DEPARTAMENT DE GENÈTICA

BASES GENÉTICAS DE LA ESQUIZOFRENIA: ASPECTOS EMOCIONALES, COGNITIVOS Y NEUROANATÓMICOS.

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Genetic bases of schizophrenia: cognitive, emotional and neuroanatomical aspects

MEMORIA PRESENTADA POR

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CERTIFICAN:

Que el trabajo de investigación recogido en esta memoria bajo el título “Bases Genéticas de la esquizofrenia: aspectos emocionales, cognitivos y neuroanatómicos”, ha sido realizado, bajo su codirección, en el Departamento de Genética de la Facultad de Biología por Dña. OLGA MARÍA RIVERO MARTÍN, licenciada en Ciencias Biológicas, para optar al grado de Doctora.

Y para que conste, en cumplimiento de la legislación vigente, firman el presente certificado en Burjassot a 15 de Mayo de 2009.

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A las personas que siento más cerca: a mi padre, a mi madre y a Juan Antonio. Esta tesis es también vuestra.

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INTRODUCTION

INTRODUCTION

Preface

Although descriptions of madness and psychotic-like behaviors have existed in many cultures since ancient times, neither of these records was of scientific or medical nature. It was necessary to wait until the late 19th century and early 20th century to see schizophrenia as an illness of neurological origin, when the German psychiatrists Kraepelin and Bleuler began to give the first descriptions of this disease. The term "schizophrenia" was originally proposed by Eugen Bleuler in 1908 to refer to a group of disorders characterized by serious cognitive, emotional and behavioral symptoms (Bleuler, 1911). However, this disease had been previously conceptualized by Kraepelin under the name Dementia praecox (see Kraepelin, 1971 for an English version), in an attempt to differentiate it from the manic-depressive insanity (currently known as bipolar disorder), a group of diseases where the psychotic symptoms were secondary to the mood changes and with better outcome than dementia praecox. Since these initial stages, the knowledge about this devastating brain disorder has progressively increased.

Because of its chronic nature, severity, early onset and psychosocial impairment, schizophrenia, and psychoses in general, imply a high human and economic cost when compared to other major chronic conditions, such as diabetes or breast cancer (UK National Health Service Executive, 1996). Moreover, when compared to some of the most important chronic conditions such as Alzheimer disease, asthma, cancer, depression, osteoporosis and hypertension, schizophrenia presents the highest prevalence-cost ratio and also one of the highest yearly costs per patient, only below Alzheimer disease (Berto *et al.,* 2000).

Thus, as it can be seen, schizophrenia is an expensive illness which starts just when the potential of the individual is at its height (Wong and Van Tol, 2003). For this reason, the consequences are especially dramatic, since behavior and personality change, preventing in many cases the development of a normal life. Therefore, there are many good reasons to study the underlying physiopathological mechanisms of schizophrenia. Particularly, the discovery that schizophrenia (as well as other psychiatric conditions) presents an important genetic component and a high heritability encouraged researchers to look for the genetic factors underlying schizophrenia. However the clinical and genetic complexity of schizophrenia makes the road long and difficult. Thus, although many advances have been made in the last decades, old questions still remain unresolved and new questions emerge every day.

1. Phenomenology and clinical aspects of schizophrenia

1.1. Diagnostic symptoms

Schizophrenia symptoms involve several domains (cognitive, emotional or behavioral) and are grouped into three main categories: positive or psychotic symptoms, deficit or negative symptoms and cognitive impairment. This three–symptom classification, widely accepted by clinicians currently, was originally developed by Liddle (1987) as an extension of the Crow's two-syndrome classification in positive and negative symptoms (Crow, 1980), which supposed an important step in schizophrenia research.

Psychotic symptoms are one of the most characteristic features of schizophrenic patients and include several clinical manifestations, generally with an episodic pattern, which are not present in normal population: hallucinations, delusions and thought disorder. **Hallucinations** consist of false perceptions in a conscious state in the absence of external stimuli which are felt as real by the person who suffers from them. In schizophrenia, auditory hallucinations (AH), and especially human voices (verbal hallucinations), are the most common form of hallucinations. Hallucinations can also be related to the content of the delusions suffered by the patient. **Delusions** or false beliefs are generally paranoid in schizophrenia and include delusions of persecution, grandiosity, external control, thoughts inserted or withdrawn from one's head, ideas of reference and mind-reading (Wong and Van Tol, 2003). The content of the delusions is directly influenced by the patient's life and culture (Tateyama *et al.,* 1993; Stompe *et al.,* 1999). **Thought disorder** consists in disorganization of thinking and behavior which affects the logical elaboration and development of ideas. Depending on the severity of these abnormalities, patients may have important problems to attend their basic needs.

Negative symptoms refer to the absence or disturbance of certain behaviours such as social interaction, motivation, expression of affect, ability to experience pleasure and spontaneous speech (Wong and Van Tol, 2003).

Finally, the **cognitive impairment** observed in most schizophrenic patients mainly affects attention, executive function and memory. The memory organisation deficits can also have implications for more general information processing (Kurachi, 2003).

All these symptoms are part of the diagnostic criteria included in the Diagnostic and Statistical Manual of Mental Disorders or DSM, currently in the fourth edition (American Psychiatric association, 1994), as well as in the International Classification of Diseases or ICD-10 (World Health Organization - WHO, 1992). A summary of the diagnostic criteria for schizophrenia can be found at table I1.

Table I1. DSM-IV diagnostic criteria for schizophrenia.

A.	***Characteristic symptoms***: At least two of the following, each present for a significant portion of time during a one-month period (or less if successfully treated): 1. delusions 2. hallucinations 3. disorganized speech 4. grossly disorganized or catatonic behaviour 5. negative symptoms (Note: Only one criterion A is required if delusions are bizarre or hallucinations consist of a voice commenting on the patient's behaviours or thoughts, or at least two voices conversing with each other).
B.	***Social/occupational dysfunction***: For an important portion of time since the onset of the disturbance, at least one major area of functioning (i.e. work, interpersonal relations, self-care) is clearly below the level achieved prior to the onset (or when the onset is in childhood or adolescence, failure to achieve the expected level).
C.	***Duration***: Continuous signs of the disturbance persist for at least 6 months. This period must include at least 1 month of symptoms (or least if successfully treated) that meet criterion A, and may include other residual periods where the signs of the disturbance may be manifested by only negative symptoms or two or more symptoms from criterion A in an attenuated form.
D.	***Schizoaffective and Mood Disorder exclusion***: Schizoaffective Disorder and Mood Disorder with Psychotic Features have been excluded since (a) no Major Depressive, Manic, Mixed Episodes have occurred together with the active-phase symptoms; or (b) if mood symptoms have occurred during active-phase symptoms, their total duration has been brief when compared to the duration of the active and residual periods.
E.	***Substance/general medical condition exclusion***: The disturbance is not caused by the direct physiological effects of a substance or a general medical condition.
F.	***Relationship to a Pervasive Developmental Disorder***: If there is a history of Autistic Disorder or another Pervasive Developmental Disorder, the additional diagnosis of schizophrenia is made only if prominent delusions or hallucinations are also present for at least one month (or less if successfully treated).

1.2. Controversies in the diagnosis of schizophrenia disorders

The wide variety of symptoms in the schizophrenic phenotype leads to a great clinical heterogeneity, with different patients having different symptoms, to such an extent that two schizophrenic patients may not overlap in the clinical manifestation of the disease. This situation is the origin of important problems when trying to define and explain schizophrenia from a biological point of view. Moreover, it is also important to remind the existence of other psychotic disorders included in the schizophrenia spectrum (schizotypal personality disorder, schizoaffective disorder and schizophreniform disorder, among others) which share an important part of their phenomenology, outcome and treatment response (Siever and Davis, 2004). Thus, it is difficult to define schizophrenia as a completely differentiated entity. Even more, although Kraepelin's classical division between dementia praecox and manic-depressive disorder is still widely used among psychiatrists, many authors doubt about the validity of this "artificial" division and defend an overlap between the different diagnosis (see figure I1), as well as a single underlying entity for the different psychoses and even for all psychiatric disorders (Crow, 1995; Cuesta and Peralta, 2001; Peralta and Cuesta, 2005).

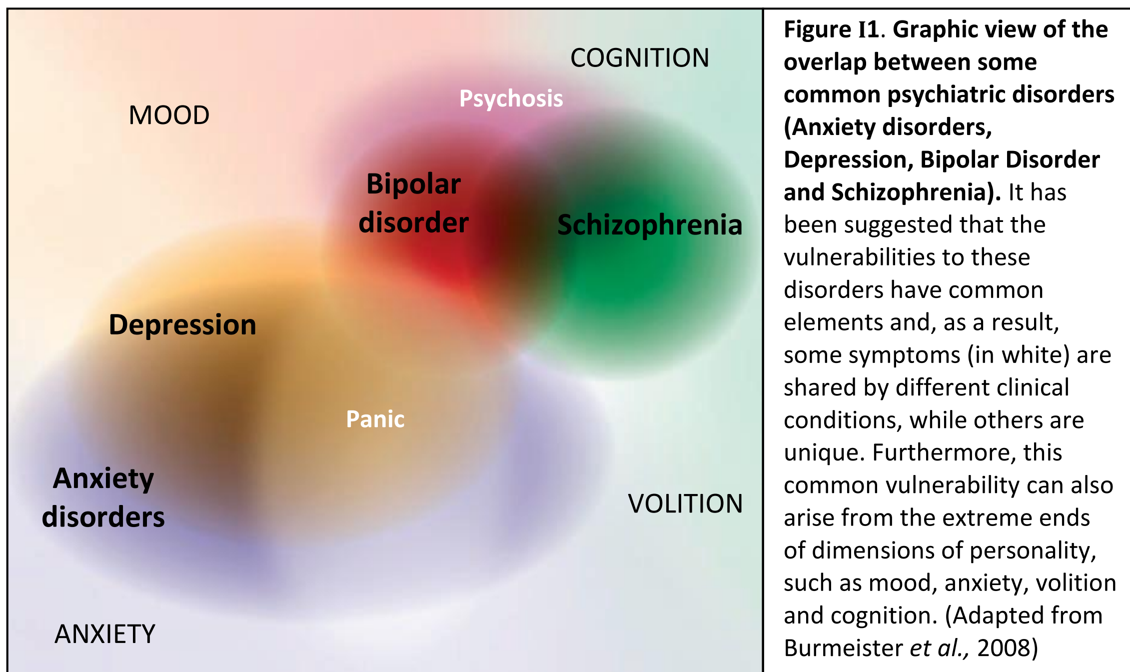


Figure I1. Graphic view of the overlap between some common psychiatric disorders (Anxiety disorders, Depression, Bipolar Disorder and Schizophrenia). It has been suggested that the vulnerabilities to these disorders have common elements and, as a result, some symptoms (in white) are shared by different clinical conditions, while others are unique. Furthermore, this common vulnerability can also arise from the extreme ends of dimensions of personality, such as mood, anxiety, volition and cognition. (Adapted from Burmeister *et al.,* 2008)

1.3. Epidemiological aspects

Since the famous World Health Organization (WHO) 10-country study (Jablensky *et al.,* 1992), the incidence of schizophrenia was considered to be similar across different cultures and countries (between 1.6% and 4.2% in all populations). Interestingly, this fact, among others, has linked schizophrenia to human evolution (Brüne, 2004). However, more recent revisions of this finding doubt this homogeneity, since it has been detected a higher incidence of schizophrenia disorders in males, urban areas (Sundquist *et al.,* 2004) and immigrant population (Cantor-Graae and Selten, 2005; Fearon *et al.,* 2006), among others. Moreover, symptoms usually develop earlier in males (in their late teens or early twenties) than in females (in the twenties and thirties) (Riecher-Rossler and Hafner, 2000). Furthermore, other environmental conditions have also been described to increase the risk for schizophrenia. Among them, some of the best studied are: maternal infections, prenatal malnutrition, maternal depression, hypoxia and neural damage, among others (Jablensky, 2003). Figure I2 shows the relative risks for some of the factors commented above. Although there are no clear signs to state or deny that these are causal factors of schizophrenia, these variations support the development of vulnerability x stress interaction models.

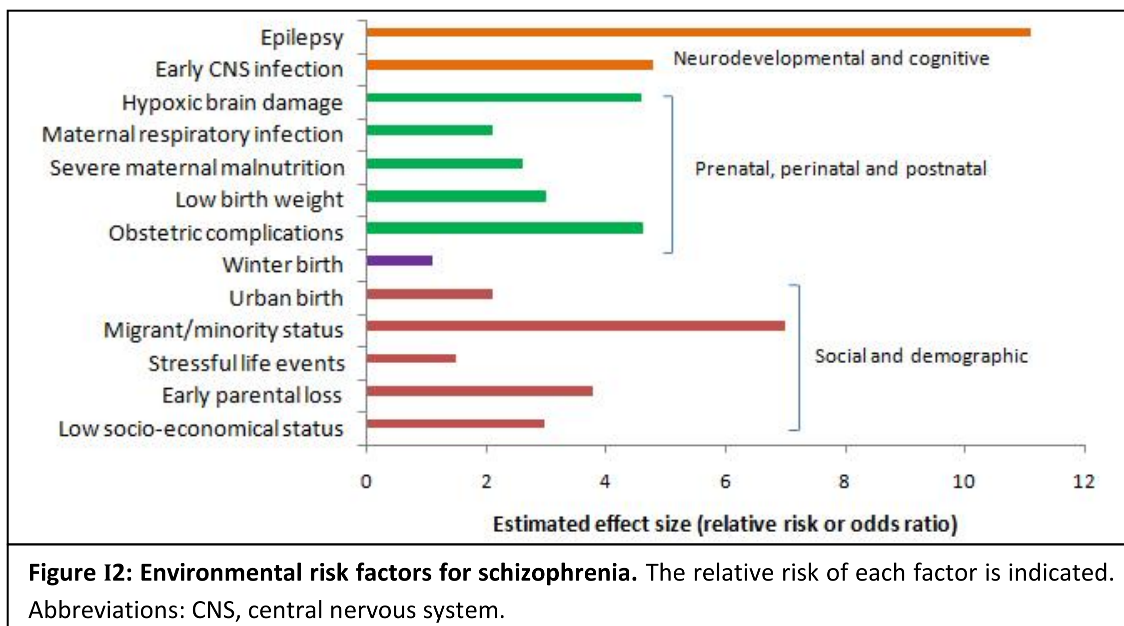


Figure I2: Environmental risk factors for schizophrenia. The relative risk of each factor is indicated. Abbreviations: CNS, central nervous system.

1.4. What neuropathological findings can tell us about schizophrenia: from brain structure to neurotransmitters.

Despite the wide variety of neuropathological findings related to the schizophrenia-spectrum psychoses in the last century, neither of these discoveries can be used as a diagnostic criterion for schizophrenia. First of all, the degree of overlap between patients and controls is too high to ensure a reliable diagnosis. Moreover, the great majority of symptoms is not specific for psychosis and can be also found in other neurological patients, thus restricting their utility in the clinical practice. Finally, we cannot forget that many of these findings, although replicated, are only partial and appear in a variable percentage of patients. And, unfortunately, many of these findings are discrete and have not even been replicated. However, all what we know about the neurobiology of schizophrenia can be the first step to understand in greater detail its physiopathological mechanisms.

1.4.1. Global macroscopic and histological abnormalities

Despite both the negative and unreplicated findings, there are some facts which can be reliably associated with the schizophrenic condition. Among them, the most important are the following (obtained from an interesting revision work from Tandon and colleagues (2008), who collected the most important evidences discovered so far):

- **Whole brain volume is reduced** in patients when compared to healthy subjects (Harrison *et al.,* 2003). Moreover, **lateral and third ventricles are enlarged** in approximately 20-30% of schizophrenic patients prior to the first psychotic episode (Elkis *et al.,* 1995; Harrison *et al.,* 2003). However, there is no correlation between ventricular size and cortical loss (Ward *et al.,* 1996). These findings are in coherence with post-mortem studies (reviewed in Wong and Van Tol, 2003).

- Structural brain abnormalities are found at illness onset, thus discarding the possibility that these abnormalities are a consequence of the progression of the disease or the neuroleptic medication.
- **Absence of gliosis** (Iritani, 2007), which is a sign of neurodegeneration. This evidence is an argument for a neurodevelopmental rather than neurodegenerative origin for schizophrenia.
- **Reductions in neuropil** (Selemon and Goldman-Rakic, 1999), which corresponds to those elements which are not the neural soma, as well as an altered localization of neuronal elements.

1.4.2. Neurophysiological abnormalities

Different neurophysiological alterations have also been found in schizophrenic patients (Tandon *et al.,* 2008):

- Abnormalities in the latencies and/or amplitudes of several **event-related potentials**, including P-50 and P-300 and the mismatch negativity (MMN).
- **Eye-movement alterations** in patients and, in a milder degree, in their healthy relatives.
- Affectation of the **sleep structure**, including shortening of the REM (rapid eye movement) sleep latency.

1.4.3. Abnormalities in the neurotransmitter systems

Studies about the mechanisms of action of antipsychotic drugs revealed that **dopamine** (DA) appeared to have a prominent role in the development of psychotic symptoms, since these drugs blocked the dopaminergic receptors. Moreover, amphetamine and cocaine, which interfere with the reuptake of DA, can also induce psychosis. These observations led to the development of the "dopamine hypothesis of schizophrenia" (Seeman, 1987), which has suffered different reformulations, in an attempt to adapt it to the new findings. The current hypothesis (Weinberger, 1987) defends the existence of a DA excess in the meso-limbic tracts, as a consequence of the hyperstimulation of D2 receptors. This DA hyperactivity would trigger the positive symptoms. Moreover, the lack of DA in the prefrontal cortex caused by the hypostimulation of D1 receptors would explain the negative and cognitive symptoms.

However, the study of **glutamate**, which is the primary excitatory neurotransmitter in the brain (Wong and Van Tol, 2003), led to the formulation of the "glutamate hypothesis of schizophrenia". This new approach considers the existence of a glutamatergic misbalance, especially in frontal regions, which would ultimately cause the dopaminergic alterations described above. This new view is supported by several evidences; the most replicated are that dopamine antagonists such as phenylcyclidine (PCP) and ketamine can induce psychotic symptoms (Halberstadt, 1995; Lahti *et al.,* 1995).

The **serotonergic system** cannot be excluded from the schizophrenia research since alterations in this system have been found in schizophrenic patients, although these abnormalities are milder than those observed in the DA system. The interest on the relationship between the serotonergic system and schizophrenia arouse out of observations about the effects of certain

drugs targeting the serotonin (5-HT) system. For example, there are important structural similarities between 5-HT and the lysergic acid diethylamide (LSD), one of the psychoactive drugs which mimics the action of serotonin, primarily at 2A receptors (Aghajanian and Marek, 2000; Geyer and Vollenweider, 2008). Moreover, it is also well known that the atypical antipsychotic drugs (Harrison, 1999; East *et al.,* 2002) have an effect on both serotonergic and dopaminergic receptors and there is also a partial effect of the selective serotonin re-uptake inhibitors (SSRIs) on the treatment of negative symptoms in schizophrenia (Silver, 2003). The effects of atypical antipsychotics led to another reformulation of the dopaminergic hypothesis as an imbalance between both neurotransmitter systems (Reveley and Deakin, 2000).

