known as miRanda by Sanger).

Box 5. Principles of miRNA-mRNA interactions



(from Filipowicz et al.[565])

MiRNAs interact with their mRNA targets by base pairing, through imperfect complementarity in most metazoan, following a set of rules that have been identified by experimental and bioinformatics analyses[586]:

- Perfect and contiguous base pairing of miRNA nucleotides 2 to 8 (seed region, shown in dark red and green), which nucleates the miRNA-mRNA association. GU pairs or mismatches and bulges in the seed region greatly affect repression. However, an A residue across position 1 of the miRNA, and an A or U across position 9 (shown in yellow), improve the site efficiency, although they do not need to base pair with miRNA nucleotides.

- Bulges or mismatches must be present in the central region of the miRNA-mRNA duplex, precluding the Argonaute (AGO)-mediated endonucleolytic cleavage of mRNA.

- There must be reasonable complementarity to the miRNA 3' half to stabilize the interaction. Mismatches and bulges are generally tolerated in this region, although good base pairing, particularly to residues 13-16 of the miRNA (shown in orange), becomes important when mathching in the seed region is suboptimal.

Other factors improving site efficacy include and AU-rich neighbourhood and, for long 3'UTR, a position that is not too far from the poly(A) tail or the termination codon, which can make the 3'UTR regions less structured and more accessible to miRNP (micro-ribonuceloptroteins) recognition. Indeed, accessibility of binding sites might have an important effect on miRNA-mediated repression. Usually, miRNA-binding sites in metazoan mRNAs lie in the 3'UTR and are present in multiple copies. Importantly, multiple sites for the same or different miRNAs are generally required for effective repression. When they are present close to each other (10-40 nucleotides apart) they tend to act cooperatively, that is, their effect exceeds that expected from the independent contributions of two single sites.

1.3.2. miRNAs in biological functions and disease

miRNAs usually have target sites in hundred of genes and abundant mRNAs are under synergistically control of more than one miRNA. Thus, miRNAs represent a highly related and interconnected regulatory network that can fine-tune the expression of their target sites. Consequently, they play important roles in most physiological processes of development and biological functions in animals[587]. There is strong evidence indicating that miRNAs are involved in the regulation of cellular processes such as cell differentiation, growth/proliferation, migration, apoptosis/death, metabolism and defense. In addition, there are several studies supporting a role of miRNAs in the pathogenesis of diverse diseases, including cancer, cardiovascular disease, stroke, neurodegenerative and psychiatric disorders, diabetes, liver disease, kidney disease and infectious disease[588, 589]. Indeed, regulatory changes affecting miRNA activity can account for functional mutations in the proteins involved in transcription, processing, and targeting of miRNAs, chromosomal alterations, epigenetic modifications, polymorphic promoter elements and polymorphisms within the miRNA itself (pri-, pre-, and mature miRNA sequences), as well as mutations in miRNA target sites[590].

Next, the evidence involving miRNAs in the control of circadian rhytms and their possible contributing role in the etiophatogenesis of psychiatric disorders will be further explained, since they are important topics in the development of this project.

1.3.2.1. miRNAs and psychiatric diseases

Since miRNAs play a central role in the CNS and, in addition, they can have a broad effect on gene expression and functional pathways, miRNAs could account as possible contributors in the etiology and pathophysiology of psychiatric disorders, which could explain, at least in part, the dysregulation

of multiple pathways in those diseases. Moreover, they could partly account for the missing heritability previously explained in psychiatric disorders. Consequently, different studies have focused their attention in the involvement of miRNAs in psychiatric disorders, although the results obtained are controversial in some cases.

In schizophrenia, Perkins *et al.*[591] found 16 miRNAs dysregulated in schizophrenic patients compared to control individuals, 15 of which were down-regulated. In addition, for several of the differentially-expressed miRNAs, the ratio of mature miRNA to pri-miRNA was lower, suggesting a disruption in miRNA biogenesis in schizophrenia. Further studies exploring the expression of different miRNAs in schizophrenia and control samples found schizophrenia-related upregulation of a large number of miRNAs: 21% of the miRNAs expressed in superior temporal gyrus, and 9.5% in dorsolateral prefrontal cortex, being only 4 miRNAs upregulated in both regions (miR-128a, miR-16, miR-20a, and miR-338)[592, 593]. Four miRNAs (miR-24, miR-26b, miR-29c, and miR-7) overlapped with the set of significantly change miRNAs from the study of Perkins *et al.*[591], although the regulation of those went in opposite directions and, in addition, changes in miRNAs biogenesis were also contrary to the first study, as the levels of mature miRNAs and miRNAs processing enzymes were significantly upregulated in the later studies[592, 593]. Stark *et al*. found that a mouse model hemizygous for a deletion of the 22q11.2 locus produced schizophrenia-like phenotypes, upregulation of pri-miRNA levels, and downregulation of mature miRNA levels in the brain[594]. In the 22q11.2 region and the deleted murine locus is contained DGCR8, the absence of which results in a bottleneck in the processing of pre-miRNAs to mature miRNAs. Thus, deletion of Dgcr8 alone was sufficient to produce schizophrenia-like behaviors in mice, being the first time that abnormal miRNA biogenesis was shown to affect cognitive

performance in mice. Other groups have associated specific miRNAs with schizophrenia. When SNP genotyping 28 brain-expressed miRNAs in three case/control populations of European ancestry, minor alleles of miR-206 and miR-198 were found over- or under-represented, respectively, in schizophrenia[595]. In another study resequencing 59 X-linked SNPs, the authors found an increase in private, ultra-rare mutations of the pri- or mature miRNA sequences in schizophrenia[596]. Zhu *et al*.[597] found by means of bioinformatics strategies that miRNA-346 targets more schizophrenia-associated genes than the expected by chance. MiRNA-346 is located in intron 2 of the glutamate receptor ionotropic delta 1 (*GRID1*) gene, previously implicated in schizophrenia, and the expression levels of both miR-346 and GRID1 were lower in schizophrenic patients compared to control individuals. Finally, 6 miRNAs are located within the 8p21-23 locus, a CNV "hot spot" linked to schizophrenia and autism[598].

BDNF, which is a strong candidate gene for schizophrenia, BD, and MDD, inhibits the effects of miR-134 on *Limk1* (LIM domain kinase 1), a regulator of synaptic morphogenesis, allowing Limk1 to be translated[599]. Moreover, several miRNAs target BDNF, such as miR-30a-5p and miR-195, which are expressed in human prefrontal cortex and were found to directly target the BDNF 3'UTR and reduce BDNF expression[600]. In addition, BDNF is indirectly regulated by miR-132: CREB-induced transcription of miR-132 results in decrease of MECP2 (methyl CpG binding protein 2), involved in Rett syndrome, and a subsequent decrease in BDNF due to depression of REST (RE1-silencing transcription factor)[601, 602]. CREB expression is reduced in schizophrenia, suggesting that miR-132 expression may also be reduced[603]. Indeed, a significant reduction in miR-132 levels in prefrontal cortex has been observed in schizophrenic and BD patients, although definite results have not been published yet[302].

Furthermore, there is some evidence of miRNAs mediating the effects of psychiatric drugs therapies. Zhou *et al.*[604] found that *in vitro* lithium and valproic acid treatment differentially regulated 37 and 31 miRNAs, respectively, with 8 miRNAs in common, and several of these miRNAs target genes being potential genetic risk factors for BD. Another study examined miRNA expression in lymphoblastoid cell lines derived from BD patients or unaffected siblings, and identified alterations in expression of several miRNAs following lithium treatment[605]. In addition, in rats, treatment with antipsychotic haloperidol upregulates 3 miRNAS: miR-199a, miR-128a, and miR-128b[591]. Finally, disruption of NMDA (N-methyl-D-aspartic acid) receptor signaling by dizocilpine, a selective NMDA receptor antagonist, was found to decrease miR-219 level in the prefrontal cortex of mice[606]. *In vivo* inhibiton of miR-219 by specific anti-miR in the murine brain caused up-regulation of its target calcium/calmodulin-dependent protein kinase II gamma subunit (CAMKII gamma). In turn, abnormal expression of CAMKII gamma resulted in malfunction of NMDA receptor signaling and alterations in relevant behavioral responses. Interestingly, the dizocilpine-induced effects on miR-219 could be attenuated by pretreating the mice with antipsychotic drugs haloperidol and clozapine.

1.3.2.2. miRNAs and circadian rhythms

Recently, miRNAs have been involved in the control of circadian rhythms in mammals. First, Cheng *et al*. identified two brain-specific miRNAs (miR-132 and miR-219-1) as modulators of endogenous circadian clock in the SCN in mice[607]. The role of both miRNAs was tested using antagomirs, which block the miRNA activity, suggesting miR-132 as a negative regulator of the light-dependent resetting of the clock and a role of miR-219 in the modulation of period length, which naturally occurs as a light-dark cycle of approximately 24 hours. Moreover, the authors functionally characterized miR-132 and miR-

219-1 within the context of circadian clock and gave experimental evidence of genes *Rfx4* and *Phlpp* as respective targets. In another study in mice, the daily cycling of expression of a number of miRNAs was found in the retina[608]. Among this subgroup of miRNAs, there were members of the miR-183/96/182 cluster, with predicted targets known to be important in the regulation of the circadian rhythms, such as adenylyl cyclase VI (*Adcy6*) and *Clock*. *Adcy6*, also expressed rhythmically, was validated as a target site for miR-182 and miR-96, being the expression of these miRNAs in anti-phase to the *Adcy6* transcript. Finally, some miRNA may not show diurnal cycling but still have a significant impact in regulation of clock, or clock-controlled proteins. For example, nocturin, a cycling deadenylase downstream of the circadian clockwork that serves as the clock output in metabolic regulation[609], has been shown to be targeted by miR-122[610], which is consistent with an earlier study suggesting that this miRNA is involved in lipid metabolism[611].

Rationale, hypothesis and objectives

Psychiatric disorders are common and high prevalent diseases in all countries, being one of the leading causes of disability worldwide with over a third of the global population meeting criteria for the major categories at some point in their life. Individuals suffering from these disorders are often subjected to social isolation, poor quality of life and increased mortality. Accordingly, mental illnesses are one of the major concerns of public health, accounting for significant economic and social costs[38].

As heritability estimations have extensively demonstrated a clear contribution of genetic components in the etiopathogenesis of psychiatric disorders[134, 135], the discovery and deep understanding of these underlying genetic factors has been a great challenge widely pursued by the scientific community for many years. The field of psychiatric genetics has brought into light the potential involvement of genes in the development of these illnesses, mainly by means of association studies interrogating SNPs[12, 195]. However, the complete map of genetic variation underlying the susceptibility of psychiatric disorders is still unknown and, after some decades of psychiatric genetics research, it has become evident the need to explore other kind of human genome variation, apart from SNPs in candidate genes, as possible contributing factors in the liability to psychiatric disorders, using alternative approaches and taking advantatge of technological advancements in this field[472].

Thus, the hypothesis underlying this thesis is that genetic variants, including not only SNPs but also the newly recognized sources of human genome variation, in concrete CNVs, affecting coding genes as well as regulatory elements, such as miRNAs and their target sites, could be involved in the genetic susceptibility to psychiatric disorders. Accordingly, we suggest that these types of genome variation in different genetic elements might play

significant roles in the etiopathology of psychiatric illnesses, through their effect in the dosage of genes pertaining to neuronal pathways important in brain function. Based on this hypothesis, and with the aim to gain insight into the genetic role of different types of human genome variation possibly contributing to the development of psychiatric disorders, the following objectives were defined:

1. To study, in a comprehensive way, the putative variation in copy number of genes pertaining to neuronal pathways involved in the pathophysiology of psychiatric disorders.

1.1. To identify CNVs contained or partially overlapping candidate genes for psychiatric disorders.

1.2. To determine whether the selected CNVs are polymorphic in a sample of subjects diagnosed of different psychiatric disorders and in a sample of control individuals.

1.3. To perform case-control association studies (using the global sample and also stratifying by diagnosis) to determine if the frequency of copy number changes differs between groups.

2. To replicate previous studies that have reported a significant association of the gene glycogen synthase kinase 3 beta (*GSK3β*) with bipolar disorder in a Spanish sample comprising patients with mood disorders, and take into account SNPs located along the genomic region and also a known CNV which partially overlaps the gene.

2.1. To select tagSNPs that capture 100% of the allelic variation in the genomic region of the gene and genotype them in a combined sample of mood disorder patients and control individuals.

2.2. To identify and quantify changes in copy number in *GSK3β* by means of quantitative real-time PCR (qPCR) in the same set of subjects.

2.3. To perform association analyses with the screened variants taking into account different diagnosis and subphenotypes of mood disorders.

3. To study the role of microRNAs (miRNAs) previously described to regulate circadian rhythms and their relationship with target genes known to pertain to the clock machinery in a sample of mood disorder patients.

3.1. To identify, through direct sequencing, new and already described allelic variants in the precursor forms of the selected miRNAs and their respective target regions in candidate selected genes.

3.2. To perform association analyses with the variants found to be polymorphic in mood disorder patients with available clinical data on phenotypes related to circadian rhythmicity such as chronotype, seasonal onset of the episodes or sleep pattern.

3.3. To perform functional studies, by means of a luciferase reporter-based system and qPCR, to investigate the possible involvement of the identified and associated miRNAs in the post-transcriptional regulation of the candidate genes.

Results

The results section of this thesis is divided in three different parts, and from each one has derived an accepted publication in international indexed scientific journals. In the first part, the copy number variation (CNV) of neuronal pathways genes involved in the pathophysiology of mental illnesses was examined in a sample consisting of patients with four different psychiatric disorders and control individuals. In the other two parts of the results, the study was narrowed on patients with mood disorders (MD) using two different approaches: the study of *GSK3β* in a candidate gene approach and, on the other hand, the exploration of miRNAs as possible contributing factors in the pathophysiology of these diseases.

3.1. Study of CNVs in psychiatric disorders

In the first part of the results, the aim was to assess the potential dose effect of CNVs in a sample of 724 patients with psychiatric disorders and 341 control individuals in a candidate gene approach, taking advantage of the sensitivity and specificity of MLPA technique[504, 612]. The study was carried out in collaboration with the following centers and research clinicians who contributed with the collection of samples and the corresponding clinical data:

- Fernando Fernández-Aranda, from the Psychiatry Department, Bellvitge University Hospital, who provided the samples of subjects with eating disorders.
- Miriam Guitart, from the Genetic Laboratory, UDIAT-Centre Diagnòstic, Fundació Parc Taulí UAB, Corporació Sanitària Parc Taulí, and Vicenç Vallès, from the Department of Mental Health, Consorci Sanitari de Terrassa, who provided samples of patients with schizophrenia diagnosis.
- Rocío Martín-Santos and Ricard Navinés, from the Neuropsycopharmacology Group IMIM-Hospital del Mar, and Marta

Torrens, from the Drug Abuse and Psychiatry Department (IAPS), Hospital Universitari del Mar, who provided samples of panic disorder patients.

- José Manuel Menchón and Pino Alonso, from the Psychiatry Department, Bellvitge University Hospital, who provided samples of patients with obsessive-compulsive disorder.
- Virginia Soria, José Manuel Crespo and Mikel Urretavizcaya, from the Psychiatry Department, Bellvitge University Hospital, who provided samples of patients with mood disorders.

The results of this study led to the publication of the following article:

Comprehensive copy number variant (CNV) analysis of neuronal pathways genes in psychiatric disorders identifies rare variants within patients.

Saus E, Brunet A, Armengol L, Alonso P, Crespo JM, Fernández-Aranda F, Guitart M, Martín-Santos R, Menchón JM, Navinés R, Soria V, Torrens M, Urretavizcaya M, Vallès V, Gratacòs M, Estivill X.

Journal of Psychiatric Research. 2010 April 14. [Epub ahead of print].

Saus E, Brunet A, Armengol L, Alonso P, Crespo JM, Fernández-Aranda F, et al. Comprehensive copy number variant (CNV) analysis of neuronal pathways genes in psychiatric disorders identifies rare variants within patients. J Psychiatr Res. 2010; 44(14): 971-8.

3.2. Candidate gene approach in mood disorders: GSK3β

The plan for this second part of the results was to test *GSK3β* as a candidate gene in mood disorders (MD), since GSK3β has long been suggested as an important player in their pathophysiology and treatment[461, 462, 613, 614]. The study was performed in collaboration with Virginia Soria, Mikel Urretavizcaya, José Manuel Crespo and José Manuel Menchón, from the Psychiatry Department, Bellvitge University Hospital, who provided samples of patients with mood disorders and collected the corresponding clinical data. Moreover, Joaquín valero, Alfonso Gutiérrez-Zotes, Lourdes Martorell and Elisabet Vilella, from the Hospital Psiquiàtric Universitari Institut Pere Mata, IISPV, Universitat Rovira i Virgili, provided samples and socio-demographic data of control individuals.

The results of the study are reflected in the following article:

A haplotype of glycogen synthase kinase-3beta is associated with early onset of unipolar major depression.

Saus E, Soria V, Escaramís G, Crespo JM, Valero J, Gutiérrez-Zotes A, Martorell L, Vilella E, Menchón JM, Estivill X, Gratacòs M, Urretavizcaya M.

Genes Brain and Behavior. 2010 July 7. [Epub ahead of print].

Saus E, Soria V, Escaramís G, Crespo JM, Valero J, Gutiérrez-Zotes A, et al. A haplotype of glycogen synthase kinase-3beta is associated with early onset of unipolar major depression. Genes Brain Behav. 2010; 9(7): 799-807.