1.4.4. Neuroimaging findings

With the arrival of brain imaging techniques three decades ago, new possibilities appeared to observe in vivo which brain features are linked to the schizophrenic condition, and to understand the underlying processes of neuropsychiatric disorders. These techniques use a wide variety of methods (Gur *et al.,* 2007), like magnetic resonance (MR), which allows the study of a) structural neuroanatomy through structural resonance imaging (sMRI), b) neurochemistry and metabolite levels by means of magnetic resonance spectroscopy (MRS) and c) the activation pattern of brain areas using functional MRI (fMRI). Moreover, there are other functional methods such us positron emission tomography (PET) and single-photon computed emission tomography (SPECT).

With regard to **Structural magnetic resonance imaging,** the more replicated evidences can be grouped into three categories:

- The **reduction of whole brain volume and enlarged ventricles** observed in schizophrenic patients by different techniques when compared to controls has also been confirmed by sMRI (Lawrie and Abukmeil, 1998; Shenton *et al.,* 2001; Steen *et al.,* 2006). This reduction is of about 3%. Interestingly, the decreased cortical volumes and enlarged ventricular spaces have been also found in relatives of schizophrenic patients (Cannon *et al.,* 1998; Lawrie *et al.,* 1999) suggesting a genetic vulnerability to the disease.

- There are also **regional gray matter reductions**, which mainly affect the medial and superior temporal lobes (see Honea *et al.,* 2005 for a meta-analysis), prefrontal cortex (PFC) and thalamus (reviewed in Tandon *et al.,* 2008). Among these areas, the most severely affected region is the medial temporal lobe (MTL), specially hippocampus and amygdala (Lawrie and Abukmeil, 1998; Honea *et al.,* 2005). It has been also argued a relationship between a) positive symptoms and both hippocampus and temporal cortex size and b) negative symptoms, cognitive impairment and PFC size (Gur *et al.,* 2007).

- However, there are also some **artifactual findings**, such as the enlargements of basal ganglia (specially the globus pallidus) due to medication with typical antipsychotics (Gur *et al.,* 2007). Likewise, drug abuse can also trigger structural changes and this fact should be also taken into account.

Abnormalities in **functional neuroimaging** phenotypes have also been found in PET as well as fMRI studies using different paradigms and neural tasks. The most consistent evidences are:

- Abnormal activation patterns in several brain regions in **cognitive and emotional tasks**, as well as in resting condition. Of special interest is the hypoactivation of the prefrontal cortex, which is also associated with the characteristic negative symptoms of schizophrenia (Weinberger *et al.,* 1986; Glahn *et al.,* 2005). Moreover, the abnormal activation of temporal and parietal areas anatomically connected with PFC has been also detected. With regard to the temporal lobe, there is an increased activity in schizophrenic patients, according to several PET and SPECT studies (see Zakzanis *et al.,* 2000 for a meta-analysis).

- Regarding **psychotic symptoms**, it has been found that hallucinating patients show changes in the activation pattern of certain cortical areas with an important role in speech and processing of auditory stimuli (Shergill *et al.,* 2000; Woodruff, 2004).

- Moreover, **negative symptoms** have been also studied through several face recognition tasks. It has been shown that, with these paradigms, there is an abnormal activation of amygdala as well as motor and premotor cortical regions. These findings could be related to the difficulties of schizophrenic patients in social interactions (reviewed in Wong and Van Tol, 2003).

Finally, **MRS studies** have detected reductions in N-acetyl aspartate (NAA, a marker for neuronal loss and glia integrity) peaks in patients with chronic schizophrenia. These reductions encompass several regions, especially temporal cortex (hippocampus) and frontal cortex (Steen *et al.,* 2005; Abbott and Bustillo, 2006), and are associated, in those patients, with cortical atrophy, cognitive impairment and negative symptoms (Keshavan *et al.,* 2000).

1.5. Are these findings enough to formulate hypotheses about the origin of schizophrenia?

Researchers now agree on several basic facts which can serve as the start point to understand schizophrenia from a biological, neurodevelopmental point of view: schizophrenia is the result of an **interaction between the biological predisposition (genetic factors) and environmental aspects**. According to this scenario, the schizophrenic brain can suffer subtle alterations during its early development (including neonatal and postnatal processes) due to genetic factors and environmental stressors. These alterations may be affecting the cytoarchitecture, neuronal connectivity and/or neuronal plasticity. However, the presence of these alterations would not guarantee the development of schizophrenia, since certain environmental factors occurring later in childhood and adolescence (for example, vital events) would be necessary to trigger the first symptoms of schizophrenia. This general hypothesis, although correct in its presentation of the facts, is, however, currently poor in content, to such extent that it could be applicable to many other complex diseases. As De Lisi recently emphasized (2008), in response to a review article from Tandon *et al.,* (2008), only a few evidences can be considered for the moment as real facts in schizophrenia research. The main efforts are now focused in achieving a deeper understanding of the genetic factors and how they interact with the environment to give the final phenotype.

2. Genetics bases of schizophrenia

The hereditary component of psychiatric disorders was already postulated by Emil Kraepelin more than a century ago. Currently, psychiatric genetics is one of the most interesting focuses in the research of mental disorders. However, a necessary condition of any genetic study is the existence of a valid, precise and measurable phenotype. Nevertheless, mental disorders lack these phenotypes, since diagnostic is only made through clinical assessment (see table I1 as a reminder). Thus, it is necessary to tackle their study with particular methodologies which may help to unravel the causative molecular processes of these pathologies.

2.1. Genetic epidemiology of schizophrenia

Genetic epidemiology studies, which include familiar, twin and adoption studies, were the first approach to the genetics of mental disorders and have been widely used to disentangle the relative contributions of both genetic and environmental factors on the liability to these diseases. Family studies can determine the familialty of a mental disorder; however, it is impossible to verify whether the observed familial aggregation is due to environmental, genetic or both causes. For this reason, twin and adoption studies are used to separate the genetic component from the shared environmental factors.

Studies of families have shown that schizophrenia runs in families. First-degree relatives of schizophrenic patients have a higher degree (between 2% and 9%) to develop schizophrenia than the general population. This risk progressively diminishes in second degree (2%-6%) and third degree relatives (2%) (Kendler and Diehl, 1993; Shih *et al.,* 2004).

Twin studies are based in the comparison among the concordance rate for certain disorder in monozygotic (MZ) and dizygotic (DZ) twins. Since MZ twins share all their genetic material, concordance is expected to be higher in those siblings. Regarding schizophrenia twin studies, MZ twins have a concordance rate around 30-50%, while DZ have a lower concordance rate for schizophrenia (approximately 10%) which is similar to non-twin siblings (McGuffin *et al.,* 1984; Kendler and Robinette, 1983). This type of approach shows clearly that schizophrenia is predominantly a genetic disease, with an heritability around 80%, higher than other complex diseases such as breast cancer (5%-60%) and Parkinson disease (13%-30%), although in these cases the genetic factors are now better understood (Burmeister *et al.,* 2008). Moreover, the fact that the concordance rate in MZ twins is not close to 100% suggests the existence of shared environmental elements probably of prenatal and postnatal origin.

Adoption studies are a valuable tool to distinguish between inherited genetic factors and environmental factors. Children of schizophrenic biological parents adopted by unaffected parent are less likely to be exposed to environmental risk factors (Wong and Van Tol, 2003). According to this hypothesis, several studies have been performed and it has been shown that adopted children whose biological mothers suffered from schizophrenia had a higher liability to this disorder than those adoptees with biological mothers who were not schizophrenic (Heston, 1966; Lowing *et al.,* 1983; Tienari *et al.,* 2003). Furthermore, the adoptive relatives of

schizophrenic children had a risk for schizophrenia similar to the overall risk in the general population (Kety, 1987).

In summary, all these studies confirm that schizophrenia presents a complex pattern of high heritability which is also influenced and modulated by non-genetic environmental factors. Moreover, this disorder (and all mental disorders in general) does not fit a Mendelian classical model with a single highly penetrating gene.

2.2. The search for the schizophrenia genes. First approach: Linkage studies.

In 1980s and 1990s, polymorphic markers began to be available for genetic analysis, and scientists began to collect large family pedigrees, in an attempt to look for the genetic loci which were linked to different pathological conditions. A single DNA marker (which can be a VNTR [variable number in tandem repeats] or a single nucleotide polymorphism [SNP]) can be used to study whether certain allele of this marker cosegregates with the disease within a family more than expected by chance. In this case, it is said that the marker shows linkage to the clinical phenotype.

The first linkage studies were performed on monogenic diseases with a classical Mendelian transmission with a high success (for examples, see Knowlton *et al.,* 1985; Guilford *et al.,* 1994). They were based on **parametric linkage analysis**, which uses the LOD score (Logarithm of Likelihood ratio) to evaluate the level of evidence. This approach can use more than one marker simultaneously (multipoint analysis). A good standard for LOD score significance is $P = 0.00005$ ($LOD \geq 3.6$), which would be equivalent to a 5% chance of randomly finding linkage (Wong and Van Tol, 2003).

Unfortunately, the parametric linkage analysis implies certain difficulties when it is moved from monogenic to complex, poligenic diseases. Firstly, larger samples and pedigrees are needed to detect the low effect genes which are supposed to be implicated in complex diseases such as schizophrenia. Moreover, to apply the parametric approach, it is necessary to have a precise knowledge of the inheritance model, the gene frequencies and the penetrance of each genotype (Strachan and Read, 2004). However, the inheritance model and the genetic contribution of each gene on the disease are not normally known in mental disorders because of the use of groups of families with probably variable diagnostics and different causative genes each of them with variable effect. There are several ways to face up to these difficulties, such as the use of families in which the disorder segregates according to a near-mendelian model; to test several inheritance models (recessive and dominant, for example); the use of affected pedigree members only in a parametric analysis; or the use of a nonparametric (model-free) method of linkage analysis. The **nonparametric method of linkage analysis** ignores unaffected people and searches for alleles or chromosomal regions shared by affected individuals. This methodology can be used within nuclear families, extended families, or even in whole populations (in this latter case they constitute association analyses, which will be tackled in the next sections).

Linkage studies on schizophrenia pedigrees have found several loci although the results are enormously controversial, since there are contradictory findings or unreplicated results.

Therefore, the evidence of the linkage studies is limited, as acceptable significance levels have been achieved in only four studies (Straub *et al.,* 1995; Blouin *et al.,* 1998; Brzustowicz *et al.,* 2000; Williams *et al.,* 2003), specifically in chromosomic regions 6p22-24, 8p21-22, 1q21-22 and 10q25.3-q26-3. However, two meta-analyses have been useful to clarify the situation. Meta-analyses are useful because they amplify low signals which are general to most works, while strong signals from only one study are somehow silenced. Badner and Gershon in 2002 found three regions which reached genome-wide signification: 8p, 13q y 22q. Moreover, Lewis and colleagues performed in 2003 another meta-analysis (Lewis *et al.,* 2003) and, in this case, only one region (2p12-22.1) was found to be linked to schizophrenia under the most stringent criteria. Curiously, this region had not been found in any single study. Furthermore, under less stringent criteria, other regions emerged: 5q, 3p, 11q, 2q, 1q, 22q, 8p, 6p, 20p y 14q. It is likely that these regions contain genes implicated in the pathogenesis of schizophrenia. Then, the next step will be to find the specific genes which lay behind the positive linkage findings and to test its real implication in the pathophysiology of schizophrenia.

However, in the light of all these controversial linkage findings, it is really difficult to state that the chromosomic regions found with this approach really contain common allelic variants which increase the liability for schizophrenia. Firstly, it is possible that families in positive linkage studies may be segregating uncommon large effect genes. Moreover, each one of the positive regions may contain multiple susceptibility genes with a low effect on schizophrenia risk, but together can account for an important increase in the risk, detectable by linkage analysis (Harrison and Weinberger, 2005). Thus, it is really necessary to apply new techniques which can be helpful to unravel all these contradictory messages from the linkage analysis.

2.3. The use of genetic polymorphisms in association studies as a new approach to tackle the genetics of complex disorders.

It has been more than thirteen years since Neil Risch and Kathleen Merikangas demonstrated that when we face up to genetic variants with a small effect on the liability to suffer certain complex disease, association studies represent a more powerful tool than linkage analysis (Risch and Merikangas, 1996). Since then, association studies in psychiatric genetics (as well as in other complex diseases) have replaced linkage analysis as the preferred approach. But how can we define an association study? Although there are several types depending on the sample of individuals we are handling (unrelated subjects, family trios…) and the response variable we want to analyze (case-control phenotype, response to treatment, age at onset…), we can briefly say that the basic aim of **association analyses** is to evaluate if certain allele or genotype from a polymorphic variant appears in the affected group in a higher frequency than expected. These polymorphisms of interest would be selected because they would be located on those genes considered previously as candidate genes, either because they are part of a pathway which is known to be related to the disease **(biological candidate genes)** or because the genes are in a linkage peak **(positional candidate genes)** (Burmeister *et al.,* 2008). Moreover, another recent alternative to find associations with a certain disease is to scan polymorphisms across the whole genome (Carlson *et al.,* 2004). These studies are called genome-wide or whole-genome association studies (GWA). Furthermore, polymorphisms can be of different

categories, for example, variable number of tandem repeats (VNTRs), insertions-deletions (indels) and, more commonly, **single nucleotide polymorphisms** (SNPs).

Association studies led to the first discoveries of genetic risk factors for common diseases, such as Alzheimer disease (Strittmatter and Roses, 1996), diabetes (Dorman *et al.,* 1990) or inflammatory bowel disease (Hugot *et al.,* 2001). Most of these first studies were focused on functional variants which were considered as putative causal variants (or candidate polymorphisms). This approach to association studies is known as the **"direct approach"** (Risch, 2000). However, the search of all putative causal risk variants can become a difficult task in terms of money and time. For this reason, there is an alternative, known as the **"indirect approach"**, in which the association between a set of polymorphisms and certain pathology is studied, without considering whether those polymorphisms are really functional or not. This approach is based in the intrinsic structure of every genome: every variable position in our genome results from a single historical mutation event and each new allele will be associated with the other alleles present in the chromosomic region when this new allele arose. All those different alleles located in the same chromosome are called a **haplotype** and new haplotypes will emerge by additional mutations or recombination processes between the maternal and paternal haplotypes (Pääbo, 2003). Interestingly, the alleles which are present on each haplotype are subject to associations between them as a result of coinheritance processes. This association of certain alleles in the population is known as **linkage disequilibrium** (LD). It has been seen that, due to the LD relationships, the human genome can be divided into haplotype blocks (Gabriel *et al.,* 2002): considerably large regions over which there is little evidence for historical recombination and within which only a few common haplotypes are observed. Thus, this LD structure can be enormously useful in association studies: since all alleles in each block are inherited together, one can choose only a few of these polymorphisms to capture all the variability in the region of study. These polymorphisms, known as **"tag" SNPs**, will represent all those other SNPs in high LD with them (called proxies) which will not be included in the study (de Bakker *et al.,* 2005). Thus, these haplotype-based methods offer a powerful approach to disease gene mapping (figure I3).

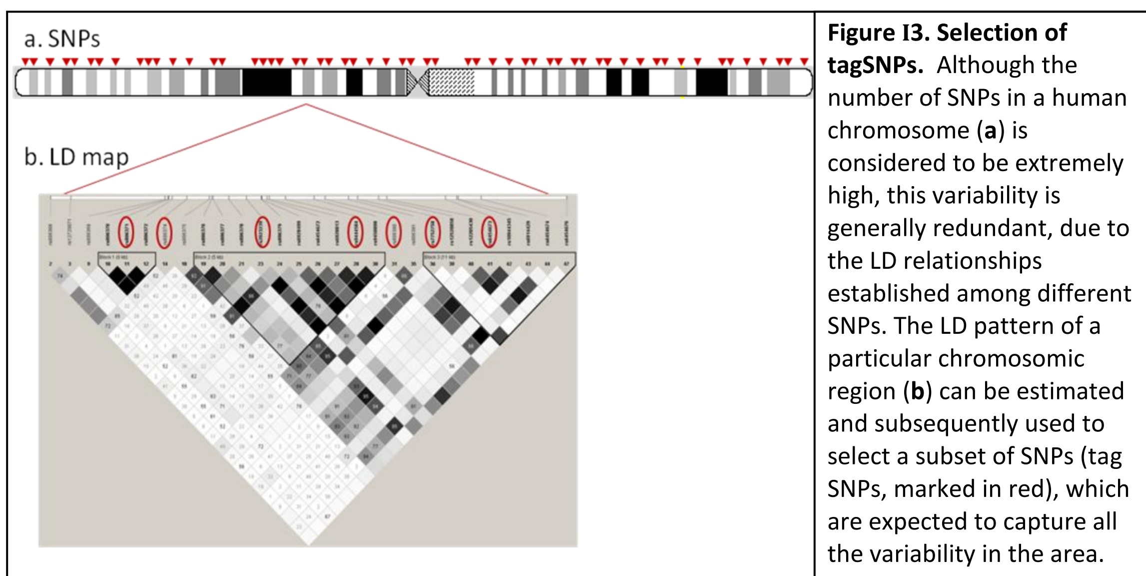


Figure I3. Selection of tagSNPs. Although the number of SNPs in a human chromosome (**a**) is considered to be extremely high, this variability is generally redundant, due to the LD relationships established among different SNPs. The LD pattern of a particular chromosomic region (**b**) can be estimated and subsequently used to select a subset of SNPs (tag SNPs, marked in red), which are expected to capture all the variability in the area.

However, this approach would have been unsuccessful without international initiatives such as the **HapMap Project** (The International HapMap Consortium, 2003). The HapMap Project meant a qualitative step forward in association studies, since its main objective is "to determine the common patterns of DNA sequence variation in the human genome and to make this information freely available in the public domain", in an attempt to accelerate the study of complex diseases. This project arose as a natural continuation of the Human Genome Project. When it was officially launched in October 2002, the number of non-redundant SNPs in the public SNP map from NCBI (dbSNP) was around 2.8 million (International HapMap Consortium, 2005); however, many regions were poorly represented. Currently, through its different phases, the project has increased the SNP coverage across the genome and the whole number of non-redundant SNPs has increased spectacularly (figure I4). Interestingly, there is also information available on some representative populations to ensure that the HapMap includes most of the common variation and some of the less common variation in different populations.

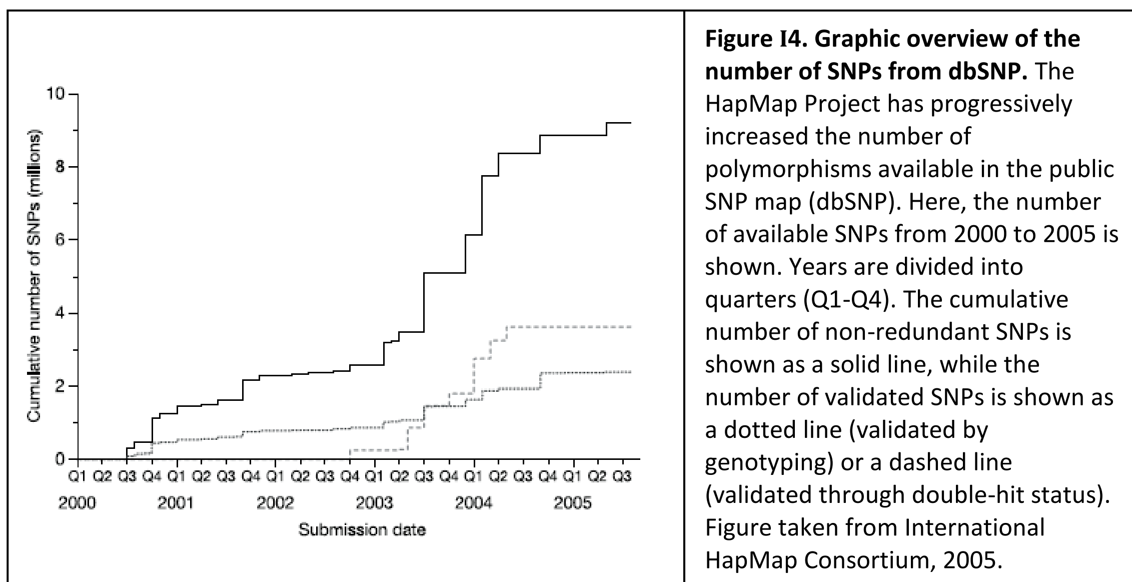


Figure I4. Graphic overview of the number of SNPs from dbSNP. The HapMap Project has progressively increased the number of polymorphisms available in the public SNP map (dbSNP). Here, the number of available SNPs from 2000 to 2005 is shown. Years are divided into quarters (Q1-Q4). The cumulative number of non-redundant SNPs is shown as a solid line, while the number of validated SNPs is shown as a dotted line (validated by genotyping) or a dashed line (validated through double-hit status). Figure taken from International HapMap Consortium, 2005.