Supplementary statistical methods

qPCR data analysis

To determine the DNA copy gain or loss, a normalization procedure was carried out on the relative quantification (R) of the target gene in comparison to the reference gene, which can be obtained using the following expression (Pfaffl, 2001):

$$R = \frac{\left(E_{Target}\right)^{Cp_{Target}}}{\left(E_{Control}\right)^{Cp_{Control}}} \qquad (1)$$

where E_{Target} is the qPCR efficiency of the target gene and $E_{Control}$ refers to the efficiency of the control gene. Usually Cp_{Target} and $Cp_{Control}$ are replaced by ΔCp_{Target} and $\Delta Cp_{Control}$ respectively, which are the deviations of the Cp values of a specific individual to the Cp value of a calibrator; however, for the purpose of comparative studies, where the same calibrator is used for all individuals, the Cp values of the calibrators may be ignored. We therefore applied a variance component model to describe the expression ratios on the log scale, which allows a decomposition of the different sources of variation due to the experimental design:

$$Cp_{gijk} \cdot \log(E_{gj}) = \mu + \beta_g + \alpha_i + \gamma_j + e_{gijk} \qquad (2)$$

where μ is the mean value across individuals, β_g is a fixed effect representing a different mean for the target gene (g=1) and the control gene (g=0), α_i is a random effect for the *i*th individual (and accounts for the between-individual variation), γ_j is a random effect for the *j*th plate (and accounts for the between-plate variation), and e_{gjik} is a random error variable (the within-individual variability). The index *k* denotes the replicate of a specific individual within a plate. The model assumes that α_i, γ_j and e_{gijk} are normally distributed variables with

mean 0 and variances σ_{α}^{2} (between-individual variance), σ_{γ}^{2} (between-plate variance) and σ_{e}^{2} (within-individual variance) respectively.

From model (2), β_g is interpreted as the average of the log relative quantification (log*R*) across all individuals, and α_i are the individual deviations from the average of the log*R*. Therefore a gain for a specific individual *i* is defined if the estimate of α_i is significantly greater than 0, and a loss is defined analogously for a specific individual *i* if the estimate of α_i is significantly lower than 0.

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3.3. miRNAs as potential regulators of circadian rhythms in mood disorders

In the last part of this thesis the aim was to study the role of microRNAs (miRNAs) as a source of genomic diversity with putative regulatory consequences in MD, since miRNAs have recently been recognized as a new layer of post-transcriptional regulation playing important roles in many physiological and pathological processes in humans[565]. The study was carried out in collaboration with Virginia Soria, José Manuel Crespo, Mikel Urretavizcaya and José Manuel Menchón, from the Psychiatry Department, Bellvitge University Hospital, who provided samples and clinical data from patients with mood disorders.

The results of this work are reflected in the following article:

Genetic variants and abnormal processing of pre-miR-182, a circadian clock modulator, in major depression patients with late insomnia.

Saus E, Soria V, Escaramís G, Vivarelli F, Crespo JM, Kagerbauer B, Menchón JM, Urretavizcaya M, Gratacòs M, Estivill X.

Human Molecular Genetics. 2010 July 23. [Epub ahead of print].

Saus E, Soria V, Escaramís G, Vivarelli F, Crespo JM, Kagerbauer B, et al. Genetic variants and abnormal processing of pre-miR-182, a circadian clock modulator, in major depression patients with late insomnia. Hum Mol Genet. 2010; 19(20): 4017-25.

SUPPLEMENTARY METHODS

Data and statistical analyses of functional assays

Analysis for qRT-PCR and Luciferase reporter assays data were carried out using linear mixed effects models (LMM) which have been shown to provide more powerful results and are more flexible than other classical analyses for different kinds of experimental design (1). The LMMs include random effects that can also account for the different sources of variation of the specific experimental design.

For qRT-PCR data, the following model was applied:

$$\log(y_{gtijk}) = \mu + \beta_g + T_t + \beta_g * T_t + \alpha_i + \gamma_j + e_{gtijk}$$

where y_{gtijk} are the normalized Cp TaqMan values (i.e., Cp values referred to the expression levels of non-transfected HeLa cells treated with Lipofectamine 2000) of the g*th* gene (target: g=1 or housekeeping: g=0), t*th* experimental condition (with t=0, 1, 2 or 3 meaning p182_wt, p182_MUT, p182_wt+pFirefly+pRenilla, p182_MUT+ pFirefly+pRenilla, respectively), i*th* transfection, *jth* reverse transcription and k*th* replicate. μ is the mean value across all experimental conditions, β_g is the gene fixed effect, T_t is the t*th* experimental condition fixed effect, $\beta_g * T_t$ is the gene-condition interaction effect, α_i is a random effect for the *i*th transfection (and accounts for between-transfection variation), γ_j is a random effect for the *j*th reverse transcription (and accounts for between-reverse transcription variation), and e_{gtjik} is a random error variable (the within-condition variability). The model assumes that α_i, γ_j and e_{gtijk} are normally distributed variables with mean 0 and variances σ_α^2, σ_γ^2 and σ_e^2 respectively.

Therefore, the exponential of the interaction term indicates the Fold Change (FC) of the normalized expression levels between different experimental conditions. For example

$\exp\{\beta_1 * T_1\}$ indicates the FC of p182_wt versus p182_MUT, and $\exp\{\beta_1 * T_3 - \beta_1 * T_2\}$ indicates the FC of p182_wt+pFirefly+pRenilla versus p182_MUT+ pFirefly+pRenilla. The FC derived from this LMM model are interpreted as the relative quantification calculated via the popular method of $2^{-\Delta\Delta C_T}$ (2).

For Luciferase reporter assays, three models were applied for each gene being considered:

$$\log(R_{tjk}) = \mu + T_t + \gamma_j + e_{gtjk}$$

Where R_{tjk} are the relative reporter activity values (Ratio of Firefly Luciferase activity / Renilla Luciferase activity) for the specific gene being analyzed of the t*th* experimental condition (with t=0, 1 or 2 meaning p182_NULL , p182_wt and p182_MUT, respectively), j*th* experimental day and k*th* replicate. μ is the mean value across all experimental conditions, T_t is the t*th* experimental condition fixed effect, γ_j is a random effect for the *j*th experimental day (and accounts for between-day variation), and e_{tjk} is a random error variable (the within condition variability). The model assumes that γ_j and e_{tjk} are normally distributed variables with mean 0 and variances σ_γ^2 and σ_e^2 respectively.

Therefore, as before, the exponential of the experimental condition term (T_t) indicates the Fold Change (FC) of relative reporter activity between different experimental conditions.

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Discussion

The use of new approaches and the study of different types of genome variability have become an essential need in the research of genetic susceptibility to psychiatric disorders, since the gene-finding efforts for these and other complex illnesses have met with limited success, especially if considering that the heritability estimates for some disorders, such as schizophrenia or bipolar disorder, are around 80% or higher[134, 135]. In fact, although genetic epidemiology has vastly demonstrated a clear genetic influence on psychiatric disorders, most of the results regarding specific genes contributing to risk are inconsistent and far from conclusive[615]. Consequently, the unraveling of factors accounting for this missing heritability in mental illnesses is a constant aim pursued by most of the current genetic studies[214]. Nowadays, it is clear that the analysis of common variants by means of conventional approaches such as association, linkage, or genome-wide association studies, represent a step further in the identification of genes and pathways underlying the susceptibility to psychiatric disorders, although being not sufficient for a complete understanding of their genetic basis. Thus, all these studies usually depict common variants that individually or in combination confer relatively small increments in risk, in accordance with the characteristic of a large number of alleles in several genes influencing the liability to complex disorders. Still, there is an unexplained fraction of heritability, which in part can account for rare variants[208]. Low frequency variants might have from moderate to substantial effect sizes, and in its study, the accumulation of several private mutations in the same gene in a group of patients and not in control individuals point to a functional importance. Nevertheless, it is hard to determine which of the multitude of variants carried by an individual are responsible for a given phenotype, especially if the causal alleles are not well characterized in terms of functional consequences[616]. Besides, non-genetic factors, such as environmental ones, play important roles in this intricate

network of factors contributing to the etiology and development of psychiatric disorders. Albeit some examples in the literature interrogating for possible gene-environment interactions in relation to mental illnesses[272, 617-620], there are still few genetic studies incorporating environmental variables, probably due to the inherent difficulties in standardizing and quantifying these kind of data, such as life events, and to the poor results obtained with most of the studies performed when collecting these variables retrospectively. Alternatively, prospective studies could help in the study of this complex interplay between genes and environment in the psychiatric genetics field with most robust results. Finally, it is important to bear in mind that structural variations and other epigenetic, transcriptional and post-transcriptional regulatory mechanisms play major roles as contributing factors to genetic susceptibility to mental illnesses, through affecting gene expression in central nervous system pathways. In this context, the present work might contribute to decipher the genetic basis of psychiatric disorders through the use of various approaches to consider different types of human genome variability affecting neuronal gene regulatory networks. Concretely, along this section, it will be extensively discussed the genetic study of SNPs, CNVs, and miRNAs in candidate regions for psychiatric disorders, focusing specifically on the results described in the present thesis.

Phenotypic features and boundaries of psychiatric disorders

Other than the problems related to the complex genetic basis of mental illnesses, the discipline of psychiatric genetics faces with phenotypic difficulties. It is widely accepted that it exists a great heterogeneity within each psychiatric category, since the diagnosis is based in clinical examination and not in any physiological test. Thus, these categorical diagnoses, although being very valuable and useful for clinical purposes, are artificial constructs which not necessarily reflect the underlying genetic factors. For example,

twin studies have suggested a genetic influence on typical *versus* atypical forms of major depressive disorder[621]. Moreover, it is not always possible to clearly define the boundaries between diagnostic categories, due to a substantial overlap between them[12]. Hence, individuals with different diagnoses can share the same symptoms, and comorbidity among psychiatric disorders is also common[16-21, 28, 29, 34-37]. For instance, one study found that the same genetic influences impact major depressive disorder and generalized anxiety disorder, but differences in environmental experiences contribute to the manifestation of different outcomes[622]. Accordingly, the definition of a homogenous psychiatric sample is an essential step for facilitating a posterior genotype-phenotype study, probably improving chances of identifying susceptibility factors. The use of endophentoypes and subphenotypes significantly contribute to this phenotypic dissection, minimizing the phenotypic heterogeneity within psychiatric patients (which, in turn, will reduce the genotypic one) and, moreover, allows a better examination of the phenotypic and genotypic overlap between different psychiatric disorders. Since subphenotypes approaches have proven successful in elucidating the genetics of breast cancer[623] and nonsyndromal deafness[624], they may also prove valuable in psychiatric genetics. Indeed, familiality has been identified in some clinical variables useful for subphenotypic analyses, such as temperament, polarity of onset, (early) age at onset, and rapid cycling in bipolar disorder patients[625-628]. Importantly, in the genetic studies, subphenotypes to be tested should be carefully chosen in accordance with well-formulated hypotheses, in order to avoid insuperable multiple testing problems. Consequently, when performing our genetic studies, we took advantage of the available clinical data to consider different subphenotypes in order to reduce, as far as possible, phenotypic and genotypic heterogeneity in the studied sample. In each study, however, we chose different subphenotypes in line with the underlying hypothesis. In

this way, when studying genomic variability in *GSK3β* gene in a sample of patients with mood disorders (MD), we interrogated for subphentoypes previously associated with the gene, such as polarity, age at onset and severity of depressive index episodes. On the other hand, regarding the study of miRNAs previously related with circadian clock regulation, we focused on circadian rhythms-related subphentoypes in our sample of mood disorder patients, including seasonality, chronotype, and early, middle and late insomnia.

Finally, another important point to take into consideration is sex differences in the prevalence of psychiatric disorders, as well as in risk and protective factors associated with psychiatric outcomes. Regarding major depressive disorder, for example, different twin studies have suggested that the genetic risk factors are not entirely the same for males and females[629], that the genetic influences of major depression are modestly stronger in women than in men[629, 630], and that lifetime prevalence is significantly higher in females than in males[13]. These sex differences could influence results arising in genetic studies and, accordingly, in all association studies performed in the present work we have introduced the covariate sex in the association analyses in order to avoid this confounding factor.

Copy number variants (CNVs) and psychiatric disorders

Since the discovery of CNVs as a common source of inter-individual variation in the genomes of healthy individuals[479, 480], several studies have pointed to a contribution of CNVs not only in rare genomic disorders, as originally thought, but also in common ones[510]. Indeed, as previously mentioned in the introduction section, CNVs have been associated with a number of complex diseases, including human immunodeficiency virus (HIV)[631], autoimmune diseases[632-639] and a spectrum of neuropsychiatric disorders[640]. Hence, as a

first step in this search for new sources of human genome variability involved in the development of mental illnesses, we aimed to screen in a comprehensive way the possible variability in copy number in different candidate genes for psychiatric disorders. Taking advantage of a previous project from our group[641], the selection of genes was based on the hypothesis that changes in one or more neurotransmitter pathways in any of their functional processes in the central nervous system (CNS) might contribute to develop a psychiatric disorder. Moreover, as above-mentioned, the different psychiatric categories do not always reflect clearly separated etiological entities, thus the same genetic pathological factors could be common susceptibility factors to different psychiatric disorders[12]. Accordingly, the 68 selected CNS candidate genes for mental illnesses totally or partially overlapping with CNVs were explored in four different populations of psychiatric disorders (mood disorders, anxiety disorders, eating disorders and schizophrenia) and in a sample of control individuals. Initially, we expected to find high frequencies of the screened CNVs in the different groups of samples analyzed since, at the moment of starting this study, most CNVs associated with complex disorders were common[631-635]. However, this was not the case in our study. On the contrary, we detected variation in copy number in only 30 out of the 68 genes predicted to overlap with CNVs. Still, although CNVs could not be confirmed in a great number of genes, MLPA was corroborated as an efficacious technique to detect copy number changes targeting specific genes, since all positive control regions were detected in the three corresponding affected control samples.

The low number of screened regions found to be variable in copy number could be due to the absence of variability in these loci or to the low frequency of these CNVs in our population. Since the screening of CNVs overlapping with our candidate genes of interest was based on a study that

used large insert clone-based CGH and SNP genotyping arrays to describe genetic variation[488], it is possible that the regions targeted by our MLPA probes, spanning around 60 nucleotides, do not correspond to the variable *loci*. That is, clone-based CGH techniques normally overestimate the CNV boundaries and their median size, because it is assumed that the whole clone region pertains to a CNV, when perhaps the variable region corresponds only to a fraction of it. In fact, more than the half of the genes per which not variation was found in our study were initially detected using clone-based CGH and, thus, the real loci affected by a CNV might have not been targeted when designing our MLPA probes. This stresses the necessity of determining the exact CNV mapping on the human genome, defining their boundaries and sizes to be able to perform more accurate studies regarding their relationship with human evolution and disease. Fortunately, with the recent technological advancement in this field, the resolution of the CNV detection has dramatically increased, as shown by different studies reporting fine-mapping of structural variants by means of using different methodologies such as pair-end mapping (PEM) and high-throughput sequencing technologies[489, 491 490]. On the other hand, it is also possible that the frequencies of CNVs not detected in the present study are very low in our population. When comparing the CNVs frequencies at the exactly genomic regions tested in the present work, considering our study as well as all previous ones available at the Database of Genomic Variants (http://projects.tcag.ca/variation/), we noticed a high variability between the different studies depending on the methodologies and populations used, being very difficult to determine the expected frequencies with certainty. Consequently, it is not possible to discard an absence or a very low frequency of these CNVs in the Spanish population.

As a consequence of the low CNV frequencies in our sample, we were not able to perform association studies considering each gene separately as we initially planned. Conversely, we first tested the overall number of genes presenting rare structural variants comparing each psychiatric category separately and also all psychiatric patients together *versus* control individuals. Apart from patients with anxiety disorders, who carried less number of different CNVs than controls individuals (although significance was lost after Bonferroni correction for multiple testing), no significant difference was found between the number of disease-specific CNV *loci* and the number of CNV *loci* in controls, either overall or by CNV type (gain or loss). Similarly, no differences were found when interrogating the number of samples carrying gains, losses or both, between psychiatric and control samples, neither when considering all patients together or separated by diagnosis category, except for anxiety disorder patients, who carried fewer changes than control individuals, being this difference not statistically significant. Nevertheless caution should be kept regarding these nominal associations in patients with anxiety disorder, since sample sizes of each independent psychiatric group are small. In fact, due to the unexpected low frequencies of detected CNVs, this study should be considered as underpowered and, accordingly, interpreted as hypothesis generating but not as hypothesis testing[642]. Hence, large samples would be needed to increase the statistical power and obtain reliable association results.

Noticeably, despite the lack of significant results, 14 changes in copy number were only present in psychiatric samples and not in the control individuals tested: *CORT*, *NTSR1*, *GNRHR2*, *GRM7*, *DLG1*, *PPP3CC*, *GDF2*, *NRG3*, *NOS2A*, *SLC6A13*, *S100B*, *GLO1*, *SSTR5* and *COMT*. However, all CNVs tested in our study are described in the public databases and previously found in general population. Moreover, our controls were not psychiatrically explored and

mental illnesses are very prevalent in the general population. In spite of these appreciable limitations, the unaffected individuals were blood donors, who are not allowed to take psychotropic drugs, thus, it is improbable that in our control samples are included individuals with major psychiatric disorders, although being not possible to rule out subclinical neuropsychiatric symptomatology or that a psychiatric disorder has not yet manifested in the younger individuals. Even so, this detection of 14 rare CNVs in psychiatric patients but not in unaffected subjects goes in line with the recently findings of a greater overall burden of rare events increasing the risk to develop some psychiatric disorders such as schizophrenia[211, 212, 283, 284, 517] and autism[210], although no significantly results arose in our study probably due to small sample sizes. Furthermore, there is previous evidence linking some of these 14 genes found only in patients with the pathophysiology of the same psychiatric disorder for which a gain or a loss have been detected. For example, in mood disorders six different genes were only variable in copy number in patients: *CORT*, *NTSR1*, *GNRHR2*, *GRM7*, *DLG1*, and *PPP3CC*. From them, the gain found in *CORT* might suggest a possible deregulation of sleep-wake cycles in this patient, since CORT has been reported to induce slow wave sleep[643], and dysfunction of circadian rhythms are involved in the etiology and development of mood disorders[88]. In addition, one mood disorder patient presented a loss in *GRM7* gene, which has been associated with major depressive disorder and bipolar disorder in two independent genome-wide association studies[196, 290]. It is also noteworthy, if considering the overlap and familial coaggregation between bipolar disorder and schizophrenia[644], that three out of the six genes variable in copy number in mood disorder patients (*GRM7*, *DLG1*, and *PPP3CC*) have been previously associated with schizophrenia[645-648], and that the three patients carrying changes in these genes were diagnosed with bipolar disorder. In anxiety disorders, only *SLC6A13* (*GABA* transporter) gene presented a loss in a