With all this information available for the scientific community, the application of several association methods on complex diseases seems to be somehow easier.

2.3.1. Types of association tests.

2.3.1.1. Association tests for unrelated individuals

Case-control association test. This approach compares two groups of unrelated individuals with similar demographic characteristics (age, sex, ethnic group…) to avoid stratification problems: one group of affected individuals (cases) and another group of healthy subjects (controls). It is also necessary that all individuals come from the same genetic population, to avoid confounding effects due to differential population ancestries, such as admixture and population stratification, among others (Langefeld and Fingerlin, 2007). This analysis is based on testing the null hypothesis of no differences in the genotypic or allelic distribution between cases and controls (Balding, 2006). If the null hypothesis is rejected, then the polymorphism is

considered to be associated with the disease. There are different ways to test this hypothesis, for example, researchers can use approaches which are based on standard contingency table tests for independence, such as the **Pearson test** and the **Fisher exact test**. Among these two possibilities, the Fisher exact test is more appropriate, particularly if the size of some of the categories is small. However, it is also computationally more demanding.

However, the study of complex disorders generally implies the study of genetic polymorphisms with an additive effect, which means that the heterozygote risk will be intermediate between the two homozygote risks. In this situation, Pearson and Fisher tests are not powerful enough to detect additive risks. Nevertheless, other alternatives exist, such as the **Cochran-Armitage test**, which tests the hypothesis of zero slope for a line that fits the three genotypic risk estimates best (Balding, 2006). However, under other models different from the additive one, the Cochran-Armitage test has a low power to detect association. Thus, other possibility to solve the problems outlined above is the **regression analysis**. When information about several genetic, phenotypic and environmental factors is available, the regression approach can adjust for all these factors. Although several regression tools exist, the **logistic regression** model should be employed for binary traits such as disease status (case or control).

Furthermore, during case-control association analyses it is also interesting to calculate other related parameters to evaluate the impact of each genetic factor on the risk. One of these measures is the **Odds Ratio** (OR), which evaluates the association between genetic susceptibility and the disease, that is, the OR associated to certain disease would be the quotient between the probability of suffering the disease and the probability of not suffering the disease, in the presence of certain genetic factor.

Finally, it should be noted that other categorical variables different from case-control status (presence or absence of auditory hallucinations, age at onset…) can be evaluated through all the methods described above.

Quantitative disease traits. Several quantitative traits, such as the scores for different clinical scales or cognitive tasks, can also be studied to search for associations with genetic polymorphisms. In those cases, simple linear regression can be used to test for an association between a genetic marker and a quantitative trait without adjusting for covariates (Langefeld and Fingerlin, 2007). Moreover, other phenotypic or genetic factors can be included in the model as covariables.

2.3.1.2. Family-based association analysis

Family-based association tests use family members as internal controls. For this reason, they are more resistant to population stratification (Langefeld and Fingerlin, 2007). Below there is a brief description of some of the most popular association tests based on families.

The **transmission-disequilibrium test** or TDT (Spielman *et al.,* 1993) is one of the most famous family-based tests and it is widely used to study the genetics of complex disorders on families. The TDT assumes that, under the null hypothesis, transmission of each allele is equally probable (Mendel's first law). Thus, this test examines how often each allele of each heterozygote parent is transmitted to their affected offspring. An overtransmission of one of

the alleles to the affected offspring is then interpreted as an association between the polymorphism and the disease. However, it is necessary that the genotypes from both parents are available to avoid biases due to allele frequencies in the population. Subsequently, new related methods such as the sib transmission/disequilibrium test (S-TDT) and Discordant Allele Test or DAT (reviewed in Langefeld and Fingerlin, 2007) were developed to overcome this problem, because these tests are based in the comparison of allele frequencies between affected subjects and their unaffected siblings.

Finally, when data about some parents and some discordant siblings is available, there are other tests which can use all this information. An example of this approach is the pedigree disequilibrium test or PDT, which combines data from the TDT and the S-TDT within families. Another variant is the **Family Based Association Test** or FBAT (Laird *et al.,* 2000), which is a generalization of the TDT, with the special feature that it can work with missing parental genotype data and relatedness among trios. This is especially useful for those diseases with a late onset, such as schizophrenia, where the obtaining of DNA samples from both parents can be particularly hard.

2.3.1.3. Haplotype association analysis

Another interesting strategy, applicable to both family-based and population-based association analyses, is to use haplotypes instead of single SNPs in the association test (Balding, 2006). This approach considers the block-like structure of the human genome. Interestingly, haplotype analysis is more powerful to detect association when several cis-acting causal variants are conferring disease risk (Balding, 2006). However, the phase is generally unknown and haplotypes must be inferred from the genotypic frequencies through different methods and algorithms. One of the most widely-used methods to calculate the haplotype frequencies is the Expectation-Maximization (EM) algorithm (see Zhao *et al.,* 2003 for a review), which obtains the maximum likelihood estimates of haplotype frequencies in the study sample. It is divided in two steps: the Estimation step, which allows the estimation of the haplotype pairs of each subject; and the Maximization step, which estimates those haplotype frequencies which maximize the probability to obtain the observed genotypes. Subsequently, other methods and modified EM algorithms have been also elaborated.

2.3.2. Results from the association analyses in schizophrenia

Since the first association studies began, a huge number of publications have emerged and many candidate genes and polymorphisms have been evaluated (figure I5). In some cases, the results have been promising and have been confirmed through other approaches.

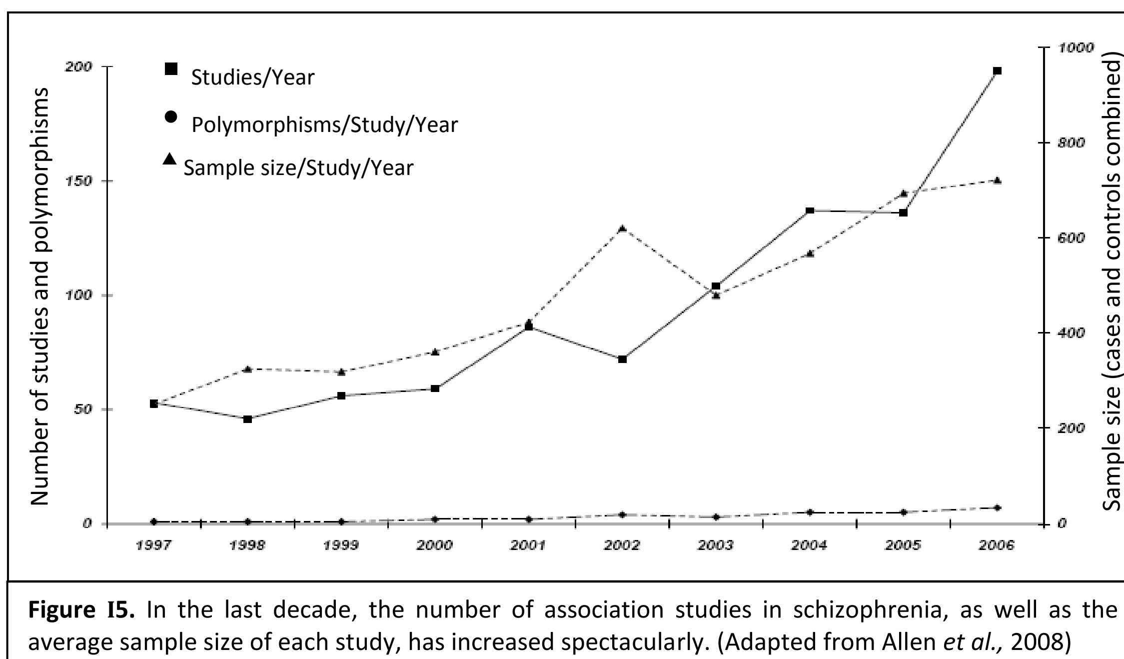


Figure I5. In the last decade, the number of association studies in schizophrenia, as well as the average sample size of each study, has increased spectacularly. (Adapted from Allen *et al.,* 2008)

There are some good revisions about this theme which can be helpful to show an overall vision of what has been done (Harrison and Weinberger, 2005; Norton *et al.,* 2006; Lang *et al*., 2007; Burmeister *et al.,* 2008). According to these revision works, the candidate genes with a stronger evidence of association with schizophrenia are the following:

- **Neuregulin 1 (*NRG1)*:** Attention was focused on this gene as a result of a study by Stefansson *et al.,* (2002), which found linkage with schizophrenia in an Icelandic sample and association with several SNPs and a seven-marker core haplotype. Interestingly, the region where *NRG1* lies (8p12-21) had been also previously related to schizophrenia in several linkage studies. Following the original Stefansson's report, association between *NRG1* gene and schizophrenia has also been found in other populations from Scotland, China and Ireland, among others (Stefansson *et al.,* 2003; Yang *et al.,* 2003; Corvin *et al.,* 2004). Moreover, association of the original core haplotype with bipolar disorder has also been detected (Green *et al.,* 2005). Thus, despite some negative findings (Iwatta *et al.,* 2004; Thiselton *et al.,* 2004), the overall results support the implication of *NRG1* in the pathogenesis of schizophrenia. However, there are discrepancies with regard to the haplotypes and SNPs implicated on each study. *NRG1* is expressed in the developing and adult human brain and is involved in several neural processes and signaling mechanisms, such as neuronal migration, myelination, synaptogenesis and neurotransmission (Harrison and Weinberger, 2005).

- **Dystrobrevin binding protein** or **dysbindin (*DTNBP1*)** is a promising schizophrenia candidate gene which maps to chromosome 6p22.3, one of the most consistently replicated schizophrenia linkage regions. Straub *et al.* (2002) reported, through a family-based analysis with individuals of Irish origin, that variation in dysbindin was associated with schizophrenia. This original finding has been replicated in different studies and samples (Numakawa *et al.,* 2004; Funke *et al.,* 2004; Vilella *et al.,* 2008) but not in other studies (Morris *et al.,* 2003). However, clear inconsistencies have also appeared, since risk and

protective haplotypes vary considerably among studies. Nevertheless, dysbindin expression is reduced in schizophrenic patients and several cis-acting variants that results in a reduction of dysbindin mRNA expression have also been discovered (Harrison and Weinberger, 2005), thus supporting the role of this gene in the etiology of schizophrenia.

- ***DAOA*** (*G72*) and ***DAAO*** genes: The *DAOA* gene (formerly known as *G72*) was cloned from the 13q linkage region and subsequently analyzed for association in a French-Canadian case control sample (Chumakov *et al.,* 2002). Interestingly, several SNPs and haplotypes in *DAOA* (and another overlapping gene called *G30*) were found to be associated with schizophrenia in this original study and replicated in other samples (Schumacher *et al.,* 2004; Zou *et al.,* 2005). In parallel with these results, associations between polymorphisms in the D-amino acid oxidase (*DAAO*) gene (interaction partner of DAOA according to Chumakov *et al.,* 2002) have also been found (Chumakov *et al.,* 2002; Schumacher *et al.,* 2004). However, some negative findings and the existence of allelic heterogeneity across studies can obscure the meaning of these associations. These two genes are involved in the metabolism of D-serine, an important element in the glutamatergic pathways (Harrison and Weinberger, 2005; Lang *et al.,* 2007).

- **Regulator of G protein signaling (*RGS4*)** gene: This gene, located in a putative linkage area at 1q22, encodes a protein, highly expressed in the central nervous system, which modulates G-protein mediated signaling via some dopamine, glutamate and muscarinic receptors (Harrison and Weinberger, 2005; Lang *et al.,* 2007). It was originally linked to schizophrenia susceptibility due to a study by Mirnics *et al.* (2001), who found a decreased *RGS4* expression in schizophrenic postmortem brains. Genetic associations between *RGS4* haplotypes and schizophrenia were subsequently found (Chowdari *et al.,* 2002; Williams *et al.,* 2004), although the evidence has been generally modest and inconclusive and in some cases the results have been negative (Sobell *et al.,* 2005). Further research is needed to elucidate the role of *RGS4* in the liability to schizophrenia.

- **Cathecol-O-methyl transferase (*COMT*)** gene: its plausability as a schizophrenia candidate gene lies on its role in dopamine catabolism and the existence of a functional variant (a valine to methionine polymorphism which affects protein activity and stability) that has been associated to schizophrenia in several studies (Egan *et al.,* 2001a; Shifman *et al.,* 2002). Moreover, this gene is located in 22q11, a region that, when hemideleted, produces velocardiofacial syndrome (VCFS), a medical condition associated with an increased risk of psychosis (Harrison and Weinberger, 2005). However, the results from association analyses are variable or even negative (see Glatt *et al.,* 2003 and Munafò *et al.,* 2005 for meta-analyses). Therefore, it seems that an effect exists, but it is modest.

- **Disrupted-in-schizophrenia 1 (*DISC1*)**: St Clair *et al.* (1990) described a Scottish family in which a balanced translocation involving chromosomes 1 and 11 [(1;11) (q42.1;q14.3)] was linked to a complex psychopathological inherited pattern, including schizophrenia, depression and mania. This fact led to the discovery of the *DISC1* gene at 1q42, a region previously linked to schizophrenia in some samples (Ekelund *et al.,* 2001; Hwu *et al.,* 2003). Different association studies subsequently found association between *DISC1* variation and schizophrenia and also with bipolar disorder (Hodgkinson *et al.,* 2004; Callicott *et al.,* 2005);

however, there are also negative findings. Moreover, in this case, significant haplotypes and SNPs also differed among studies although the involvement of this gene in the pathophysiological mechanisms of schizophrenia seems very probable. Its role in development, plasticity, neurite outgrowth and membrane trafficking of receptors also support this hypothesis (Harrison and Weinberger, 2005; Lang *et al.,* 2007).

Table I2 summarizes the current evidences coming from different approaches supporting the role of the genes presented above on the vulnerability to schizophrenia.

Table I2. Evidences supporting some promising schizophrenia susceptibility genes.

GENE	Locus	Linkage to SCZ	Association to SCZ	Altered expression in SCZ	Expression in PFC	Chromosomic aberration linked to SCZ	Biological plausability
NRG1	8p12-21	Yes*	Multiple studies	Yes	Yes	No	Yes
DAOA (G72)	13q32-34	Inconsistent	Multiple studies	Not known	Inconclusive	No	Inconclusive
DAAO	12q24	Inconsistent	Some studies	Yes	Inconclusive	No	Yes
RGS4	1q21-22	Yes*	Multiple studies	Yes	Yes	No	Yes
COMT	22q11	Yes*	Some estudies	Yes	Yes	Yes	Yes
DISC1	1q42	Yes	Multiple studies	Yes	Yes	Yes	Yes
DTNBP1	6p22	Yes*	Multiple studies	Yes	Yes	No	Yes

*linkage to SCZ supported by meta-analytic approaches.
Abbreviations: SCZ, schizophrenia; PFC, prefrontal cortex;
Information obtained from: Harrison and Weinberger, 2005; Sullivan, 2005; Sawamura and Sawa, 2006; Madeira *et al.,* 2008.

However, other candidate genes have also been explored as interesting and plausible candidate genes, although the evidences are currently less clear than for the genes presented above. Among them, we should highlight a) **genes related to the dopaminergic hypothesis**, such as the dopamine receptors (*DRD2* and *DRD3*), the dopamine and adenosine 3',5'-monophosphate (cAMP)-regulated phosphoprotein of 32 kD (*DARPP-32*) and the Brain-derived neurotrophic factor (*BDNF*) (Lang *et al.,* 2007); b) more **genes linked to the glutamatergic hypothesis of schizophrenia**, including the metabotropic glutamate receptor 3 (*GRM3*), among others (Harrison and Weinberger, 2005; Norton *et al.,* 2006); c) genes involved in the **response to antipsychotic medication**, such as the serotonin receptor 2A (*HTR2A* gene) (Norton and Owen*,* 2005); and finally, d) other genes involved in the **Phosphatidylinositide 3-kinase (PI3K) pathway** (Lang *et al.,* 2007), which is known to have a role in neuronal growth and migration, axonal sprouting and cell survival. This group of genes includes Reelin (*RELN*), *BDNF*, *AKT1 (*the most important downstream target of PI3K) and other well-known genes such as dysbindin and *NRG1*.

In summary, many genes have been associated to schizophrenia disorders; however, the results remain somehow inconsistent and controversial, even for the most replicated genes, due to the existence of negative findings, as well as differences among the positive studies, where the lack of consistent replication of the same markers and haplotypes is a normal issue. One extreme example of this situation is the dysbindin gene, where every one of the five major haplotypes in this gene has been reported to be associated with schizophrenia in at least one study (Mutsuddi *et al.,* 2006). For all the reasons exposed above, **genome-wide**

association (GWA) studies appeared very recently in the scientific panorama of schizophrenia as a powerful alternative based in high-throughput genotyping chips elaborated with the information given by the HapMap project. Until now, several GWA studies have been performed (table I3).

Table I3. Genome-wide association studies in schizophrenia.

Study	Design	Population	Number of SNPs	Sample size (SCZ/C)	Significant results (genes or regions)[a]
Mah *et al.,* 2006	Case-control	USA	25494	320/325	Yes (*PLXNA2*)
Lencz *et al.,* 2007	Case-control	USA	439511	178/144	Yes (*CSF2RA*, *IL3RA*)
Sullivan *et al.,* 2008	Case-control	USA	492900	738/733	No
O'Donovan *et al.,* 2008	Case-control	Mixed	362532	7308/12834	Yes (*ZNF804A*, 11p14.1, 16p13.12)
Kirov *et al.,* 2008	Family-based	Bulgaria	433680	574/1753	Yes (*CCDC60*, ***RBP1***[b])
Shifman *et al.,* 2008a	Case-control	Israel	510552	660/2771	Yes (***RELN***[b])

a Only the regions with the strongest associations are indicated.
b These genes had been previously considered as candidate genes for SCZ.
Abbreviations: SNP, Single Nucleotide Polymorphism; SCZ, schizophrenics; C, controls.

Unfortunately, despite some significant findings, the associated genes are different on each study and neither of the most important candidate genes previously found in the "classical" association studies has been replicated with the GWA approach. The situation is also quite similar with regard to GWA bipolar disorder studies (Burmeister *et al.,* 2008). Moreover, other very recent large scale studies and meta-analyses in schizophrenia could not replicate (Sanders *et al.,* 2008) or replicated partially (Allen *et al.,* 2008) some of the original findings in association studies of schizophrenia. Thus, at this point, scientists are divided on their opinions about the utility of the association studies to find schizophrenia susceptibility genes. The next section will evaluate some of the problems which may explain the inefficacy of the association approach.

2.3.3. Inconsistencies and limitations in association studies.

Several reasons can be invoked to explain the inconsistencies and difficulties derived from both case-control and family-based association analysis in the study of the genetics of schizophrenia. Firstly, there is evident **heterogeneity at a genetic level**. The most striking heterogeneity is found between different studies of the same candidate gene, where positive and negative associations coexist in the literature. This fact could be due to differences in the sample ascertainment or the existence of different risk factors depending on the origin of the sample. However, the heterogeneity can be also clearly observed across positive studies, since there is a **lack of strict replication**, that is, the risk alleles and haplotypes vary across studies. By contrast, association studies of other complex diseases, such as Type 1 and Type 2 diabetes mellitus or age-related macular degeneration, have produced consistent and strictly replicated findings (Sullivan, 2005). Perhaps schizophrenia has a high genetic heterogeneity, in contrast with these other pathologies. However, it is also plausible that many of the positive findings are actually **false positives** (type I error), due to the use of **stratified samples** of different genetic origin which could seriously distort the results. Moreover, **false negatives** (type II error) can also account for a considerable part of the inconsistencies, in coherence with the

widely accepted hypothesis of genetic risk variants with a very small effect. A high level of genetic heterogeneity (with many rare risk variants) would also favor the increase of type II errors. However, this type II error could be avoided with **larger samples**.

Moreover, **epigenetic mechanisms** should be also considered, since they may regulate the expression of several genes in the central nervous system (Petronis, 2004; Tsankova *et al.,* 2007). As a result, epigenetic processes may regulate complex behaviors without changes in the DNA sequence. Furthermore, changes in the epigenetic pattern can partially account for the existence of abnormalities in psychiatric disorders, such as depression or schizophrenia (Petronis, 2004).

Nevertheless, the genetic heterogeneity is not the only confounding element in psychiatric genetics, since the high **clinical complexity** of schizophrenia (with the existence of many symptoms which can vary among patients or during the course of the illness) also makes the comparisons among studies extremely difficult. Finally, risk **environmental factors** cannot be forgotten and undervalued but, unfortunately, the majority of schizophrenia association studies do not consider them in the analyses. These environmental factors can modulate the risk for schizophrenia independently or by means of interactions among different environmental factors, but also through **gene x environment interactions** (Caspi and Moffitt, 2006). Several recent studies have begun to show the importance of epistatic processes to properly understand the pathophysiology of schizophrenia and other mental disorders (Caspi *et al.,* 2003, 2005).

Thus, it is necessary to tackle alternative strategies to resolve the inconsistencies of the association approach.

3. Returning to the symptom: the alternative phenotypes in psychiatric genetics.

As we have seen, the phenotypic complexity of schizophrenia together with the probable lack of biological validity of the current nosology of mental disorders are limiting factors in understanding the genetic basis of schizophrenia. Therefore, new strategies should be defined. A critical issue in clinical psychiatry is that, despite the advances in neuropsychiatry in the last decades, the symptom is still poorly understood. Thus, to focus on the symptom instead of a syndromic phenotype appears to be a valid approach. As a result, during the last decade many researchers have begun to focus on other study phenotypes in an attempt to approach psychiatry to biology (table I4).

Table I4. Some alternative phenotypes used in schizophrenia genetic studies.

Phenotype	Examples	Advantages	Disadvantages
Broad phenotype	- Unique psychosis - Unique Mental Disorder	- Identification of common risk factors - Reconsideration of the nosology	Phenotypic differences cannot be explained
Extreme phenotypes	- VCFS - Chronic hallucinators - Catatonic psychosis	Consistent results in other complex disorders	It only explains a small fraction of the patients
Clinical subtypes	- Positive symptoms - Disorganized SCZ	Closer to the traditional psychopathology	Doubtful biological validity
Dimensions of personality	- Schizotypy - Schizotaxia	Continuum with normal personality	Doubtful biological validity
Neurophysiological alterations	- P50 and P300 - Eye movement	- Closer to genotype - Biological validity - Study through animal models	Not specific for schizophrenia
Neuroimaging phenotypes	- Hypofrontality - Brain size	Closer to the genotype	Not specific for schizophrenia
Hallucinations	- Auditory hallucinations	- High specificity -Close to the clinical diagnosis - Studied through neuroimaging	Episodic nature
Cognitive impairment	- Working memory - Attention	- Continuum with normal cognition - Studied through neuroimaging - Knowledge about its physiology	Not specific for schizophrenia

Abbreviations: SCZ, schizophrenia; VCFS, velocardiofacial syndrome.

Among the different phenotypes which could be chosen, one of the most widespread approaches is the deconstruction of schizophrenia in simpler elements to increase power and simplify the genetics. At this point, several interrelated concepts (usually treated as synonyms), such as **alternative phenotype, intermediate phenotype or endophenotype**, arise. All of them are based on the same principle: since genes do not encode for psychopathology, we have to deal with the study of other phenotypes which are closer to the biological processes underlying psychiatric diseases. The reason to use these phenotypes would lie in the belief that these entities involve fewer genes than the whole disorder (Gottesman and Gould, 2003).

The concept of **endophenotype** was formulated more than thirty years ago by Gottesman and Shields (1973); however, its popularity only grew in the last decade as a result of the difficulties to find the underlying genetic causes of psychiatric disorders. It was originally defined as an internal trait which is presumably causally closer to the gene than the pathology as a whole and can be detected through biochemical or microscopic tests (figure I6).

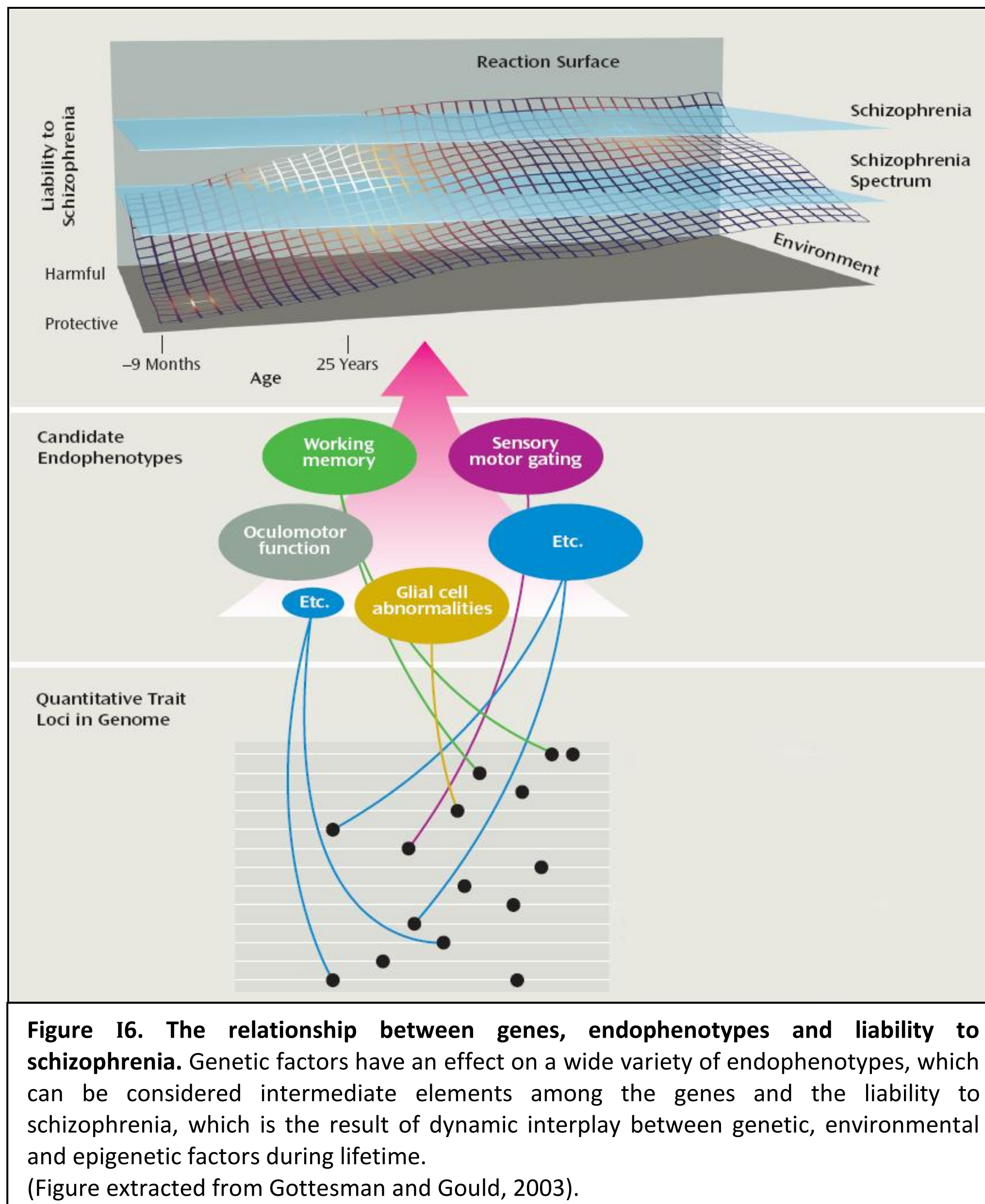


Figure I6. The relationship between genes, endophenotypes and liability to schizophrenia. Genetic factors have an effect on a wide variety of endophenotypes, which can be considered intermediate elements among the genes and the liability to schizophrenia, which is the result of dynamic interplay between genetic, environmental and epigenetic factors during lifetime.
(Figure extracted from Gottesman and Gould, 2003).

Although there is some controversy about which phenotypes are meaningful, the following are some necessary criteria (Gottesman and Gould, 2003; Prathikanti and Weinberger, 2005; Bearden and Freimer, 2006):

- The endophenotype co-segregates with the illness in the population.
- It is heritable and detectable in family members of individuals with disorders associated with that phenotype at a higher rate than in the general population.
- It should be associated with the causes rather than consequences of the disorder.
- They should have good reliability, validity and good psychometric properties.
- It should be distributed continuously (or even normally) in the general population.
- It must be stable over time and can be present even when the disease is not active (state independent).

According to these criteria several clinical measures have been found to be useful in schizophrenia research. These include several cognitive deficits related to executive function and attention, electroencephalogram (EEG)-evoked potential abnormalities and eye movement disfunctions, among others (Prathikanti and Weinberger, 2005; Flint and Munafò, 2007).

In contrast with the endophenotype concept, but at the same time closely related to it, we consider that the **alternative phenotype** particularly refers to those clinical symptoms which can be used as selection criteria instead of the traditional DSM or ICD criteria. These phenotypes are useful for several reasons. Firstly, they allow a more precise selection of patients for molecular genetic studies and facilitate the collection of more homogeneous patient samples. Secondly, an alternative phenotype should represent a simpler pathopshysiological pathway, which can be studied by different approaches with probably more chances of success than the schizophrenia phenotype as a whole.

Neuroimaging phenotypes also need a special mention since they can be excellent endophenotypes which are really closer to the genotype than a behavioral phenotype. Moreover, they can also be useful tools to investigate other alternative phenotypes or endophenotypes such as cognitive dysfunction or auditory hallucinations.

For the present study, two alternative phenotypes have been chosen: auditory hallucinations and cognitive impairment.

3.1. Auditory hallucinations in psychoses.

Hallucinations have been defined as sensorial experiences in a conscious state in the absence of an external sensorial stimulus with characteristics of a real perception, since they are vivid, substantial, and located in external objective space (David, 2004; Sanjuán, 2006). Several studies have shown that hallucinations are phenomenologically heterogeneous events as they can be of different sensorial nature: visual, auditory (verbal or musical), olfactory, gustatory, tactile, kinesthetic, hypnagogic (at sleep onset), hypnopompic (during awakening) or even multimodal. Moreover, hallucinations also present phenomenological variations across clinical populations and may be clearly influenced by the person's mood, emotional experiences suffered in the past and cultural beliefs (Aleman and Larøi, 2008). Interestingly, hallucinations can be present even in healthy individuals with no psychiatric or neurological disorders and it has been hypothesized that there is a continuum between the perception of normal population and the perception of psychiatric patients; consequently, hallucinations should be viewed as a dimensional phenomenon rather than a categorical event.

Auditory hallucinations (AH) are the most common type of hallucinations in psychotic patients, with prevalence between 50% and 70% in schizophrenic patients (Slade and Bentall, 1988; Andreasen and Flaum, 1991). Although they may involve simple sounds, AH in psychosis generally consist of a voice or voices **(verbal hallucinations or "phonemes")** speaking about the subject's thoughts aloud, commenting the individual's behavior or giving instructions or commands (Aleman and Larøi, 2008). Interestingly, voices can be located inside or outside the head and this location can change over time. Content is also variable and, despite patients

usually describe AH as negative, they can also be perceived as positive or even pleasurable (Sanjuán *et al.,* 2004a; Aleman and Larøi, 2008).

However, the **dimensional nature** of auditory hallucinations is also a critical issue. Traditionally, different dimensions of AH have been considered (Oulis *et al.,* 1995; Nayani and David, 1996; Stephane *et al.,* 2003): a) physical dimension (which includes aspects such as intensity, localization and complexity of voices); b) emotional influence; c) insight (which refers to the belief about the origin of voices) and d) grade of control of AH. Among them, we consider that the **emotional component** has been particularly undervalued. Emotional factors may have a significant role in the hallucinatory experience of hearing voices. Firstly, certain states of high emotionality have been described as preceding psychosis (Freeman and Garety, 2003) and it has been also described an increase in anxiety prior to the onset of hallucinations. Secondly, auditory hallucinations in psychotic patients generally involve a highly emotional content (Nayani and David, 1996; Smith *et al.,* 2006), which seems to be correlated with the general emotional state (presence of depression, lower self-steem). Finally, the emotional response triggered by AH (which includes depression and anxiety states) may have important consequences in the development of the psychotic outbreaks. It has been also suggested that AH would be associated with auditory affective processing deficits (Rossell and Boundy, 2005).

In conclusion, the multidimensional characteristics of AH must be evaluated through appropriate **assessment strategies**, which include specific hallucination scales such as the Psychotic Symptom Rating Scale (PSYRATS), the Launay-Slade Hallucination scale (LSHS), the Beliefs About Voices Questionnaire (BAVQ) and the Verbal Hallucinations Questionnaire (reviewed in Aleman and Larøi, 2008).

To better understand the underlying processes controlling the hallucinatory experience, it is important to investigate which differential processes are taking place in the brain when a subject is suffering from AH. With this purpose, many **neuroimaging studies** have been performed, although these approaches generally do not take into account the dimensional nature of auditory hallucinations. With regard to **structural studies**, the best replicated finding is a reduction of gray matter (GM) volumes of the temporal lobe, especially the left superior temporal gyrus (STG), which is negatively correlated with the severity of hallucinations (reviewed in Aleman and Larøi, 2008). Interestingly, this region includes the primary auditory cortex. Moreover, volume reductions in other areas have been also reported in some studies. These areas include the dorsolateral prefrontal cortex (DLPFC), cerebellum and left inferior supramarginal gyrus (involved in speech perception). Moreover, connectivity studies through resonance diffusion tensor imaging (Hubl *et al.,* 2004) have found alterations in hallucinatory patients compared to controls in different white matter tracts located predominantly in left areas. Regarding **functional neuroimaging studies,** hallucination-related activity has been found in those areas related to the normal processing of auditory stimuli (Silbersweig *et al.,* 1995; Woodruff, 2004; Aleman and Larøi, 2008), such as those left regions involved in prosodic aspects of language, the emotional response and verbal monitoring. Some of the most replicated areas are the left superior temporal cortex, Broca's area, thalamus, basal ganglia and anterior cingulate cortex. In summary, the neuroimaging findings support the existence of a distributed network of cortical and subcortical areas involved in the whole hallucinatory experience. Of remarkable interest is the use of functional neuroimaging to study particular

dimensions of auditory hallucinations, such as the emotional response to voices. Regarding this issue, our group has developed an **auditory-emotional fMRI paradigm** (further explained in the Materials and Methods section) to evaluate the impact of emotional words on brain activation in hallucinatory patients and healthy subjects. In this study (Sanjuán *et al.,* 2007), patients showed stronger activation of prefrontal areas, temporal cortex, insula, cingulate and amygdala. The latter finding is coherent with the hypothesis that hyperactivation of the amygdala may be related to the affective components of positive symptoms of schizophrenia (Aleman and Kahn, 2005).

In the light of all these results, we consider auditory hallucinations to be a valid and **useful alternative phenotype** in psychiatric genetics for the following reasons:

- AH are the most frequent and characteristic feature of psychotic patients.
- AH can be easily identified and measured through different methodologies (clinical scales, neuroimaging protocols…).
- Despite the existence of AH in other types of patients, the phenomenon of "hearing voices" has very particular features in psychotic patients (table I5).
- Its importance for the clinical diagnostic and prognostic is high.

Table I5. Clinical features of auditory hallucinations in psychotic patients.

Form of hallucinations	Voices in second or third person speaking to the patient or talking between them about the patient.
Physical features	Variable frequency. Normal volume and variable location (inside or outside the patient's head).
Content of hallucinations	Generally negative content. Sometimes perceived as neutral or positive.
Triggering factors	Stress, drug abuse, poor response to medication.
Emotional response	Very high, normally with anxiety, irritation or anger. Some patients feel them as pleasurable.
Insight	Low level of insight: delusional explanations of hallucinations.

Information extracted from González *et al.,* 2003a.

Our hypothesis is that auditory hallucinations in psychotic patients are consequence of disturbances in specific neurobiological circuits related to the different dimensions of AH. The integrity of those circuits would be also mediated by **genetic factors**. The idea of genetic factors implicated in AH is not new. A classical study from Rosenthal and Quinn (1977) showed a unique case of monozygous quadruplets concordant for auditory hallucinations and schizophrenia. The authors concluded that a genetic predisposition to AH should be considered. However, until now, only a few studies have been focused on the **molecular genetics of hallucinations**, and most of them have been performed in neurological patients. Table I6 shows a summary of those studies.

Table I6. Molecular genetic studies of auditory hallucinations.

Study	Disease	Type of hallucinations	Gene	Polymorphism	Sample size
Malhotra *et al.,* 1998	SCZ	Auditory	*SLC6A4* (5-HT transporter)	5-HTTLPR	50 p
Wei & Hemmings, 1999	SCZ	Auditory	*CCK-AR* (CCK receptor)	779C/T	210 p
Tachikawa *et al.,* 2001	SCZ	Auditory	*CCK-AR* (CCK receptor)	-81A/G	87 p/100 c
Sanjuán *et al.,* 2004b	SCZ	Auditory	*CCK-AR* (CCK receptor)	779C/T	105 p
Sanjuán *et al.,* 2006a	SCZ	Auditory	*FOXP2*	rs2396753	186 p
Holmes *et al.,* 1998	Alzheimer	Auditory/Visual	*HTR2A/HTR2C* (5-HT receptors)	T102C/Cys23Ser	211 p
Goetz *et al.,* 2001	Parkinson	Visual	*DRD3* (dopamine receptor)	Ser9Gly	88 p
Wang *et al.,* 2003a	Parkinson	Visual	*CCK* (cholecystokinin)	-45C/T	160 p
Limosin *et al.,* 2004	Alcoholism	Visual	*DAT* (dopamine transporter)	VNTR	64 alcoholic women
Fujii *et al.,* 1999	Parkinson	Undetermined[a]	*CCK* (cholecystokinin)	-45C/T	116 p
Okubo *et al.,* 2002	Alcoholism	Undetermined[a]	*CCK-AR* (CCK receptor)	-85C/G	201 p/98 c
Chang *et al.,* 2004	Alzheimer	Undetermined[a]	*ApoE* (Apolipoprotein E)	ε4 allele	135 p
Goldman *et al.,* 2004	Parkinson	Undetermined[a]	*CCK* (cholecystokinin)	-45C/T	86 p

Note: CCK is a neuropeptide whose function is directly related to the DA system.
a No information about the modality of hallucination is provided by the authors.
Abbreviations: SCZ, schizophrenia; 5-HT, serotonin; p, patients; c, controls.