patient, being not variable in any of the unaffected subjects screened. Remarkably, the neurotransmitter GABA has been suggested to be underlying the pathophysiology of these disorders and, in fact, some drug treatments for anxiety act on the GABA system[649]. Finally, four different genes were variable in schizophrenic samples and not in controls: *S100B*, *GLO1*, *SSTR5*, and *COMT*. It is noticeable that *S100B* gene has been previously associated with schizophrenia[650] and that we have detected a CNV in this gene in three schizophrenic patients, two of them presenting a gain, in accordance with a quantitative meta-analysis reporting higher serum concentrations of S100B in schizophrenic patients compared to control individuals[651]. Then, another schizophrenic patient carried a deletion at *COMT* gene, with previous studies reporting this gene and a deletion of 22q11 region (containing *COMT*) associated with schizophrenia[652, 653]. Moreover, a haplotype in the gene implicated in the disorder has been also associated with lower expression of COMT mRNA[654].

All together, these results could imply an initial approach to screen the existence of CNVs in psychiatric disorders in a comprehensive way, allowing the further selection of genes to be studied in a more accurate way in larger samples. However, another important limitation of this study insurmountable due to the nature of our sample is that parent's samples of affected individuals were not available. Therefore, we cannot distinguish between inherited and *de novo* events, which could give further support for their potential involvement, in case of being *de novo* CNVs only found in patients, in the pathophysiology and development of psychiatric disorders.

The sample of patients with mood disorder (MD)

In the next sections of this thesis, we focused the study of the human genome variability in genes and regulatory regions in mood disorders (MD).

In this way, and since the number of patients with MD studied was larger, we could define and explore better their phenotypic characteristics, using subphenotypes to homogenize the sample and, consequently, studying in depth and more accurately the genotype-phenotype relationship.

The MD sample used in our studies consisted of a clinically well-defined and characterized number of patients with major depressive disorder (MDD) and bipolar disorder (BD). All patients were recruited from the outpatient and inpatient section of Psychiatry Department at the "Hospital Universitari de Bellvitge", and were diagnosed by experienced psychiatrics using the Structured Clinical Interview (SCID)[655] according to the Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition (DSM-IV)[656] for MD. The fact that all patients were recruited and diagnosed in the same psychiatric department prevents from increasing sample heterogeneity due to different phenotype assessment. In other words, large sample sizes are needed in the study of psychiatric disorders in order to facilitate the discovery of common and rare alleles genetically contributing to their etiopathology, and pooling different data sets is a good strategy to increment sample size. Nevertheless, because of the impossibility in obtaining an objective experimental diagnosis, using different clinical sample sets could contribute in increasing phenotypic heterogeneity. In spite of this, a drawback of this sample is that the studied cases were recruited in a tertiary referral center for adult care and, consequently, they may differ from community-based cases due to selection bias; this could affect the clinical characteristics and the age distribution of our sample, making difficult the possible comparisons with other studies.

On the other hand, samples from patients with MD used in this thesis present no comorbidities, since the exclusion criteria were being under 18

years of age, presenting additional past or present psychiatric diagnoses other than MDD or BD, past or present history of psychoactive substance abuse except for nicotine, and severe medical disease. Moreover, the availability of other clinical data for MD patients, apart from the main diagnosis, is a further advantage which allows a more homogeneous classification of MD using different subphenotypes. In this way, the 21-item Hamilton Depression Rating Scale (HAM-D)[124], administered by a psychiatrist during the depressive index episode, was used to assess the baseline severity of the index depressive episode and to measure early, middle and late insomnia. All patients completed as well the Spanish versions of the Seasonal Pattern Assessment Questionnaire (SPAQ)[125] and the Horne-Östberg Morningness-Eveningness Questionnaire (MEQ)[127] to assess seasonality and chronotype, respectively. Other sociodemographic and clinical variables, such as age at onset of the first mood episode, were obtained retrospectively through a direct interview based on self-report data, so a recall bias cannot be discarded. Nevertheless, in order to minimize this possibility, the assessment of these more controversial clinical data was done throughout a best-estimate procedure, involving at least two clinical investigators who blindly reviewed all the information derived from the clinical interviews with the patients and with key informants, and also patients' medical records. Besides, clinical data such as age, sex, and polarity of the disorder have been used as a covariate in the association analyses performed in order to avoid possible confounding problems in regard with these variables since, for example, MDD is known to be more frequent in women than in men[13] and our controls are not matched by sex with patients.

GSK3β as a candidate gene in MD

The first step to study the genetics underlying MD was a candidate gene approach interrogating for genomic variability in regards to SNPs and a CNV

in the *GSK3β* gene. Association analyses are powerful tools when alleles of small risk effect are expected to be the ones underlying the studied disease[268], as it is the case for MD and complex disorders in general. Indeed, throughout the years association analyses based on candidate gene approaches have yielded hundreds of positive results in many complex diseases, although most of them are not robust and need replication and further confirmation in independent samples[195]. Accordingly, our aim was to study *GSK3β* gene in our sample of MD, since there is great evidence supporting *GSK3β* as a strong candidate gene for MD and, in addition, it has been previously associated with different MDD and BD phenotypes, as reviewed in the introduction section. Concretely, from all the clinical data available in relation to our sample of MD patients, and in order to prevent excessive multiple testing, we chose to study polarity (MDD or BD diagnosis), age at onset of the disorder, and severity of the depressive index episode, since all of them have been previously associated with this gene[436, 439]. We first attempted to study genomic variability along *GSK3β* region through a conventional approach of TagSNPs selection and genotyping. Then, we also quantified gains and losses of a CNV partially overlapping with the gene and previously associated with BD[443], being the first time that *GSK3β* gene is studied considering both SNPs and CNV in a sample of MD patients.

Regarding CNV analysis, we could detect variation in the CNV overlapping with the *GSK3β* gene, confirming it is a common CNV in both patients and controls, but not replicating the association found by Lachman *et al.*[443] of a higher number of gains in BD, as well as finding no significant associations with any of the other tested phenotypes. Again, the poor resolution of the CNV breakpoints when described by means of array-based CGH technologies, as it is the case for variation_0035[479], can lead to overestimation of the CNV size. Thus, variation_0035 is supposed to encompass a region of *GSK3β* as

well as to include *NR1I2* and the 3' region of *C3orf15* genes and, indeed, the concrete region amplified in the study of Lachman *et al.*[443] did not target *GSK3β* gene but *NR1I2.* Consequently, they could probably be detecting variability in *NR1I2* gene but not in *GSK3β,* which makes sense with the posterior description of two other smaller CNVs within this same variation_0035 involving the *NR1I2* gene[232, 487]. As in our study we specifically targeted the *GSK3β* gene, we cannot discard the detection of different CNVs in the two different analyses and, thus, it raises the possibility of an involvement of the CNV encompassing the *NR1I2* gene in the development of MD.

Focusing on SNP analysis, we could not replicate the association between the gene and BD patients previously reported in another study[439]. This lack of replication could be due to the fact that, in the original report, the association was with the subgroup of BD-II female patients, which accounted only for 57 patients, hence, a very small sample size. In our study, we did not have enough statistical power to detect any association within our BD-II female patients (n=43). Besides, we found a significant higher risk of developing a MDD at an early age at onset in patients carrying the G allele of the SNP rs334555, which is located in the *GSK3β* promoter region. An association with the same phenotype was also found when considering a haplotype, containing this same SNP, which partly encompasses the promoter and intron 1 region of *GSK3β.* Indeed the -50T/C (rs334558) SNP, also located in the promoter region of *GSK3β,* has been previously associated with age at onset in BD[436]. Although this polymorphism was not included as a TagSNP in our analysis and we could not establish the linkage disequilibrium (LD) with our significant SNP because it was not genotyped in the European ancestry (CEU) families of the HapMap project, it is located within the same LD block where our associated SNP and haplotype lye. Thus, despite not

finding a significant association with BD in our study, probably due to the lack of statistical power, our results give further evidence of the involvement of this genomic region encompassing the promoter and intron 1 of *GSK3β* in an earlier onset of MD. With regard to possible functional roles of variants within the associated haplotype, it is noticeable that one nearby SNP (rs6438552), which is in moderate LD with our associated SNP (D'=0.67), causes alternative splicing[657] and, moreover, it is associated with reduced grey matter volumes in some brain areas in MDD patients[658]. On the other hand, this functional SNP was not associated in any of our analyses, probably because of differences in LD of the region in populations used in the two studies and due to both polymorphisms are tagSNPs, which could indicate that another variant in LD with both polymorphisms could be the real causative one of the associated phenotypes.