Therefore, a deeper study on the genetic basis of auditory hallucinations is still necessary. We also consider that auditory hallucinations could be a good environment to study the emotional dysfunction suffered by schizophrenic patients.

3.2. Cognitive dysfunction in schizophrenia.

Cognitive impairment has been traditionally described as one of the most severe symptoms of schizophrenia, which accounts for much of the psychosocial disability found in these patients (Breier *et al.,* 1991; Green, 1996; Lewis, 2004). Both global and specific cognitive deficits in schizophrenic patients can be found in the literature. These deficits also have a high prevalence in **adult patients**. In an exhaustive meta-analysis, Heinrichs and Zakzanis (1998) reported that 61%-78% of schizophrenic patients scored below the normal median on all cognitive tests, while the largest effects were found in verbal memory, some measures of the intelligence quotient (IQ), as well as attention and word fluency. With regard to **children and adolescents with schizophrenia**, cognitive functions are also clearly compromised, with a low full-scale IQ and reduced ability for information processing, as well as deficits in attention and several types of memory, including working memory and episodic memory.

As a result of all these observations, some logical questions arise: are these cognitive deficits a consequence of the psychotic state, and if so, does the cognitive dysfunction worsen together with the progression of the illness? Several studies support the hypothesis that cognitive alterations are **not dependent on positive symptoms of schizophrenia and are relatively stable as the illness progresses** (Bedwell *et al.,* 1999; Elvevåg and Goldberg, 2000). Moreover,

some cognitive deficits, especially related to attention and working memory, can be also found prior to the onset of the disease (Davidson *et al.,* 1999; Cannon *et al.,* 2002a; Lewis, 2004)

Working memory seems to be one of the key elements to understand the cognitive dysfunction in schizophrenia, since many studies have reported an abnormal functioning of this system in schizophrenic patients (Goldberg *et al.,* 1987; Goldman-Rakic, 1994; Silver *et al.,* 2003). Working memory can be defined as a limited capacity system which maintains, stores and works with relevant information, through complex processes involving thinking, language and goal-directed behavior (Baddeley, 2003). Working memory is in fact extremely complex, since the current hypotheses suggest that it really involves many systems related to attention, storage of peripheral information and executive function (Baddeley, 2003; Tan *et al.,* 2007). This memory system is also necessary for a wide range of cognitive abilities, such as reasoning, learning and comprehension.

Currently, there is a great interest in studying the cognitive impairment of schizophrenic patients in depth. This interest has been partially motivated by the fact that, unless cognitive deficits in schizophrenic patients are potentially treatable, the current antipsychotic drugs used to treat the positive symptoms of schizophrenia have been found to be useless with regard to cognitive symptoms and, as a result, there is an intense search of drugs which can relieve some of the cognitive alterations suffered by those patients. Therefore, a deep study of the neurobiology of cognitive processing in schizophrenia is necessary to achieve this goal. Table I7 shows some cognitive and neuroanatomic traits frequently studied in psychiatry.

Table I7. Cognitive and neuroanatomic measures studied in schizophrenia research.

MEASURE	COGNITIVE DOMAIN
Neurocognitive tasks	
California Verbal Learning Test	Verbal memory
Logical memory	Verbal memory
Visual reproduction	Visual memory
Wisconsin Card Sorting Test	Executive function
N Back task	Working memory
Trailmaking Test, A and B	Attention and executive function
Digit symbol	Processing speed
Spatial span	Working memory and attention (visual)
Digit span	Working memory and attention (verbal)
Perceptual span	Sustained attention and visual search
Verbal fluency	Language
Neuroanatomic measures	
Gray matter density	
White matter volume	
Hippocampus size	
Frontal lobe volume	
Prefrontal activity[a]	Working memory, attention
Hippocampal activity[a]	Working memory, episodic memory

a While performing an specific neurocognitive task that engages this brain region.
Information extracted from Bearden and Freimer, 2006.

Interestingly, cognitive abilities can be considered as some of the most valid and useful endophenotypes for schizophrenia genetics research. Several reasons support this idea:

- **Cognitive abilities** (memory, processing speed, attention, overall IQ…) seem to be **highly heritable in humans**. Evidences from twin studies corroborate this hypothesis in healthy subjects (McClearn *et al.,* 1997; Devlin *et al.,* 1997) and schizophrenic patients (Cannon *et al.,* 2000; Toulopoulou *et al.,* 2007). Moreover, several cognitive abnormalities related to schizophrenia have been found in healthy siblings of affected individuals with a higher prevalence than in normal population, thus implying a higher relative risk for those siblings (Keefe *et al.,* 1997; Kremen *et al.,* 1994; Cannon *et al.,* 2000; Egan *et al.,* 2001b). Particularly, several family (Park *et al.,* 1995; Conklin *et al.,* 2000) and twin (Goldberg *et al.,* 1988; Cannon *et al.,* 2000) studies also support the heritability of working memory deficits in schizophrenia.

- **Functional neuroimaging techniques** can be considered a powerful tool which allows the correlations of the poor performance in cognitive tests with alterations in certain neural systems. There are many evidences that the prefrontal cortex (PFC), and especially the DLPFC, is involved in human working memory (see Tan *et al.,* 2007 for a review). Many studies have also found a prefrontal cortical physiological dysfunction in schizophrenic subjects compared to controls. These abnormalities have been also found in healthy relatives and twins of schizophrenic patients, as well as in subjects with an At Risk Mental State (ARMS) (Fusar-Poli *et al.,* 2007). What these functional findings mainly show is a reduced blood oxygenation level-dependent (BOLD) activation in the PFC of schizophrenic patients (Callicott *et al.,* 1998; Carter *et al.,* 1998; Stevens *et al.,* 1998; Barch *et al.,* 2001; Perlstein *et al.,* 2001). Moreover, in those studies using the N-Back task (which is known to activate storage and executive processes related to working memory), schizophrenic subjects exhibit a decrease in performance accuracy and reaction time, which normally correlates with a decrease in prefrontal activation. This pattern is also specific to schizophrenia when compared to other psychiatric disorders (reviewed in Tan *et al.,* 2007). Thus, these findings suggest that abilities on working memory tasks in schizophrenia may reflect differences in **capacity limitation** between patients and controls. Capacity limitation refers to a decrease in the performance as the memory load increases. Thus, the activity of prefrontal regions would adjust to a nonlinear "inverted-U" shaped physiological response (Callicott *et al.,* 1999). A representation of this activation pattern can be found in figure I7.

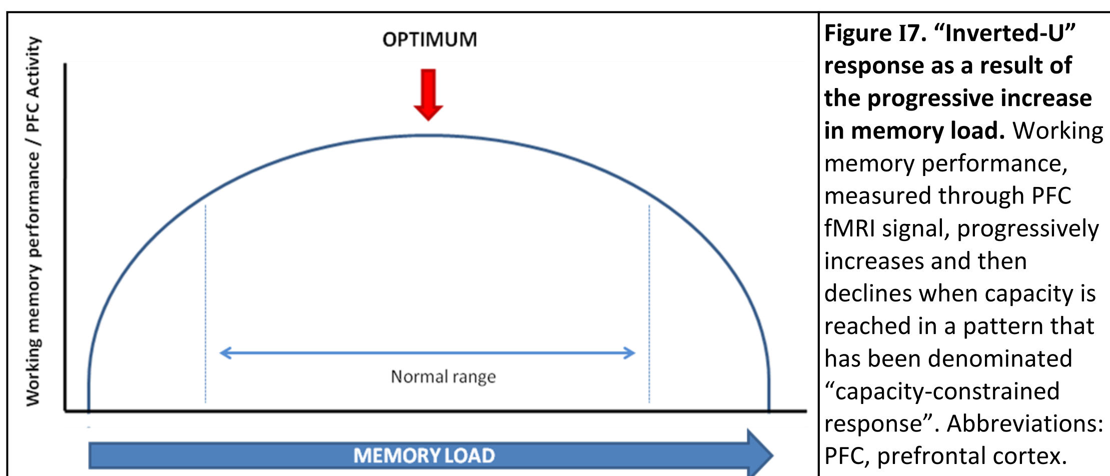


Figure I7. "Inverted-U" response as a result of the progressive increase in memory load. Working memory performance, measured through PFC fMRI signal, progressively increases and then declines when capacity is reached in a pattern that has been denominated "capacity-constrained response". Abbreviations: PFC, prefrontal cortex.

- **Structural neuroimaging** can also supply valuable information with regard to cognition in schizophrenic patients. It is well established that there is a clear relationship between intelligence and regional brain volumes (Toga and Thompson, 2005). Moreover, gray matter density and brain morphology are highly heritable traits (Bartley *et al.,* 1997; Cannon *et al.,* 2002a; Thomson *et al.,* 2001) and neocortical gray matter volumes are also heritable and correlate with several cognitive domains (Thomson *et al.,* 2001). Furthermore, several volume deficits seem to be related to genetic liability to schizophrenia (Cannon *et al.,* 1998; Cannon *et al.,* 2002b; Goldman *et al.,* 2008; Honea *et al.,* 2008).

As a result of the previous findings related to cognition and risk for schizophrenia, **imaging genetics** appears to be one of the most powerful strategies to study the underlying mechanisms of cognitive dysfunction affecting schizophrenic subjects. Although behavioral and cognitive tasks can give valuable information per se, these phenotypes are undoubtedly less sensitive to detect the effect of a risk allele of small effect, since they are specially away from genotype (Meyer-Lindenberg and Weinberger, 2006; Roffman *et al.,* 2006). By contrast, neuroimaging captures biological processes which are closer to the genotype. Thus, imaging phenotypes are expected to be more directly predicted by the genotype than a pure behavioral or clinical phenotype. In conclusion, the combination of both cognitive tasks and neuroimaging tools are becoming essential elements to elucidate the genetic mechanisms of cognitive dysfunction in schizophrenia.

Some examples of imaging genetics applied to the study of cognitive deficits in schizophrenia will be briefly described now. One of the most studied genes is *COMT*, because of its clear relationship to the dopaminergic hypothesis of schizophrenia. Several studies have been performed to elucidate how the functional Val158Met polymorphism influences on working memory phenotypes (reviewed in Meyer-Lindenberg *et al.,* 2006; Roffman *et al.,* 2006; and Tan *et al.,* 2007). *COMT* genotypes were found to account for approximately 3-4% of the variance in performance of tasks involving prefrontal cortex (such as the Wisconsin Card Sorting test or WCST), with a poorer performance for the Val-allele carriers when compared to the Met-allele carriers (Egan *et al.,* 2001a; Nolan *et al.,* 2004; de Frias *et al.,* 2006). This effect has been found even in healthy subjects. Subsequently, an effect of *COMT* variation has been seen in fMRI studies, since Val-allele carriers present a higher N-Back prefrontal activation than Met-allele carriers, suggesting that those subjects with the Val-allele are less efficient (Egan *et al.,* 2001a; Meyer-Lindenberg *et al.,* 2006). Likewise, these effects increased as memory load increases, adjusting to an inverted-U response (Mattay *et al.,* 2003).

Furthermore, other genes apart from *COMT* have also been reported to influence cognition and risk for schizophrenia (Roffman *et al.,* 2006; Tan *et al.,* 2007). Some examples are the *GRM3* gene, which encodes the metabotropic glutamate receptor 3; the *DAOA* (*G72*) gene; and the Ser-allele of the polymorphism Ser704Cys from *DISC1*.

4. Schizophrenia candidate genes selected for this study.

In the present study, several issues have been taken into account to select many different candidate genes. Basically, the selection has been focused on several interrelated aspects of schizophrenia: **auditory hallucinations, cognitive dysfunction and emotional response**.

Firstly, we are especially interested in those genes which could have a role in the vulnerability to **auditory hallucinations and/or cognitive dysfunction in schizophrenia**, which are the two alternative phenotypes we have decided to focus. However, another aspect of schizophrenia, the **emotional response**, has been considered of special interest, given its implication for the prognosis and outcomes of the pathological manifestations of schizophrenia. Particularly, the **emotional response to auditory hallucinations** is a critical but unappreciated aspect in the pathophysiology of AH.

On the basis of these criteria, nine genes have been included in this study. Some of them belong to systems which have been traditionally related to schizophrenia, while others represent new approaches to tackle the study of the genetics of schizophrenic processes.

4.1. Serotonergic system genes.

Serotonin or 5-hydroxytryptamine (5-HT) is a **monoamine neurotransmitter** (figure I8a) diversely present in plants and animals (Jonnakuty and Gragnoli, 2008). In mammals, it is mainly synthesized in the serotonergic neurons of the central nervous system (Fuller and Wong, 1990) and the enterochromaffin cells of the gastrointestinal tract (Furness and Costa, 1982). 5-HT synthesis requires the presence of the essential amino acid L-tryptophan, which is hydroxylated to 5-hydroxy-L-tryptophan (5-HTP) via the enzyme tryptophan hydroxylase (figure I8b). This first step constitutes the rate-limiting step in the synthesis of serotonin (Jonnakuty and Gragnoli, 2008).

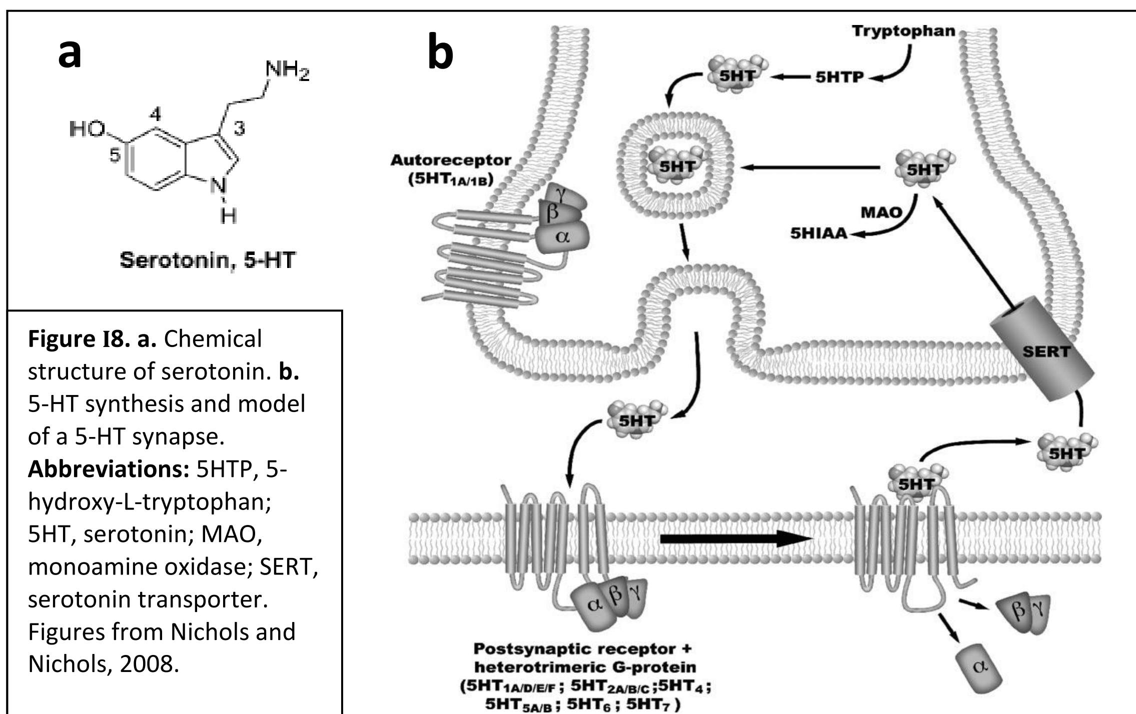


Figure I8. a. Chemical structure of serotonin. **b.** 5-HT synthesis and model of a 5-HT synapse. **Abbreviations:** 5HTP, 5-hydroxy-L-tryptophan; 5HT, serotonin; MAO, monoamine oxidase; SERT, serotonin transporter. Figures from Nichols and Nichols, 2008.

In mammalian species, serotonin has an important role in the central and peripheral nervous system. However, only a small fraction of total body serotonin is produced in the central nervous system (CNS), particularly in specialized groups of cell bodies known as the **raphe nuclei**, located in the brainstem reticular formation (Maurer-Spurej *et al.,* 2004). From the brainstem, serotonergic neurons project to virtually every part of the CNS (cortex, amygdala, hippocampus, cingulated gyrus, cerebellum…). Serotonin is produced within axon terminals and stored in secretory granules until it is released in response to an action potential from serotonergic neurons into a synapse and then diffuses across the synapse to activate postsynaptic 5-HT receptors (figure I8b). Here, the action of serotonin is terminated by reuptake via a specific transporter (SERT) located in the membrane of presynaptic terminals. Once again in the presynaptic neuron, it is metabolized by the monoamine oxidase (MAO) enzyme to 5-hydroxyindole acetic acid (5-HIAA).

As a result of its widespread projection pattern, serotonin plays a **key role in the regulation of several behavioral, physiological, and cognitive behaviors** (Jacobs and Azmitia, 1992), including motor function, memory, cognition and emotionality, as well as many endocrine functions (temperature regulation, sleep, food intake, sexual activity…). However, serotonin, like other monoamine neurotransmitters, also plays an initial role in regulating brain development and can also set its own terminal density, a phenomenon known as autoregulation of development (Whitaker-Azmitia, 2001).

The ubiquitous expression of serotonin receptors throughout the brain (Baumgarten and Grozdanovic, 1997) explains its multifunctional role in all the physiological systems explained above. The **serotonin receptor family** is larger than any other family of G-protein coupled (GPCR) neurotransmitter receptors: 13 receptors with seven transmembrane domains, which transduce signals through coupling to guanine nucleotide-binding regulatory proteins. In addition, there is one ligand-gated ion channel, the 5-HT3 receptor (Cravchik and Goldman, 2000; Hoyer *et al.,* 2002). Serotonin receptors are classified according to their structure, transduction signal, and pharmacology (Côté *et al.,* 2004). The earlier discovered 5-HT1, 5-HT2, 5-HT3, and 5-HT4 receptors have been studied extensively and their functional properties are the best understood of all the serotonin receptors. By contrast, 5-HT5, 5-HT6, and 5-HT7 receptors have been cloned within the last decade and their physiology is poorly understood (Cravchik and Goldman, 2000; Jonnakuty and Gragnoli, 2008).

Serotonin has long been implicated in the **pathophysiology of psychiatric disorders** such as depression, anxiety, aggressive behavior, obsessive–compulsive disorder (OCD), eating disorders and drug abuse (Giacalone *et al.,* 1968; Mehlman *et al.,* 1994; Bellivier *et al.,* 1998a; Bellivier *et al.,* 1998b; Lucki, 1998; Mann *et al.,* 2001). The serotonin system is one of the main targets for pharmacologic treatment of several psychiatric disorders and hallucinogens such as LSD and psilocybin inhibit the peripheral actions of 5-HT. This led to the hypothesis that brain 5-HT activity might be altered in psychiatric disorders. This hypothesis was strengthened by the introduction of tryciclic antidepressants and MAO inhibitors for the treatment of major depression and the finding that those drugs affect 5-HT and noradrenaline metabolism (Glowinsky and Axelrod, 1964). Moreover, selective serotonin reuptake inhibitors (SSRIs), which target the serotonin transporter, are used in the treatment of depression, OCD and panic disorder (Nutt *et al.,* 1999). 5HT2 receptors have also attracted interest as potential

pharmacologic targets for the treatment of schizophrenia, mainly due to the fact that some atypical antipsychotics show high 5HT2 to D2 receptors affinity ratio, relative to typical antipsychotic drugs (Meltzer *et al.,* 1989). 5-HT_{2A} receptor also mediates the effects of several hallucinogens. **Abnormalities in the serotonin system have also been found in schizophrenia**. In several cortical regions of postmortem schizophrenic brains, there have been reports on increased density of 5-HT_{1A} receptors (see Bantick *et al.,* 2001, for a review). This finding has been confirmed by PET studies (Tauscher *et al.,* 2002). Moreover, reduced densities of 5-HT_{2A} receptors (for review, see Dean, 2003) have been found in cortical regions of postmortem schizophrenic brains. Several abnormalities in serotonergic binding sites have been demonstrated in suicide victims such as reduced levels of 5-HT transporters and increased levels of 5-HT_{1A} receptors (Arango *et al.,* 1995) in the ventrolateral prefrontal cortex, and increased 5-HT_{2A} receptor binding in the PFC (Arango *et al.,* 1997). Finally, Hernandez and Sokolov (1997) found an approximately four-fold increase of serotonin transporter mRNA in Brodmann area 9 and a two-fold decrease in Brodmann areas 21 and 22 in a group of schizophrenic patients compared to controls.

Now, we will focus in the three serotonergic system genes which have been studied in this doctoral thesis work: the serotonin transporter gene (*SLC6A4*), the tryptophan hydroxylase 2 gene (*TPH2*) and the serotonin receptor 2A gene (*HTR2A*).

4.1.1. The serotonin transporter gene.

The serotonin transporter (SERT) is a protein of 630 aminoacids which is predicted to contain **twelve hydrophobic transmembrane** (TM) domains (figure I9). This solute carrier is part of the genic family of Na^+- and Cl^-- dependent **neurotransmitter sodium:symporters (NSS) for monoamines**, which also includes other cotransporters such as the dopamine transporter (DAT) and the norepinephrine transporter (NET). Its mechanism of action depends on the existence of a Na^+-K^+ gradient, and it is based on conformational changes as a result of the simultaneous binding of serotonin, Na^+ and Cl^- (Murphy *et al.,* 2004).

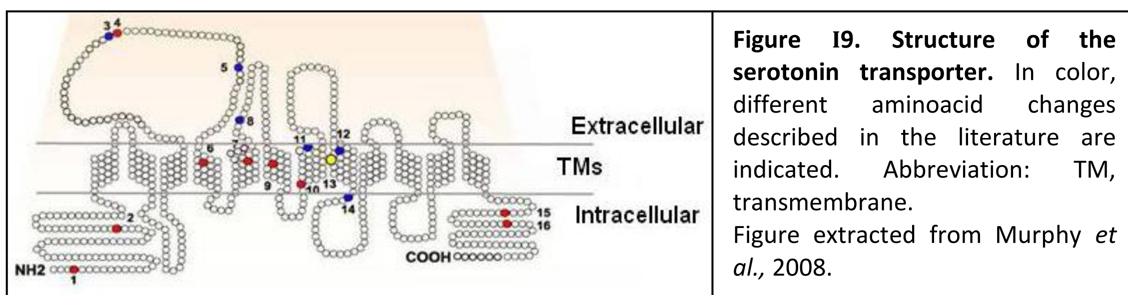


Figure I9. Structure of the serotonin transporter. In color, different aminoacid changes described in the literature are indicated. Abbreviation: TM, transmembrane.
Figure extracted from Murphy *et al.,* 2008.

The **human serotonin transporter gene** (official name: *SLC6A4*; other names: *5-HTT* or *SERT*) maps to chromosome 17q11.2, where it spans approximately 38 kb (figure I10). It is composed of 16 exons, including several alternative non-coding exons (1A, 1B and 1C), which, together with several alternative promoters and polyadenilation signals, have an important role in the regulation of gene expression (Lesch *et al.,* 1994; Bradley and Blakely, 1997; Ozsarac *et al.,* 2002). In addition to several regulatory domains which control selective expression in

serotoninergic neurons, transcriptional activity of *SLC6A4* gene is mainly modulated by a polymorphic repetitive element in the 5' regulatory region (**5-HTT- Linked Polymorphic Region or 5-HTTLPR**) located approximately 1.4 kb upstream of the transcription start site (figure I10). It consists of a deletion in a GC-rich region of repetitive elements, which mainly originates two alleles: a short allele (S) with fourteen repeat elements and a long allele (L) with sixteen repeat elements (Heils *et al.,* 1996). However, alleles with up to twenty repeats can be also found in a low frequency. Comparison of different mammalian species confirmed the presence of the 5-HTTLPR in platyrrhini and catarrhini (hominoids, cercopithecoids) but not in prosimian primates and other mammals (Lesch *et al.,* 1997). As a result, it has been speculated that the 5-HTTLPR region originated from a progenitor viral DNA or transposable element which may have been introduced into the genome around 40 million years ago (Lesch and Mössner, 1998).

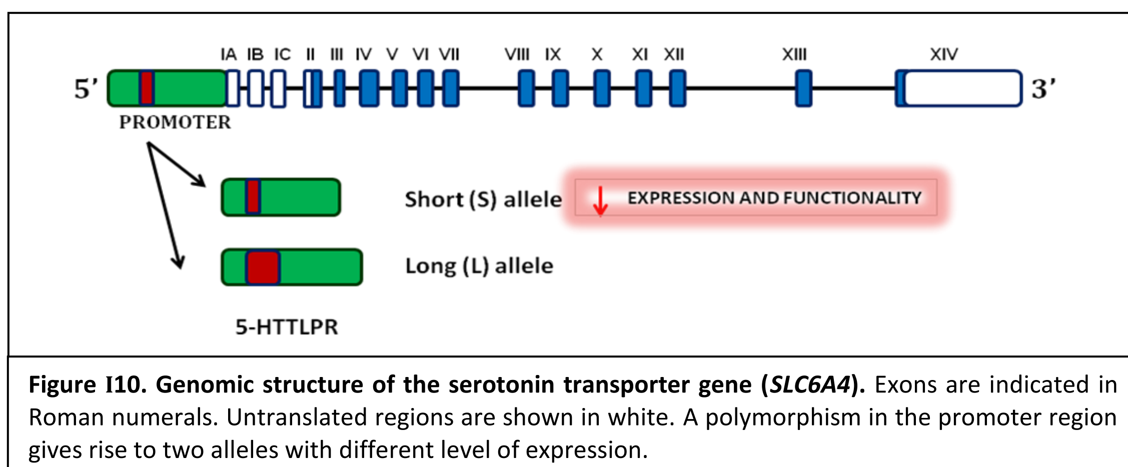


Figure I10. Genomic structure of the serotonin transporter gene (*SLC6A4*). Exons are indicated in Roman numerals. Untranslated regions are shown in white. A polymorphism in the promoter region gives rise to two alleles with different level of expression.

Interestingly, **5-HTTLPR regulates the transcriptional activity of the *SLC6A4* gene** and also affects protein expression and function. The S allele has been associated with lower *SLC6A4* expression and function when compared to the L allele (figure I10). This fact has been confirmed by several approaches, such as analysis of the promoter region in cell cultures, postmortem analysis of mRNA levels in the raphe complex of human brain, platelet serotonin uptake, responsivity of the serotonergic system to pharmacologic challenge tests with SSRIs and mood changes related to tryptophan depletion (Lesch *et al.,* 1996; Little *et al.,* 1998; Greenberg *et al.,* 1999; Mortensen *et al.,* 1999; Eichhammer *et al.,* 2003).

Moreover, **two recently discovered SNPs (rs25531 and rs25532),** located inside the 5-HTTLPR region, have been also reported to modulate the transcriptional activity of *SLC6A4* (Hu *et al.,* 2006; Wendland *et al.,* 2006, 2008). Of special interest is SNP rs25531, a relatively frequent A/G substitution immediately upstream of 5-HTTLPR that changes the consensus sequence for the activator protein 2 transcription factor (Kraft *et al.,* 2005). The combination of this SNP with the 5-HTTLPR variation generates four main alleles in the promoter region: the L_A allele with high activity, and the L_G, S_A and S_G alleles, which result in lower transcriptional levels and less serotonergic activity (Kraft *et al.,* 2005; Hu *et al.,* 2006; Gallinat *et al.,* 2007).

Additional variants at the *SLC6A4* locus have been also described. Among them, one of the most frequently studied is a 17-bp variable number of tandem repeats (VNTR) located in intron 2 (known as STin2), which seems to act as an enhancer during embryonic development (MacKenzie and Quinn, 1999). STin2 has two common alleles of 10 and 12 repeats. However, rare alleles of 7 and 9 repeats have been also found (Lesch *et al.,* 1994). Although the evidences are less than for the 5-HTTLPR variation, it has been reported that STin2 variation also affects *SLC6A4* expression (Hranilovic *et al.,* 2004). Other polymorphisms at the *SLC6A4* gene which have been previously studied include several common SNPs (Battersby *et al.,* 1999; Kim *et al.,* 2002; McCauley *et al.,* 2004) as well as rare functional SNP variants located in the coding region that change the structure or function of the serotonin transporter (reviewed in Murphy *et al.,* 2008).

The existence of several functional variants at the *SLC6A4* locus, as well as the unique and limiting role of the serotonin transporter in serotonin uptake, has focused the attention on this gene since the last decade. Great efforts have been made to know the role of the serotonin transporter genetic variation in several behavioral traits and psychiatric conditions. As a result, a large number of studies have related this gene to **several neuropsychiatric disorders**, especially anxiety-spectrum and mood disorders, such as bipolar disorder (Cho *et al.,* 2005), obsessive-compulsive disorder (Bengel *et al.,* 1999), suicidal behavior (Li and He, 2007), major depression (Kraft *et al.,* 2005; Levinson, 2006) and high neuroticism (Lesch *et al.,* 1996). These findings come from different approaches, including studies in humans, non-human primates and mice.

With regard to human studies, there is a large body of evidence about the implication of the short, low-expressing allele of 5-HTTLPR in **affective disorders**. Lesch *et al.* first reported in 1996 an association of the short allele with **anxiety related-traits** (neuroticism, anxiety and harm avoidance) in a large sib-pair sample. Subsequently, many other single studies (Mazzanti *et al.,* 1998; Willeit *et al.,* 2003; Jacob *et al.,* 2004, among others) and meta-analyses (Anguelova *et al.,* 2003) replicated this original finding. Moreover, other studies have found a relationship between the S allele of 5-HTTLPR and **other related behavioral traits**, such as shyness (Battaglia *et al.,* 2005), aggressiveness (Beitchman *et al.,* 2006) and suicidal behavior (Courtet *et al.,* 2004). It is also worth mentioning the work from Caspi and colleagues (2003), who found that the short allele of 5-HTTLPR predisposed individuals to **depression in response to life events**. Since then, these promising results have been replicated (Eley *et al.,* 2004; Kaufman *et al.,* 2004; Grabe *et al.,* 2005; Kendler *et al.,* 2005; Cervilla *et al.,* 2007). Studies with primate models also support this hypothesis (Bennett *et al.,* 2002; Champoux *et al.,* 2002). Furthermore, several fMRI studies have shown that the S allele of 5-HTTLPR produces an **increased amygdala response to fearful stimuli** in healthy subjects (Hariri *et al.,* 2002, 2005). Furthermore, higher amygdala activation associated with the s allele was found in psychiatric patients (Furmark *et al.,* 2004; Domschke *et al.,* 2006) as well as in phobic-prone healthy subjects (Bertolino *et al.,* 2005).

With regard to **mice studies**, the results from investigations with mice having one or no copies of the serotonin transporter gene (*Slc6a4* +/- and -/-, respectively) suggest that variation at *SLC6A4* gene can impact on the vulnerability to complex psychiatric and behavioral disorders, such as polysubstance abuse, REM sleep disorders and anxiety disorders, among others

(reviewed in Holmes *et al.,* 2003; Murphy *et al.,* 2008). An extraordinarily parallelism in several behavioral and neuroendocrine traits has been found between *Slc6a4* +/- mice and human subjects with anxiety-spectrum disorders (reviewed in Holmes *et al.,* 2003), thus supporting the role of serotonin transporter in emotionality. In conclusion, with regard to anxiety-related disorders, the use of *Slc6a4* +/- mice is very useful, since they resemble the S/S genotype from 5-HTTLPR.

However, the relationship between the genetic variation of *SLC6A4* gene and **schizophrenia-spectrum disorders** is still a matter of debate. Some studies have reported alterations related to the serotonin transporter in schizophrenic patients, such as reduced 5-HT reuptake sites in distinct brain areas of schizophrenics detected in postmortem radioligand binding studies (Joyce *et al.,* 1993; Naylor *et al.,* 1996) or a significant increase of *5-HTT* mRNA in the frontal and temporal cortex of schizophrenic subjects (Hernandez and Sokolov, 1997). However, association studies with *SLC6A4* polymorphisms (mainly 5-HTTLPR and STin2) have given inconsistent results. Table I8 is a summary from the SchizophreniaGene database (http://www.schizophreniaforum.org/res/sczgene/default.asp), which stores information about all the association studies of schizophrenia. As it can be seen, the results are mainly negative. However, recent meta-analyses have found evidences of association between the serotonin transporter STin2 polymorphism and schizophrenia, but not with 5-HTTLPR (Fan and Sklar, 2005; Allen *et al.,* 2008; Shi *et al.,* 2008).

Table I8. Summary of the association studies between the *SLC6A4* gene and risk for schizophrenia performed until now.

ETHNIC GROUP	NUMBER OF STUDIES	CONTROL SAMPLE SIZE[a]	SCZ SAMPLE SIZE[a]	RESULTS
Case-control studies				
Caucasian	16	236 (ranging from 62 to 587)	170 (ranging from 39 to 684)	1 positive 2 trend 13 negative
Asian	8	186 (ranging from 103 to 362)	181 (ranging from 90 to 338)	2 positive 6 negative
Other/mixed	1	15	12	Negative
Family-based studies				
Caucasian	3	236 (ranging from 206 to 266)	121 (ranging from 103 to 139)	2 positive 1 negative

a, the mean value and the range are indicated.
Abbreviation: SCZ, schizophrenia.
Information obtained from the SCZgene database (Allen *et al.,* 2008).

With regard to **auditory hallucinations** in schizophrenic patients, there is only one study from Malhotra *et al.* (1998), who reported an association between a higher intensity of hallucinations and the L/L genotype from the 5-HTTLPR polymorphism. Lesch (1998) hypothesized that the L/L individuals (with high levels of serotonin uptake and reduced synaptic 5-HT concentration) may be suffering a hypersensitivity of those serotonin subsystems which are activated by hallucinogenic stimuli.

For all the premises presented above, we consider that the genetic variation of serotonin transporter could have a role in the vulnerability to schizophrenia and the emotional alterations suffered by schizophrenic patients.

4.1.2. *HTR2A* gene.

The 5-HT_{2A} receptor belongs to the GPCR-type serotonin receptor family, which are also classified as "type A" rhodopsin-like receptors (Nichols and Nichols, 2008), all of them presenting highly conserved motifs within the seven transmembrane (TM) regions (figure I11), a strong evidence for an evolutionary relationship.

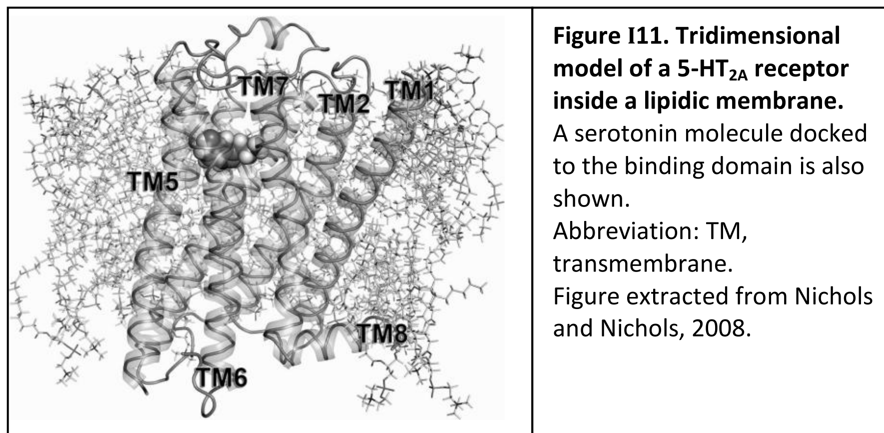


Figure I11. Tridimensional model of a 5-HT_{2A} receptor inside a lipidic membrane. A serotonin molecule docked to the binding domain is also shown. Abbreviation: TM, transmembrane. Figure extracted from Nichols and Nichols, 2008.

5HT_{2A} receptors are especially abundant in human cortical regions, particularly in frontal and temporal areas, and less abundant in basal ganglia (Pazos and Palacios, 1985; Pazos *et al.,* 1985). By contrast, they are rare at the thalamus (Wong *et al.,* 1987). This localization of 5-HT_{2A} receptors in the CNS is consistent with those neuroanatomical structures believed to be involved in the pathophysiology of schizophrenia. Higher resolution localization studies allowed locating the 5-HT_{2A} receptors to the heads and necks of dendritic spines (Jakab and Goldman-Rakic, 1998; Miner *et al.,* 2003).

The serotonin receptor 5HT_{2A} is encoded by the *HTR2A* gene, which is located in the long arm of chromosome 13 (13q14-q21). The *HTR2A* locus (figure I12) spans over 20 kb and consists of three exons separated by two introns (Chen *et al.,* 1992). The predicted 471 amino acid sequence of the human protein shares a high level of similarity with the mouse and rat 5HT2 receptors (Chen *et al.,* 1992).

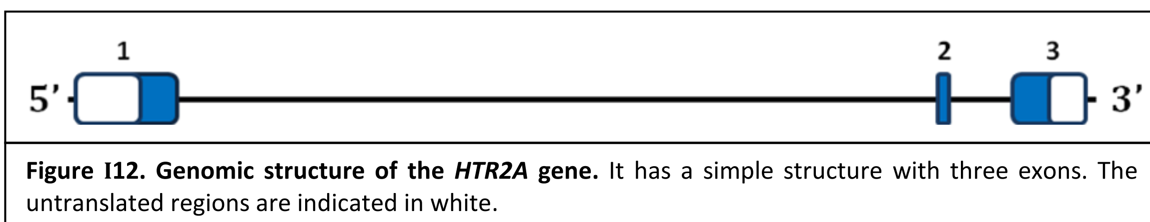


Figure I12. Genomic structure of the *HTR2A* gene. It has a simple structure with three exons. The untranslated regions are indicated in white.

Several polymorphisms along the *HTR2A* sequence have been described in the literature. The most important are the following:

- A thymidine to cytosine (T→C) silent polymorphism (known as T102C or rs6313) within the first exon of the gene which does not alter the predicted amino acid sequence of the receptor. This polymorphism has been the focus of most research. Remarkably, in human postmortem studies, the expression of $5HT_{2A}$ receptors in temporal cortex has been found to be about 20% less for the C allele than for the T allele (Polesskaya and Sokolov, 2002).
- An adenine to guanine (A→G) polymorphism at position -1438 in the putative promoter region (named -1438A/G or rs6311) of the *HTR2A* locus. This polymorphism is in perfect linkage disequilibrium with T102C.
- A histidine to tyrosine amino acid substitution at position 452 (known as his452tyr or rs6314), which seems to affect the agonist-induced desensitization process (Nichols and Nichols, 2008) as well as calcium mobilization (Cravchik and Goldman, 2000).