Finally, our control sample was randomly selected from the general population and screened to discard for the presence of any psychiatric disorder. Moreover, no evidence of population stratification between our samples was found, which could have meant the introduction of bias into analyses due to confounding effects. Besides, as previously commented on, larger sample size might be needed to confirm positive genetic associations, especially if considering the low number of homozygous patients for the G allele in our sample. Above all, this result is very interesting because it may reflect the genetic penetrance of the illness, if taking into consideration that the age at onset of mood disorder episodes is suggested to be heritable and influenced by genetic factors[659], and that an early onset has been associated with poor outcome[660, 661] and increased morbid risk in relatives[662, 663], as well as being a bipolarity predictor[664] since increases the risk of switching from MDD to BD[665]. Thus, further research could shed light on the potential role of *GSK3β* genetic variation associated with an earlier onset of MDD in their use

as a marker to identify clinical subtypes of mood disorders, which might be close to bipolar spectrum, present a poor outcome and, consequently, could benefit from specific treatments.

miRNAs as genetic susceptibility factors in MD

After performing a candidate gene approach, the aim was to explore the potential relationship between MD and microRNAs (miRNAs), since the recently discovery of their importance in regulatory mechanisms of gene expression in several human physiological processes as well as in pathological ones has opened a new field to explore the missing heritability in complex diseases. Indeed, miRNAs have been shown to present very important roles in the regulation of brain function and plasticity, which indicates their potential contribution in the development of psychiatric disorders. First, some miRNAs have been identified as brain-specific (expressed only in brain) or brain-enriched (expressed at higher levels in brain than in other tissues)[666-671]. Second, there is strong evidence of miRNAs related to basic mechanisms of brain and neural function. For example, different studies suggest involvement of some miRNAs in neuronal differentiation[672-674], and another study showed the up-regulation of several miRNAS when inducing long-term potentiation (LTP) and long-term depression (LTD) suggesting a critical role of miRNAs in synaptic plasticity[675]. Moreover, other studies support a role of proteins involved in miRNA processing, such as Dicer, DGCR8, FXR1 (fragile X mental retardation, autosomal homolog 1) and MOV10 (Moloney leukemia virus 10, homolog), in mechanisms of brain plasticity. Concretely, Dicer down-regulation causes molecular, morphological and physiological alternations in brains of zebrafish, fly and mouse models[676-678], and mutations in *Drosophila dfmr1* (homolog of the human *FXR1* gene) and *armitage* (homolog of the human *MOV10* gene) led to structural brain changes and behavioral

abnormalities[679-682]. Apart from these and other studies pointing to miRNAs involvement in the regulation of brain development and structural plasticity, several miRNAs have been shown to be dysregulated in some psychiatric disorders, as well as some studies have indicate a regulatory role of miRNAs in circadian rhytmicity, as previously explained in the introduction section.

For all these reasons, in the last section of this thesis we explored genetic variants in the five miRNAs and some of their target genes previously involved in clock regulation[607, 608], under the hypothesis of their potential involvement in the pathogenesis of MD. When performing the mutational screening in miR-132, miR-219-1, and the cluster miR-183/96/182 in MD patients and control individuals, only the variant rs76481776 in the pre-miR-182 was found to be common in both groups analyzed. Five further changes were found in the pre-miR-96 and pre-miR-182 but with minor allele frequencies (MAF) lower than one, thus, with not enough statistical power with our sample size to perform association analyses. However, three out of these five rare changes were only found in MD patients and not in control individuals, which is in agreement with the hypothesis of rare alleles underlying, at least partly, the genetics of complex disorders[209]. In relation to the common SNP rs76481776, to better homogenize our sample and to go into potential circadian clock regulation in depth, we took advantage of the clinical data available to test different circadian-rhythms related subphenotypes in our association analyses, namely seasonality, diurnal preference and early, middle and late insomnia. We found that MD patients carrying the T allele of rs76481776 SNP have a significant higher risk to present late insomnia. In fact, previous studies have shown that SNPs or mutations affecting mature miRNAs or their precursor forms could be associated with different human diseases, such as cancers, hypertension, asthma or neuropsychiatric disorders[590, 683]. Some examples of changes in

pre-miRs associated with common diseases could account for a SNP in pre-miR-196a2 that was found to be associated with survival in individuals with non-small-cell lung cancer[684], and the same polymorphism together with a SNP in pre-miR-499 being associated with a significant increased risk of breast cancer susceptibility[685]. Focusing on neurodevelopmental disorders, one mutation in the pri-miR-222 and two in the pre-miR-222 have been found in patients with X-linked mental retardation[686] and with X-linked non-syndromic mental retardation[687], respectively, co-segregating with the affection status in the last study. With respect to psychiatric disorders, a common SNP in the pri-miR-130b was found in schizophrenic patients and control individuals, and, although not associated with the disorder, it was predicted to alter transcription factors binding[688]. Then, another study found nominal associations between brain-expressed miRNAs and schizophrenia for rs17578796 and rs1700 located in mir-206 and mir-198, respectively[595]. Finally, Feng *et al.*[596] reported a significantly higher accumulation of ultra-rare variants in the precursor and mature miRNAs in schizophrenic patients compared to control individuals, and they further demonstrated that most of the rare mutations detected have a likely impact on regulatory functions. For example, a variant in the mature sequence of miR-18b had decreased activity when compared to the wild type miRNA, and a mutation in the pre-miR-502 led to a decreased level of mature miRNAs.

Consequently, these above-mentioned findings demonstrate that changes in the pri-, pre- and mature miRNAs can potentially influence the expression of different genes, affecting in this way miRNA function and leading to pathological consequences. In addition, apart from changes in microRNAs genes, genetic variations in miRNAs binding sites have also been reported to be associated with human diseases, including psychiatric disorders. First, one study identified one variant in the binding site of miR-189 in the 3'UTR of

SLITRK1 in Tourette syndrome and obsessive-compulsive symptoms, resulting in a modest higher inhibition of gene expression[689]. Then, a common variant in the miR-96 binding site in the *HTR1B* gene was found responsible for an attenuated repressive activity of the miRNA and, moreover, individuals with the homozygous genotype for the variant allele reported a higher incidence of conduct-disorder behaviors[690]. More recently, another study found different haplotypes containing this same mutation causing lower inhibition of gene expression associated with greater anger and hostility in men[691]. Furthermore, a polymorphism in the 3'UTR of *DRD1* gene associated with nicotine dependence was recognized to be within a binding site for miR-504 and the causative variant of a significant expression difference between the two alleles[692]. Finally, Muiños-Gimeno *et al*.[693] identified two new rare variants in the 3'UTR of *NTRK3* gene in patients with panic disorder. These mutations were located in functional target sites for different miRNAs, and both of them significantly altered the miRNA-mediated regulation of *NTRK3*, resulting in recovery of gene expression.

In accordance with these examples of possible gene expression regulation through variants in the miRNAs binding sites, we also performed a mutation screening in the 3'UTR regions of *RFX4*, *PHLPP*, *CLOCK* and *ADCY6* genes predicted to encompass binding sites for the five miRNAs studied in this work and previously involved in circadian rhythms regulation. Notwithstanding the fact that no variants were found in any of the binding target sites, four variants were found in the 3'UTR of *CLOCK* gene in our sample, all of them being near the miR-182 target site. First, rs1801260 SNP was found to be common in our sample of MD patients, although it was not associated with any of the circadian-rhythms phenotypes tested. Interestingly, this variant has been previously associated with evening preference and delayed timing of the sleep-wake cycle in healthy individuals[694, 695] and with early, middle

and late insomnia, diurnal activity pattern and insomnia evolution during antidepressant treatment in BD patients[434, 438, 444]. As the genotype frequencies in the different studies are very similar, the negative association between the rs1801260 variant and insomnia in our sample could account for a type II error due to a smaller sample size in our study leading to a false-negative result. Alternatively, in case of population differences in the linkage disequilibrium (LD) of the region, another untyped functional variant in LD with the rs1801260 SNP could be the responsible of the insomnia phenotype. Second, rs76334428 was found in 12 MD patients, 8 of whom presented insomnia, and another rare variant (rs80230756) was found in one MD patient with late insomnia. Finally, two MD patients with severe late insomnia carried another rare mutation (rs70965446), which noticeably was found in a previous study in one MD patient with sleep disturbances but not in healthy subjects[696]. Overall, considering these rare variants in the near region of miR-182 binding site in the *CLOCK* gene encountered in MD patients, and despite the absence of evidence of a possible functional role for any of the mutations, it might be hypothesized that they could influence the accessibility of the miRNA-RISC complex or the coordination of miRNA with other regulatory elements, which has been previously hypothesized for SNPs near a miRNA binding site[590].