These three variants, as well as other polymorphisms of the *HTR2A* gene have been associated with many different psychiatric conditions, such as schizophrenia (Williams *et al.,* 1996; Inayama *et al.,* 1996; Lohmueller *et al.,* 2003), mood disorders (Enoch *et al.,* 1999), suicidal behavior (Li *et al.,* 2006), impulsivity (Nomura and Nomura, 2006), obsessive-compulsive disorder (Enoch *et al.,* 1998; Walitza *et al.,* 2002), eating disorders (Nishiguchi *et al.,* 2001), alcohol dependence (Nakamura *et al.,* 1999) and late-onset Alzheimer disease (Holmes *et al.,* 1998). There is an especial interest in understanding the potential role of the genetic variation at *HTR2A* locus in the pathogenesis of schizophrenia. Many evidences indicate that this gene may be really important. As it has been previously described, 5-HT_{2A} agonists (including LSD, psilocin and mescaline) possess potent hallucinogenic properties in humans that are highly correlated with their receptor affinities (Glennon *et al.,* 1984); moreover, the 5-HT_{2A} receptor has a high affinity for the atypical antipsychotic drugs such as clozapine, risperidone, and olanzapine and it is thought to have a role in the therapeutic activity of these drugs (Worrel *et al.,* 2000).

Until now, several association studies on the genetics of schizophrenia involving *HTR2A* polymorphisms have been performed. Moreover, several alternative phenotypes have been also studied, including a formal diagnosis (Williams *et al.,* 1996), earlier onset and poorer outcome (Joober *et al.,* 1999) and susceptibility to tardive dyskinesia (Segman *et al.,* 2001). However, the results still remain controversial, because of the high number of negative findings (table I9). Unfortunately, this uncertainty is also present when meta-analytic approaches are used: although three meta-analyses (Williams *et al.,* 1997; Lohmueller *et al.,* 2003; Abdolmaleky *et al.,* 2004) found an association of the 102C allele with schizophrenia, this finding could not be confirmed by another recent meta-analysis (Li *et al.,* 2006). By contrast, the most promising results come from studies which evaluate the response to antipsychotic drugs, especially clozapine, depending on *HTR2A* genotype (Arranz *et al.,* 1995, 1998; Ellingrod *et al.,* 2003; Anttila *et al.,* 2007). Data suggest that 102C, Tyr452 and -1438G alleles are associated with a poor antipsychotic response, since these variants are more frequent in patients who do not respond to the clozapine medication (known as non-responders). However, a proportion of studies focused on the response to clozapine have still produced contrary results (Nimgaonkar *et al.,* 1996; Lin *et al.,* 1999).

Table I9. Summary of the association studies between the *HTR2A* gene and risk for schizophrenia performed so far.

ETHNIC GROUP	NUMBER OF STUDIES	CONTROL SAMPLE SIZE[a]	SCZ SAMPLE SIZE[a]	RESULTS
Case-control studies				
Caucasian	28[b]	241 (ranging from 57 to 2002)	225 (ranging from 37 to 1870)	22 positive 3 trend 16 negative
Asian	15	146 (ranging from 55 to 523)	158 (ranging from 31 to 471)	2 positive 13 negative
Other/mixed	6	224 (ranging from 37 to 936)	161 (ranging from 47 to 536)	2 positive 4 negative
Family-based studies				
Caucasian	6	236 (ranging from 104 to 548)	272 (ranging from 63 to 919)	1 positive 5 negative
Asian	2	224	146 (ranging from 112 to 179)	All negative
Other/mixed	2	346 (ranging from 104 to 709)	249 (ranging from 52 to 651)	All negative

a the mean value and the range are indicated.
b some studies have analyzed more than one sample.
Abbreviation: SCZ, schizophrenia; N/A, not applicable.
Information obtained from the SCZgene database (Allen *et al.,* 2008).

The relationship between the *HTR2A* gene and both emotionality and affective disorders is another interesting issue. The serotonergic system has been considered to be involved in the neurophysiology of emotionality; thus, the serotonin receptor $5HT_{2A}$ appears to be an interesting candidate gene for mood disorders, such as bipolar disorder, major depression (including response to antidepressants), anxiety-spectrum disorders and many others. However, the situation is similar to schizophrenia studies, with a mix of negative and positive results which is difficult to be interpreted (Golimbet *et al.,* 2002; Anguelova *et al.,* 2003; McMahon *et al.,* 2006). However, other study (Weisstaub *et al.,* 2006) demonstrated that global disruption of $5\text{-}HT_{2A}$ receptor signaling in mice reduces inhibition in conflict anxiety paradigms without affecting fear-conditioned and depression-related behaviors.
In conclusion, despite the uncertainties, we consider that *HTR2A* could be involved in the pathogenesis of both auditory hallucinations and emotional disturbances in schizophrenic patients.

4.1.3. *TPH2* gene.

Tryptophan hydroxylase (TPH) is the key regulator of serotonin biosynthesis. Utilizing BH4 and O_2 as co-substrates and Fe^{2+} as a cofactor, TPH hydroxylates tryptophan to 5-hydroxytryptophan (Carkaci-Salli *et al.,* 2006). Finally, decarboxylation of 5-hydroxytryptophan by amino acid decarboxylase generates serotonin (figure I13). TPH belongs to a superfamily of aromatic amino acid hydroxylases that include phenylalanine and tyrosine hydroxylase (Fitzpatrick, 1999). There are two tryptophan hydroxylase (TPH) isoforms, TPH1 and TPH2, which act as rate-limiting enzymes in serotonin synthesis (Walther and Bader 2003; McKinney *et al.,* 2005). The **gene for the brain-specific isoform** of tryptophan hydroxylase was discovered 6 years ago and designated *TPH2* (Walther and Bader, 2003). The TPH2 isoform is exclusively expressed in the brain in a circadian rhythm and is responsible for the neuronal 5-

HT synthesis. The human *TPH2* gene spans approximately 100 kb, consists of 11 exons and maps on chromosome 12q21.1 (figure I14).

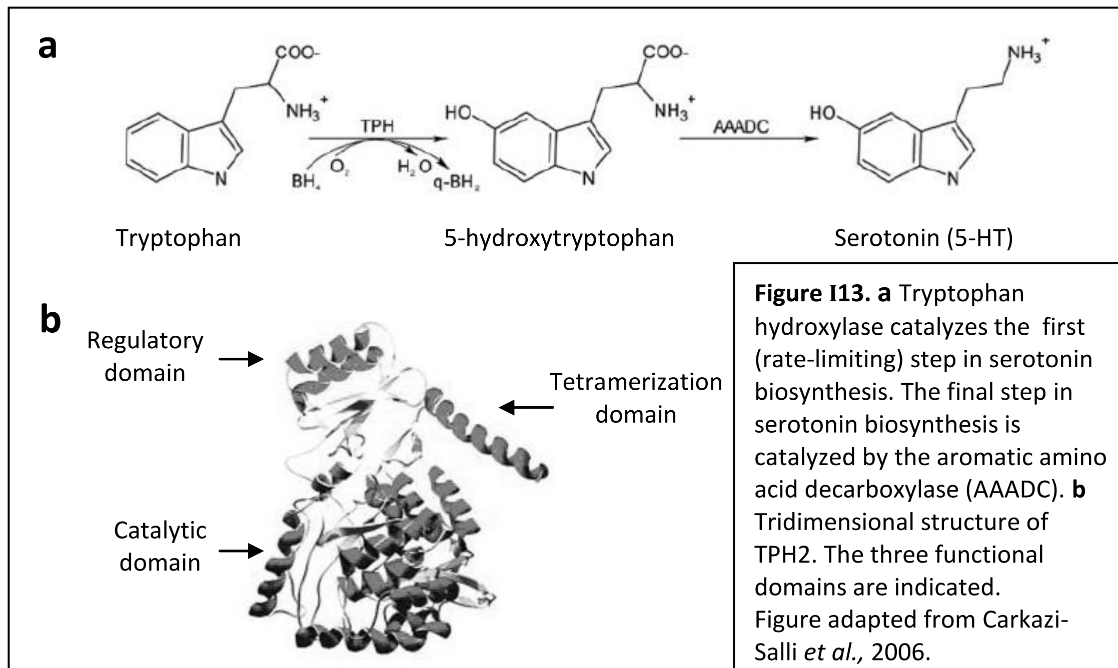


Figure I13. a Tryptophan hydroxylase catalyzes the first (rate-limiting) step in serotonin biosynthesis. The final step in serotonin biosynthesis is catalyzed by the aromatic amino acid decarboxylase (AAADC). **b** Tridimensional structure of TPH2. The three functional domains are indicated. Figure adapted from Carkazi-Salli *et al.,* 2006.

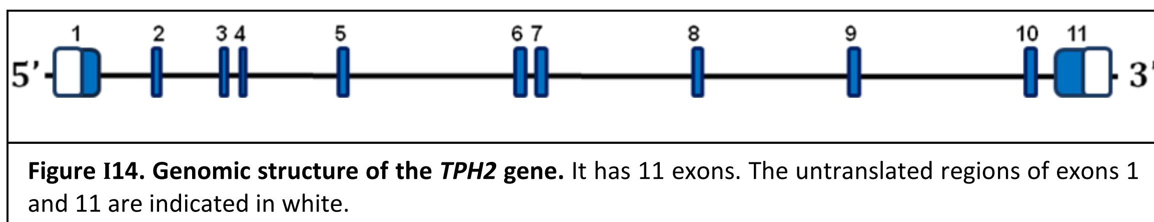


Figure I14. Genomic structure of the *TPH2* gene. It has 11 exons. The untranslated regions of exons 1 and 11 are indicated in white.

TPH2 enzyme is highly homologous to TPH1 (>70% identity in amino acid sequence), and both have a particular high sequence identity within the COOH-terminal catalytic domain (Walther and Bader, 2003). TPH2 is also highly conserved between mammalian species. However, the brain isoform is more soluble and has different kinetic properties than TPH1 (McKinney *et al.,* 2005).

Post-mortem studies confirmed the presence of *TPH2* mRNA in the human frontal cortex, hippocampus, amygdala, hypothalamus, and thalamus but not in peripheral organs (Zill *et al.,* 2004a). The two initial studies revealing the existence of TPH2 (Walther and Bader 2003; Côté *et al.,* 2003) concluded that the expression of the two isoforms is mutually exclusive, designating TPH1 as the peripheral form and TPH2 as the neuronal subtype. However, this is still a matter of debate, as several subsequent studies have also reported a detectable *TPH1* expression in raphe neurons of the rat (Patel *et al.,* 2004; Malek *et al.,* 2005), the mouse (Gundlah *et al.,* 2005) and humans (Zill *et al.,* 2007). By contrast, Gutknecht *et al.* (2008) have not found significant *TPH1* expression in the human and murine raphe.

The identification of *TPH2* genetic variants with a functional effect on enzymatic activity could be an interesting approach to study the neurobiology of emotional behaviors, as well as the vulnerability to phsychiatric disorders with emotional alterations, such as depression, anxiety, suicidality and even schizophrenia. According to this idea, several variants have been analyzed up to now. One of the polymorphisms which has received more attention is T−703G (rs4570625), due to its functional implications that we will review later.

Until now, variation of *TPH2* has been implicated in several affective disorders. The first SNP and haplotype analyses of the *TPH2* locus revealed evidence for association of *TPH2* variants with **depression** (Zill *et al.,* 2004b; Zhang *et al.,* 2005) as well as **bipolar I and II disorders** (Harvey *et al.,* 2004). Zhou *et al.,* (2005) reported a haplotype-based association with **major depression, anxiety disorders, and suicidal behavior** in different ethnic populations. Interestingly, this haplotype was predictive of lower 5-HIAA concentrations in cerebrospinal fluid. Moreover, investigation in postmortem brain samples of bipolar and schizophrenic patients revealed higher concentrations of *TPH2* mRNA in the **bipolar group** in comparison with controls (De Luca *et al.,* 2005a). Measurement of *TPH2* mRNA in the brainstem of **depressed patients who had committed suicide** also demonstrated greater expression in the dorsal and median raphe nuclei when compared to control subjects (Bach-Mizrachi *et al.,* 2006). Furthermore, Cichon *et al.* (2008) found association between **bipolar disorder (BPD)** and several SNPs located in a haplotype block covering the 5' regulatory region of the gene, as well as a rare, non-synonymous SNP, (Pro206Ser) which affects thermal stability and solubility of the enzyme. Lin and colleagues also reported in 2007 (Lin *et al.,* 2007) a significant association of a *TPH2* haplotype and **BPD.** They also showed that allelic alteration of polymorphisms in the promoter region and exon 2 of *TPH2* caused a remarkable loss of functionality in promoter and enzyme activities. Finally, Haghighi and colleagues (Haghighi *et al.,* 2008) identified a novel TPH2 short isoform with a truncated catalytic domain. An exploratory study revealed association between a novel SNP located in exon 6 of this short isoform and **major depression or suicide.**

Evidences that *TPH2* variation may play a role in cognition comes from studies that have implicated *TPH2* variants in the etiology of disorders which affect executive control, such as **attention-deficit hyperactivity disorder** (ADHD) and **obsessive–compulsive disorder** (OCD). Several studies have reported transmission disequilibrium of *TPH2* promoter variants in children and adolescents with ADHD, as well as a preferential transmission of a haplotype linked to the transcriptional control region of *TPH2* in early onset OCD (Walitza *et al.,* 2005; Mössner *et al.,* 2006). Subsequently, Strobel and colleagues (2007) measured the impact of *THP2* variation on behavioral measures in a cognitive control task in **healthy subjects**. They found that individuals lacking the rare *TPH2* T allele from rs4570625 were not faster than T allele carriers, but committed fewer errors and were less variable in responding. They also suggested that genetic variants associated with higher negative emotionality may have beneficial effects on some cognitive functions. More recently, Baehne and colleagues (2008) studied the effect on prefrontal brain function of two *TPH2* SNPs (including SNP rs4570625) in large samples of adult **ADHD** patients and healthy controls while performing a continuous performance test (CPT) during recording of an ongoing electroencephalogram (EEG). Interestingly, **ADHD** risk alleles of both polymorphisms affected prefrontal function during response inhibition in both healthy controls and ADHD patients.

Furthermore, there have been also associations with **personality traits**: Gutknecht and colleagues (Gutknecht *et al.,* 2007) also reported an overrepresentation of T allele carriers of the functional polymorphism in the upstream regulatory region of *TPH2* (SNP G-703T, rs4570625) in both patient groups from clusters B and C from personality disorders. Reuter *et al.,* (2007) also showed that the T/T genotype from the same SNP was associated with the temperament trait harm avoidance.

With regard to **schizophrenia**, apart from the first work from De Luca *et al.,* (2005a), who could not find differences in mRNA expression of *TPH2* in schizophrenic patients, the remaining studies which have analyzed the relationship between schizophrenia (and suicidal behavior in SCZ patients) have been negative (De Luca *et al.*, 2005b, 2006; Higashi *et al.,* 2007).

Finally, **functional magnetic resonance imaging (fMRI)** was employed to provide evidence that acute tryptophan depletion, which results in a transient reduction of brain 5-HT, as well as SNP G-703T (rs4570625), appear to bias the responsiveness of the amygdala, a structure with an important role in the modulation of emotionality (Brown *et al.,* 2005; Canli *et al.,* 2005a). Finally, Herrman and colleagues (2007) also found, through the measurement of event-related potentials (ERPs) during a passive emotional picture perception task, that both the 5-HTTLPR and the *TPH2* SNP rs4570625 genotypes modulate the sensory encoding of affective stimuli during early steps of visual processing and revealed additive effects of these 2 genes in the serotonergic control of emotion regulation.

Thus, it can be hypothesized that *TPH2* variation can have a role in the emotional processing of auditory hallucinations in schizophrenic patients.

4.2. *NOS1* gene.

Nitric oxide (NO) is a pleiotropic messenger molecule functionally linked to glutamatergic transmission (Garthwaite, 2008). Entry of Ca^{2+} through a NMDA channel binds to calmodulin and stimulates NO synthesis (Baba *et al.,* 2004). NO can also form covalent linkages with many targets, including guanylate cyclase (GC) (Dawson and Snyder, 1994), which synthesizes cyclic guanosine monophosphate (cGMP) in response to NO. NO and cGMP have various reversible excitatory or inhibitory effects on neuronal excitability and synaptic transmission (Garthwaite, 2008).

NO has been implicated in a **wide range of neural functions**. In the vertebrate CNS, NO appears to have roles as a gaseous messenger/neurotransmitter influencing local blood flow, learning and memory, neurodevelopment, feeding, sleeping, reproductive behavior, and sensory and motor function (Garthwaite, 2008). Depending on the circuit, NO may be produced pre- or postsynaptically and it has been suggested that it could provide a simultaneous signal to both pre- and postsynaptic elements, with importance in coordinating responses on the two sides of the synapse. Like other receptors, NO receptors are heterodimers with two known isoforms that, although widespread, have a differing cellular distribution in the brain and also at the subcellular level (Gibb and Garthwaite, 2001; Mergia *et al.,* 2003).

NO is synthesized by **nitric oxide synthase** (NOS). NOS enzymes represent a family of cytochrome P_{450}-like flavohemeproteins that catalyze the oxidation of L-arginine to form L-citrulline and NO (Bredt *et al.,* 1991; Stuehr and Ikedo-Saito, 1992). Two main isoforms with distinct functional and structural features exist (Bredt *et al.,* 1991; Garthwaite, 2008): neuronal (nNOS or NOS-I) and endothelial (eNOS or NOS-II). A third, inducible, type (iNOS) is rarely present normally but can be expressed in numerous cell types (prototypically in macrophages, mainly in microglia in the CNS) in response to immunological challenge. The three isoforms have distinct functional and structural features.

NOS-I is the main source of NO in excitable tissue and can be found in up to 1% of all neurons (Snyder and Ferris, 2000). In addition to **regulation by Ca^{2+}/calmodulin**, NOS-I has several putative sites for phosphorylation (Garthwaite, 2008). CaMKII, a kinase found at synapses together with NMDA receptors and NOS-I, was found to phosphorylate the enzyme and inhibit NO formation by 50%, probably by affecting Ca^{2+}/calmodulin binding (Hayashi *et al.,* 1999; Komeima *et al.,* 2000). The protein kinase Akt (novel candidate gene for schizophrenia) can also phosphorylate NOS-I (Rameau *et al.,* 2007). Recent evidence indicates that NOS-I can also bind to the serotonin transporter in the plasma membrane in such a way that **serotonin uptake couples to NO formation** (Chanrion *et al.,* 2007). Thus, the regulation of NOS-I activity in neurons is really complex.