Clock genes have an important role in circadian rhythmicity and sleep homeostasis, both processes contributing to sleep timing and structure[697]. Indeed, defects in clock genes causing changes in their expression are known to be implicated in circadian rhythms disorders and sleep disturbances[698]. For example, one study showed that in a patient with circadian rhythm sleep disorder the expression profile of clock genes, including *CLOCK*, was normalized by treatments such as light therapy, exercise therapy, and medicinal therapy, which resulted in synchronized sleep-wake cycle to a 24

hour[699]. Furthermore, sleep, sleep deprivation and wakefulness induce extensive and divergent brain gene expression[700-703], as well as alter a number of miRNA levels, which can be either up or downregulated depending on the brain area[704]. This possible regulation of sleep homeostasis by means of particular miRNAs and their effects in their target sites goes in agreement with the significant association found in the pre-miR-182 with late insomnia in MD. Furthermore, we aimed to study the possible functional consequences of rs76481776 in the pre-miR-182 associated with late insomnia in MD patients since, as previously reviewed, it has been demonstrated that mutations in pre-miRs might lead to potential functional effects. We first found that pre-miR-182 carrying the rs76481776 mutation overexpressed the mature form of miR-182 when compared with the wild-type precursor form. Then, in a further step, we explored the possible effect of this variant rs76481776 in pre-miR-182 in the regulation of three predicted target genes, finding that the mutated precursor caused a significant reduction in luciferase activity when testing the 3'UTR of *ADCY6*, *CLOCK*, and *DSIP* genes, all of them being involved in the regulation of circadian rhythms, including the sleep/wake pattern. However, in order to confirm and better interpret the role of the rs76481776 mutation in pre-miR-182 in sleep regulation, more functional experiments, especially focused on *in vivo* models, should be performed.

Altogether, our results are the first ones in giving evidence of miRNAs variations causing effects in the regulation of the molecular clockwork in MD patients, which is in agreement with a fine-tune regulation of the expression levels of clock genes, influencing in this way the control of sleep-wake cycles. Hence, the present results also indicate that for a better understanding of circadian rhythms and clock genes regulation is not enough to study transcription factors and modulators of the mammalian circadian clock, as

most works studied by the moment but, on the contrary, it is also essential to consider other mechanisms such as post-transcriptional regulation by miRNAs and, probably, also post-translational regulation and epigenetic factors.

Overall, in this thesis we took advantage of different genetic approaches to study putative genetic factors underlying psychiatric disorders from different points of view. The ultimate goal of this line research is to improve understanding of etiopathology of human disease, so that more effective diagnosis, treatment and prevention can be developed.

First, through the study of CNVs as a new source of human genome variation in our sample of patients with psychiatric disorders it became evident that, apart from the existence of common CNVs previously found in other studies[510], there is a great abundance of rare CNVs in the human genome at least in the candidate genes for psychiatric disorders tested in our sample, which is in agreement with a number of previous reports[210-212, 283, 284, 517]. Therefore, the analysis of this considerable proportion of rare CNVs in the genome could represent an extensive source of genomic variability which could lead to a better definition and understanding of pathophysiological pathways in common disorders, such as psychiatric ones. However, one major requirement for developing these types of studies is the availability of large homogenous samples, which is not always feasible in psychiatric disorders due to the difficulty in their collection and accurate and reliable diagnosis from a biological point of view. Moreover, as demonstrated with our two studies analyzing CNVs, it is essential the definition of accurate CNV boundaries with the use of high-resolution methodologies. Fortunately, nowadays the recent advancements of high-throughput sequencing technologies allow a fine-mapping of the studied CNVs and provide more precise definition of boundaries.

On the other hand, as demonstrated in our study of *GSK3β* in MD, more conventional approaches, such as the study of candidate genes by means of interrogating SNPs in association studies, can shed light not only in the

recognition of gene effects in disease susceptibility, but also in linking a gene with a specific symptom or characteristic of the disorder if considering intermediate phenotypes, such as the age at onset in MDD patients. Nevertheless, one of the main challenges of association studies is to perform large enough studies, with replication samples, to achieve unequivocal statistical significance. For this purpose, meta-analysis can be a useful tool to determine the contribution of genetic variants analyzed by different studies in the development of a concrete phenotype.

Furthermore, apart from the genetic study of coding genes, it should be worth to consider regulatory factors such as miRNAs, which can account for complex regulatory networks able to fine-tune the activity of entire biological pathways[302]. For this reason, the deep knowledge of miRNAs dysregulation in psychiatric diseases could widely contribute to the understanding of molecular mechanisms underlying mental illnesses. Focusing on the results obtained in our study of miRNAs in MD, they would suggest that miRNA system might represents an additional step in the fine-tune modulation of circadian rhythms, indirectly by acting on clock-controlled proteins or directly targeting clock genes, such as the ones of our study involved in the regulation of sleep/wake patterns.

Conclusions

1. Changes in copy number of candidate genes in psychiatric disorders could account, in a number of cases, for contributing factors in the pathophysiology and development of psychiatric disorders through affecting gene expression in central nervous system pathways.

- 30 out of the 68 candidate genes analyzed for mental illnesses were variable in copy number in a sample of 724 psychiatric patients and 341 control individuals confirming the existence of CNVs in these loci.

- The frequencies of changes found per gene were low in both cases and controls (>3.5%).

- No significant overall burden of rare CNVs in psychiatric disorders was found in comparison to unaffected subjects.

- Fourteen genes were variable in copy number only in patients with psychiatric disorders but not in control individuals screened.

2. Study of genetic variability of *GSK3β* gene in mood disorders: the promoter and intron 1 region of *GSK3β* gene might be involved in an earlier onset of major depressive disorder (MDD).

- A CNV overlapping with *GSK3β* gene is variable in both MD patients and control individuals, but not associated with polarity, age at onset and severity of the disorder in our population.

- The SNP rs334555, located in intron 1, and a haplotype containing this same polymorphism and encompassing a region of promoter and intron 1 of *GSK3β* gene, were associated with an earlier age at onset of MDD in our population. Thus, patients carrying the G allele of the common variant

rs334555 have a higher risk of developing major depressive disorder at an earlier age.

3. Mechanisms of post-transcriptional regulation by miRNAs could be involved in the control of circadian rhythms in mood disorder (MD) patients, fine-tuning miRNAs target genes implicated in the control of sleep and wakefulness.

- MD patients carrying the T allele of the rs76481776 polymorphism located in the pre-miR-182 have a significant higher risk of presenting late insomnia.

- Two (rs77586312 and rs75953509) and one (rs41274239) rare variants in the pre-miR-182 and the pre-miR-96, respectively, were only found in MD patients but not in unaffected individuals in our study.

- Rs76481776 polymorphism in pre-miR-182 leads to an overexpression of the mature form of miR-182 in comparison to the wild-type form of the precursor when performing quantitative real-time PCR experiments.

- Pre-miR-182 carrying the variant allele of rs76481776 caused a significant reduction in luciferase activity compared to the wild-type form when testing the 3'UTR of *ADCY6*, *CLOCK* and *DSIP* genes in luciferase assays.

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Abbreviations

3’ UTR	3’ untranslated region
5-HT	5-hydroxytryptamine (serotonin)
ACC	Anterior cingulated cortex
aCGH	Array-based comparative genomic hybridization
ADCY6	Adenylate cyclase 6
AGO	Argonaute
ALDH2	Aldehyde dehydrogenase 2
AN	Anorexia nervosa
ANK3	Ankirin G
APA	American Psychiatric Association
APOE	Apolipoprotein E
ARNTL	Aryl hydrocarbon receptor nuclear translocator-like
BAC	Bacterial artificial chromosome
BD	Bipolar disorder
BDNF	Brain-derived neurotrophic factor
BHLHB2	Basic helix-loop-helix domain containing, class B, 2
BMAL1	=ARNTL, aryl hydrocarbon receptor nuclear translocator-like
BN	Bulimia nervosa
bp	Base pair(s)
C3orf15	Chromosome 3 open reading frame 15
CACNA1C	Calcium channel voltage-dependent L type alpha 1C subunit
CAMKII gamma	Calcium/calmodulin-dependent protein kinase II gamma subunit
CCG	Clock-controlled genes
CEU	U.S. residents of northern and western European ancestry
CGH	Comparative genomic hybridization
CHB	Unrelated Han Chinese individuals from Beijing, China
CLOCK	Circadian locomotor output cycles KAPUT
CK1ε	= CSNK1ε, casein kinase 1ε
CNS	Central nervous system
CNV	Copy number variant
COMT	Catechol-O-methyltransferase
CORT	Cortistatin
crasiRNA	Centrosome-associated RNA
CREB1	cAMP responsive element binding protein 1
CRH	Corticotropine-releasing hormone
CRY1,2	Cryptochrome1,2
CSF	Cerebrospinal fluid
DA	Dopamine

DAOA	D-amino acid oxidase activator
DECIPHER	Database of chromosomal imbalance and phenotype in human using Ensembl resources
DGCR8	DiGeorge syndrome critical region gene 8
DICER	Dicer 1, ribonuclease type III
DLG1	Discs, large homolog 1 (Drosophila)
DNA	Deoxyribonucleic acid
DRD1-5	Dopamine receptor 1-5
DSM	Diagnositic and Statistical Manual of Mental Disoders
dsRNA	Double-stranded RNA
DZ	Dizygotic
ECT	Electroconvulsive shock therapy
EDNOS	Eating disorders not otherwise specified
ECARUCA	European cytogeneticists association register of unbalanced chromosome aberrations
FXR1	Fragile X mental retardation, autosomal homolog 1
GABRA2	Gamma-aminobutyric acid receptor alpha 2 subunit
GDF2	Growth differentiation factor 2
GNB3	Guanine nucleotide-binding protein beta 3
GNRHR2	Gonadotropin-releasing hormone (type 2) receptor 2
GRID1	Glutamate receptor ionotropic delta 1
GLO1	Glyoxalase I
GRM7	Glutamate receptor, metabotropic 7
GSK3β	Glycogen synthase kinase 3β
GTP	Guanosine-5'-triphosphate
GWAS	Genome-wide association study
HAM-D scale	Hamilton rating scale for depression
HGP	Human Genome Project
HIV	Human immunodeficiency virus
HPA axis	Hypothalamic-pituitary-adrenal axis
HTR1A	5-hydroxytryptamine (serotonin) receptor 1A
HVA	Homovanillic acid
ICD	International Classification of Disease
JPT	Unrelated individuals from Tokyo, Japan
Kb	Kilobase
LD	Linkage disequilibrium
LIMK1	LIM domain kinase 1
lncRNA	Long non-coding RNA

LTD	Long-term depression
LTP	Long-term potentiation
MAF	Minor allele frequency
MAO (A)	Monoamine oxidase (A)
MAPH	Multiplex amplifiable probe hybridization
Mb	Megabases
MD	Mood disorder
MDD	Major depressive disorder
MDE	Major depressive episode
MECP2	Methyl CpG binding protein 2
MEQ	Horne-Östberg Morningness-Eveningness Questionnaire
miRNA	microRNA
miRNP	Micro-ribonucleoproteins
MLGA	Multiplex ligation-dependent genome amplification
MLPA	Multiplex ligation-dependent probe amplification
moRNA	microRNA-offset RNA
MOV10	Moloney leukemia virus 10, homolog
mRNA	Messenger RNA
MSY2	=YBX2, Y box binding protein 2
MSY-RNA	MSY2-associated RNA
MTHFR	Methylene tetrahydrafolate reductase
MZ	Monozygotic
NA	Noradrenaline / norepinephrine
N. Accumbens	Nucleus accumbens
ncRNA	Non-coding RNA
NMDA	N-methyl-D-aspartic acid
NOS2A	Nitric oxide synthase 2, inducible
NPAS2	Neuronal PAS domain protein 2
NR1I2	Nuclear receptor subfamily 1, group I, member 2
NRG3	Neuregulin 3
nt	Nucleotide
NTRK3	Neurotrophic tyrosine kinase receptor type 3
NTSR1	Neurotensin receptor 1 (high affinity)
OCD	Obsessive-compulsive disorder
PAR	Promoter-associated RNA
PCR	Polymerase chain reaction
PD	Panic disorder
PER1-3	Period1-3

PEM	Pair-end mapping
PFC	Prefrontal cortex
PHLPP	PH domain and leucine rich repeat protein phosphatase
piRNA	PIWI-interacting RNA
PMP22	Peripheral myelin protein 22
Pol II-III	RNA polymerase II-III
PPP3CC	Protein phosphatase 3, catalytic subunit, gamma isoform (calcineurin A gamma)
pre-miRNA	Precursor miRNA
pri-miRNA	Primary miRNA
qPCR	Quantitative real-time PCR
REST	RE1-silencing transcription factor
REV-ERB	= NR1D1, nuclear receptor subfamily 1, group D, member 1
RFLP	Restriction fragment lenght polymorphism
RFX4	Regulatory factor X, 4
RISC	RNA-induced silencing complex
RLC	RISC loading complex
RNA	Ribonucleic acid
ROMA	Representational oligonucleotide microarrays
RORA	RAR-related orphan receptor A
RORE	Retinoic acid-related orphan receptor response elements
rRNA	Ribosomal RNA
S100B	S100 calcium binding protein B
SCID	Structured Clinical Interview
SCN	Suprachiasmatic nucleus
sdRNA	Sno-derived RNA
siRNA	Small interfering RNA
SLC6A13	Solute carrier family 6 (GABA transporter), member 13
SLC6A3	Solute carrier family 6 (dopamine transporter), member 4.
SLC6A4	Solute carrier family 6 (serotonin transporter), member 4.
snoRNA	Small nucleolar RNA
SNP	Single nucleotide polymorphism
SNRIs	Serotonin-norepinephrnie reuptake inhibitors
SPAQ	Seasonal Pattern Assessment Questionnaire
SSR	Short sequence repeat
SSRI	Selective serotonin reuptake inhibitors
SSTR5	Somatostatin receptor 5
STR	Short tandem repeat

TDT	Transmission disequilibrium test
tel-sRNA	Telomere small RNA
TH	Tyrosine hydroxylase
TIMELESS	Timeless homolog (Drosophila)
tiRNA	Transcription initiation RNA
TPH2	Tryptophan hydroxylase 2
TRBP	TAR RNA-binding protein
tRNA	Transfer RNA
TSS	Transcription start site
TU	Transcription unit
VIP	Vasoactive intestinal peptide
VNTR	Variable number of tandem repeats
WHO	World Health Organization
WMH	World Mental Health
xiRNA	X-inactivation RNA
YAC	Yeast artificial chromosome
YRI	Yoruba people of Ibadan, Nigeria

Annex

List of publications

1. Saus E, Soria V, Escaramís G, Vivarelli F, Crespo JM, Kagerbauer B, Menchón JM, Urretavizcaya M, Gratacòs M, Estivill X.
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Communications to scientific meetings

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59th American Society of Human Genetics MEETING. Honolulu, 20-24 October 2009. Hawaii. USA

- Association study of the CNV overlapping GSK3β (glycogen synthase-kinase 3β) gene with mood disorders.
  E. Saus, V. Soria, G. Escaramís, J.M. Crespo, J. Valero, A. Gutiérrez-Zotes, L. Martorell, E. Vilella, J.M. Menchón, X. Estivil, M. Urretavizcaya, M. Gratacòs

V Congreso Latinoamericano de Nutrición. 15-19 November 2009. Santiago de Chile. Chile

- Copy number polymorphism in the salivary amylase gene (AMY1): range of variation in the Chilean population.
  J Santos, E. Saus, M. Gratacòs, X Estivill.

XVIIth World Congress of Psychiatric Genetics. San Diego. 4-8 November 2009. California. USA

- CNV overlapping GSK3β (glycogen synthase kinase-3β) gene and its association with Mood Disorders phenotypes in Spanish Population.
  E. Saus, V. Soria, G. Escaramís, J.M. Crespo, J. Valero, A. Gutiérrez-Zotes, L. Martorell, E. Vilella, J.M. Menchón, X. Estivill, M. Urretavizcaya, M. Gratacòs

The 10th International Meeting on Human Genome Variation and Complex Genome Analysis. September 2008. Toronto. Canada.

- Genetic variation of glycogen synthase kinase-3β (GSK3β human gene and susceptibility to mood disorders in the Spanish population.
  M. Gratacòs, V. Soria, E. Saus, F. Vivarelli, J.R. González, J. Valero, E. Martínez-Amorós, M. Bayés, A. Gutiérrez, J.M. Crespo, L. Martorell, E. Vilella, A. Labad, J.M. Menchón, J. Vallejo, X. Estivill, M. Urretavizcaya.

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- Sequence analyses of miRNAs and some of their targets as circadian clock modulators in Mood Disorder (MD) Patients.
  E. Saus, V. Soria, F. Vivarelli, J.M. Crespo, J.M. Menchón, X. Estivill, M. Urretavizcaya, M.Gratacòs.

- Association study of the RAR-related orphan receptor A (RORA) human gene with Mood Disorders.
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- Association study of the glycogen synthase kinase-3 (GSK3 gene with Mood Disorders.
  M. Gratacòs, V. Soria, E. Saus, F. Vivarelli, J.R. González, J. Valero, E. Martínez-Amorós, M. Bayés, A. Gutiérrez, J.M. Crespo, L. Martorell, E. Vilella, A. Labad, J.M. Menchón, J. Vallejo, X. Estivill, M. Urretavizcaya.

- Additional Support for the Association of SLITRK1 var321 and Tourette syndrome.
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- Comprehensive Copy Number Variant (CNV) analysis of neuronal pathways genes in psychiatric disorders.
  E. Saus, A. Brunet, M. Gratacòs, J.R. González, L. Armengol and X. Estivill on behalf of the Psychiatric Genetics Consortium.

XVth World Congress on Psychiatric Genetics. October 2007. New York. USA

- Combined Family Based and Case-Control association study of Neurotrophin Signalling Genes in four Eating Disorders European Populations.
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