The human gene encoding NOS-I (*NOS1*) is an **extremely complex gene** located at chromosome 12q24.3 (Hall *et al.,* 1994; Zhang *et al.,* 2004). It is structured in two regions (figure I15a): a 110 kb region containing 28 protein-coding exons (named 2 to 29) and another 130 kb region, called the "variable region", which includes at least 12 distinct first non-coding exons (designated as exon 1a to 1l). Transcription of those alternative first exons is driven by at least 6 alternative promoters resulting in a tissue-specific expression pattern (Bros *et al.,* 2006). The promoter region of exon 1f is mainly expressed in the basal ganglia, but also the hippocampus and cortex. Remarkably, this exon contains a highly polymorphic dinucleotide repeat, termed ***NOS1* Ex1f-VNTR**, with 180 – 210 CA units, 33 bp downstream of the TATA box (Reif *et al.,* 2006). The most common alleles are 182, 184, 192, 200, 202 and 204 repeats (figure I15b). Although *NOS1* exon 1f is highly conserved across different species, the promoter repeat element appears to be exclusive to humans. To facilitate genetic studies the polymorphism is generally dichotomized in short (180-196 repeats) and long (198-210 repeats) alleles. Together with *MAO*-uVNTR and 5HTTLPR, *NOS1* ex1f-VNTR is a type of promoter VNTR with effects on gene expression. Reif and colleagues (personal communication) demonstrated that the short variant is associated with decreased transcriptional activity of the *NOS1* exon 1f promoter and alterations in the neuronal transcriptome of human postmortem brains, including altered expression of *RGS4* and *GRIN1*, which had been previously linked to psychiatric disorders. Apart from *NOS1* Ex1F-VNTR, other polymorphisms have been described and analyzed in the literature.

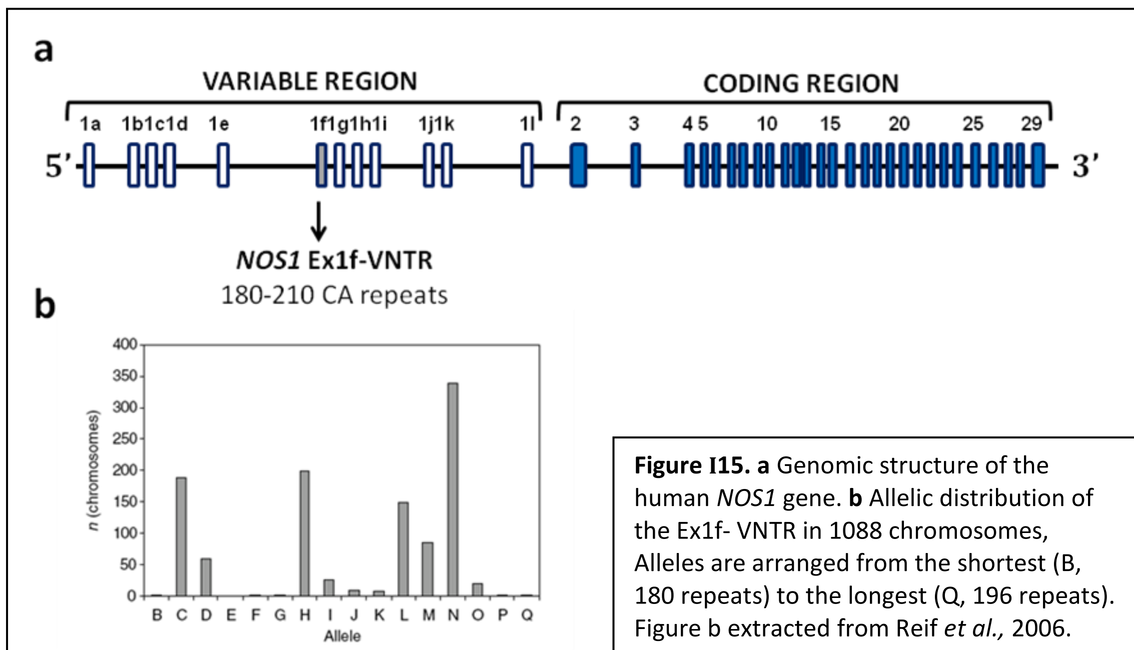


Figure I15. a Genomic structure of the human *NOS1* gene. **b** Allelic distribution of the Ex1f- VNTR in 1088 chromosomes, Alleles are arranged from the shortest (B, 180 repeats) to the longest (Q, 196 repeats). Figure b extracted from Reif *et al.,* 2006.

NOS-I has been linked to several behavioral traits and severe psychiatric and neurologic pathologies. With regard to dimensional behavioral traits, it has been linked to murine behavior by numerous pharmacological studies. Likewise, knockdown of the *Nos1* gene in mice results in behavioral changes, such as learning deficits, decreased anxiety, impulsivity and increased aggressiveness (Nelson *et al.,* 1995). Very recently, Reif and colleagues (personal communication) studied a high cohort of 3500 adult subjects and showed that the *NOS1* Ex1f-VNTR is associated with impulsivity-related traits, such as hyperactive and aggressive behaviors. Furthermore, carriers of the *NOS1* Ex1f-VNTR short alleles appear to have an increased risk to develop Alzheimer's disease, with evidence for *NOS1* x *ApoE4* interaction (Galimberti *et al.,* 2008).

With regard to psychiatric disorders, evidence is accumulating that NO may be affected in the **pathophysiology of schizophrenia**. Altered populations or distribution of NOS-containing neurons in schizophrenia have been reported in frontal (Akbarian *et al.,* 1993a; Baba *et al.,* 2004) and temporal (Akbarian *et al.,* 1993b) cortex, hypothalamus (Bernstein *et al.,* 1998), cerebellum (Karson *et al.,* 1996) and striatum (Lauer *et al.,* 2005). Moreover, alterations of the NO system in plasma and platelets of schizophrenics have been also found (Das *et al.,* 1995). Remarkably, NOS-I is involved in several signaling cascades likely involved in the pathogenesis of schizophrenia. First, NO can function as the second messenger of the NMDA receptors. Second, the adaptor protein of NOS-I to the NMDA receptor, CAPON, has been shown to be associated with schizophrenia (Brzustowicz *et al.,* 2004; Zheng *et al.,* 2005). Moreover, the nitrinergic system is also tightly linked to DA and 5-HT neurotransmission: NO can inhibit monoamine transporters by nitrosylation, which results in increased synaptic availability of monoamine neurotransmitters (Kiss and Vizi, 2001). However, there are conflicting data with regard to the direct NO effects on these transporters: it has been shown that the NO donor compound SNAP increases SERT function (Kilic *et al.,* 2003). Moreover, NOS-I knockout mice feature clear alterations in serotonergic function, with an impaired 5-HT turnover,

desensitization of 5-HT receptors and decreased expression of the serotonin transporter in the brain stem neurons (Chiavegatto *et al.,* 2001).

Association studies also support the role of the *NOS1* gene in schizophrenia and other psychiatric disorders. The first studies studying single *NOS1* markers found associations with bipolar disorder and schizophrenia (Detera-Wadleigh *et al.,* 1999; Morissette *et al.,* 1999; Bailer *et al.,* 2000, 2002; Shinkai *et al.,* 2002) although some negative results exist (Liou *et al.,* 2003). However, other studies failed to detect association with bipolar disorder (Buttenschon *et al.,* 2004) or major depression (Yu *et al.,* 2003). More recently, Fallin *et al.* (2005) performed a genotypic screen of 64 genes on families of Ashkenazi Jewish descent origin with bipolar disorder I or schizophrenia and found that *NOS1* showed evidence of association with both disorders. Reif and colleagues (2006) found association of Ex1f VNTR and a mini-haplotype with schizophrenia and reported that promoter polymorphisms impacted on prefrontal functioning. Moreover, two other recent studies (Tang *et al.,* 2008; O'Donovan *et al.,* 2008) have also reported an association between *NOS1* polymorphisms and schizophrenia.

Therefore, NO appears to be a promising candidate to be involved in the pathophysiology of schizophrenia, and its interaction with the serotonergic system could also implicate both systems in the manifestation of several behavioral traits.

4.3. *STMN1* gene.

Stathmin (Sobel, 1991), also known as Oncoprotein 18 (Hailat *et al.,* 1990), is a member of a protein family that regulates the **dynamics of microtubule (MT) polymerization/ depolymerization** during different phases of the cell cycle (Belmont and Mitchison, 1996; Gavet *et al.,* 1998) in a phosphorylation-dependent manner: this ubiquitous cytosolic protein interacts with tubulin heterodimers and prevents them from forming microtubules by stablishing a T2S complex of one stathmin (S) and two tubulin (T) α/β heterodimers (Jourdain *et al.,* 1997) (figure I16). After phosphorylation of specific serine residues by cell surface receptor kinase cascades and cycle-dependent kinases (Gavet *et al.,* 1998; Cassimeris, 2002), stathmin releases tubulin, allowing MT formation. Stathmin has also been suggested to act as an intracellular relay for extracellular signals (Sobel, 1991) and many proteins, apart from tubulin, have been identified as target/partners for stathmin (Li and Cohen, 1996; Maucuer *et al.,* 1995; Maucuer *et al.,* 1997).

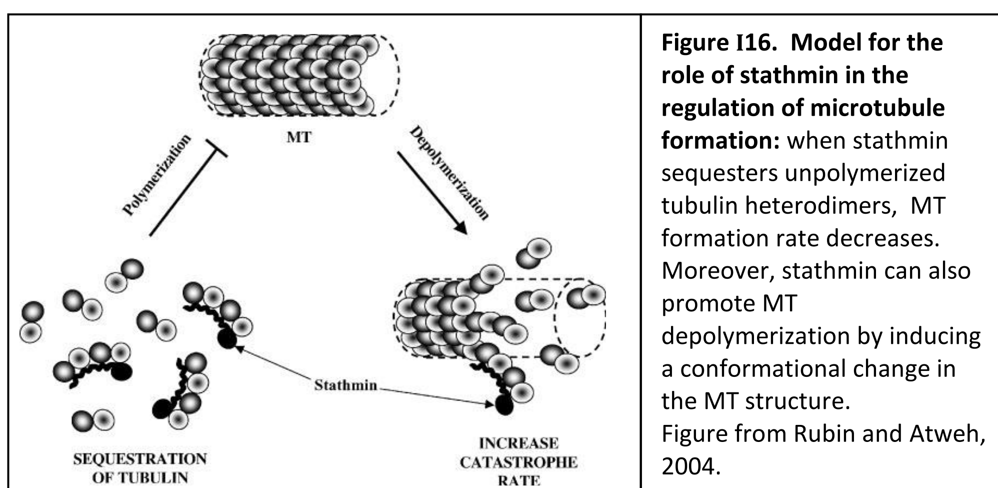


Figure I16. Model for the role of stathmin in the regulation of microtubule formation: when stathmin sequesters unpolymerized tubulin heterodimers, MT formation rate decreases. Moreover, stathmin can also promote MT depolymerization by inducing a conformational change in the MT structure.
Figure from Rubin and Atweh, 2004.

In humans, the **stathmin gene** (known as *STMN1*) is a small gene that maps to chromosome 1 (1p36.1-p35). Alternatively spliced transcript variants varying in the 5' UTR region but encoding the same protein of 149 aminoacids have been identified (figure I17). Stathmin protein is highly conserved among vertebrates (Koppel *et al.,* 1990). The **protein structure** of stathmin could be divided into two domains, an N-terminally located phosphorylation-regulatory domain and a C-terminally located microtubule-interacting coiled-coil domain (Mori and Morii, 2002; Ozon *et al.,* 2002).

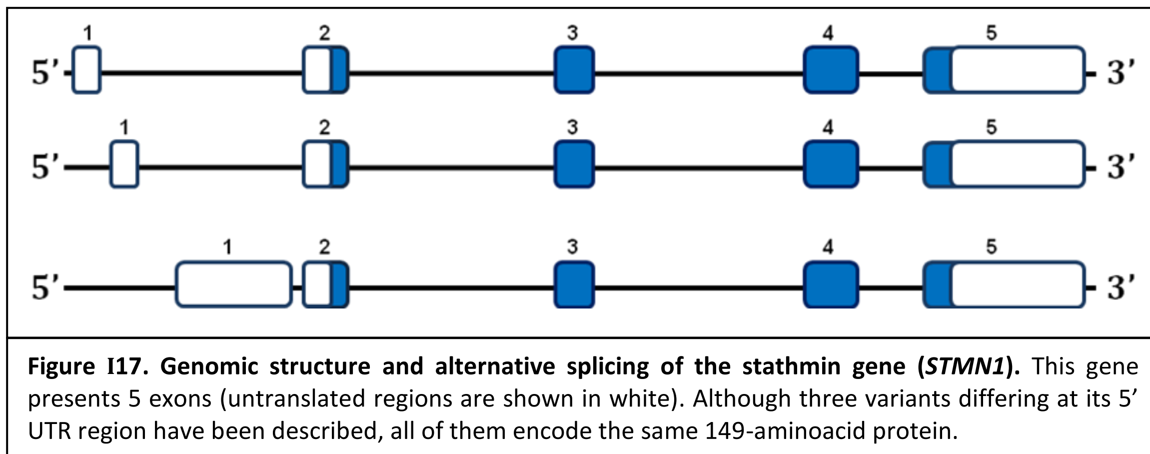


Figure I17. Genomic structure and alternative splicing of the stathmin gene (*STMN1*). This gene presents 5 exons (untranslated regions are shown in white). Although three variants differing at its 5' UTR region have been described, all of them encode the same 149-aminoacid protein.

Stathmin has a key role in different cellular processes. First, a huge number of studies support the relationship between stathmin expression and/or phosphorylation and **regulation of cellular proliferation**. Stathmin was first identified as a 17-kDa cytosolic protein that is rapidly phosphorylated when leukemic cells are induced to differentiate and cease to proliferate. Similar observations were also made in carcinoma cell lines and in other solid tumors like breast and ovarian cancer (reviewed in Rubin and Atweh, 2004). Moreover, in rodent and humans, stathmin was reported to be highly expressed in tissues with a high proliferation rate such as testis and hematopoietic cells. Stathmin is critically important not only for the formation of the mitotic spindle when cells enter mitosis but also for the morphological changes associated with cytokinesis and the entry into a new cell cycle (Rubin and Atweh, 2004). When cells enter mitosis, stathmin is switched off by phosphorylation, allowing microtubules to assemble into a mitotic spindle. Subsequently, the reactivation of stathmin in the later stages of mitosis allows the disassembly of the mitotic spindle and the exit from mitosis. Moreover, several findings support a role for the downregulation of stathmin expression in the physiological regulation of endomitosis and polyploidzation.

However, in addition to their well known role in mitosis, interphasic microtubules are essential for many other cellular processes such as intracellular transport, cell motility, cell polarity, and maintenance of cell shape. **Cell migration** is an important example. It is a complex cellular behavior which involves coordinated changes in the cytoskeleton and the controlled formation and dispersal of adhesion sites (Giampietro *et al.,* 2005). As a result, stathmin expression appears to be crucial in migrating cells. Stathmin was found to be expressed at high levels in migrating cells of the rat olfactory system (Camoletto *et al.,* 1997; Giampietro *et al.,* 2005). Jin

et al. (2004) have also shown that decreased stathmin levels in these cells inhibit their mobilization from the subventricular zone (SVZ) of rat brain. The relationship between stathmin levels and migration is also supported by studies with epithelial cells (Niethammer *et al.,* 2004), as well as studies on tumoral cell lines in which stathmin levels correlate with an invasive phenotype (Walter-Yohrling *et al.,* 2003).

Stathmin functions in the **development, maturation, and functional regulation of the CNS** (Curmi *et al.,* 1999) have a special interest for the present study. Stathmin is highly expressed during embryogenesis and progressively declines during the development of the nervous system (Koppel *et al.,* 1990; Amat *et al.,* 1991; Pellier-Monnin *et al.,* 2001). Interestingly, the high expression of stathmin in postmitotic neurons suggests that stathmin may have a distinct function in neurons that is not related to proliferation. Moreover, the expression pattern in germinal areas of the adult CNS with neurogenic and gliogenic potential (olfactory epithelium, dentate gyrus of the hippocampus and SVZ of the rat brain) (Amat *et al.,* 1991) clearly suggests the idea that this protein is involved in processes of differentiation (Camoletto *et al.,* 1997). The level of stathmin expression was also shown to be highly increased during neurite outgrowth and synapse formation, supporting a role for stathmin in neuronal differentiation (Di Paolo *et al.,* 1997). Moreover, an abnormal stathmin expression has been detected in **pathological conditions**. Stathmin gene expression is severely affected in the brains of patients with Alzheimer disease (Jin *et al.,* 1996). It is also of interest to note that stathmin is downregulated in the brains from patients with Down syndrome (Cheon *et al.,* 2001). Liu *et al.,* (2005) also reported the increased expression of stathmin in the brains of patients with multiple sclerosis.

An intriguing finding is that, despite the wide variety of functions which had been attributed to stathmin, the inactivation of this gene in mouse did not produce any apparent major phenotype (Schubart *et al.,* 1996), probably due to the redundant functions of some of the stathmin family proteins. However, several recent reports from Dr. Shumyatsky's lab have revealed that in the normal adult brain of the mouse, stathmin is highly expressed in the lateral nucleus (LA) of the amygdala as well as in the thalamic and cortical structures that send information to the LA about the conditioned (learned fear) and unconditioned stimuli (innate fear) (Shumyatsky *et al.,* 2005). Coherently, the inactivation of stathmin affects long-term potentiation (LTP) in the cortico-amygdala and thalamo-amygdala pathways, although synaptic transmission and NMDA-receptor function is normal. Moreover, as expected, inactivation of stathmin gene in mouse has a marked impact on those behaviors dependant on **neural circuitry of fear and threat assessment of the environment**: knockout mice exhibit decreased memory in amygdala-dependent fear conditioning and fail to recognize danger in innately aversive environments (Shumyatsky *et al.,* 2005). Moreover, this deficiency in fear processing also leads to improper threat assessment, which consequently affects innate maternal care and adult social interactions. Stathmin -/- females are a clear example: they lack motivation for retrieving pups and are unable to choose a safe location for nest-building (Martel *et al.,* 2008). Remarkably, stathmin -/- females have an enhancement in social interactions (Martel *et al.,* 2008). In conclusion, stathmin is a necessary element for the induction of LTP in afferent inputs to the amygdala and is essential in regulating both innate and learned fear, which has consequences in related behaviors such as innate maternal care and social interactions.

These latest results open new possibilities for the study of stathmin function in the CNS and places stathmin as an interesting candidate to be involved in psychiatric disorders where threat assessment and social interactions are affected. Emotional dysfunctions and schizophrenia are good examples of this type of disorders.

4.4. *ASPM* gene.

From an evolutionary point of view, the high increment of cognitive abilities during *Homo sapiens* evolution may have favoured the appearance of certain pathologies which are associated to cognitive alterations, such as schizophrenia (Crow, 1997; Horrobin, 1998). This idea generates an **evolutionary-genetic paradox** which could also explain how the frequency of such a deteriorating mental disease is maintained across time in all human populations. According to this hypothesis, those genes which have been subject to an accelerated evolution in the human lineage may have been involved in the development of certain human features, including those of cognitive nature, as well as other features, such as speech. As a consequence of this important role in human evolution, such genes could also be implicated in the emergence of psychiatric diseases, such as schizophrenia. One gene included in this study, *ASPM*, is directly related to neurodevelopment and suffered positive selection in the human lineage. For these reason, it has been considered as an interesting candidate gene for schizophrenia.

ASPM (abnormal spindle-like microcephaly-associated) gene (formerly known as *MCPH5*) is known to play an **important role in cortical development**. The first evidence in this direction was the finding that protein-truncating mutations in this gene were implicated in autosomal recessive primary microcephaly (MCPH), a condition in which the head circumference is less than three standard deviations (SDs) below the age-related mean (Ross and Frias, 1977). Although the whole brain is affected by this reduction, the cerebral cortex seems to be the most severely affected region in these patients. The *ASPM* gene is the locus which explains the highest number of microcephaly cases (Bond *et al.,* 2002). It is predicted that all *ASPM* mutations detected in MCPH patients truncate the protein, because of the presence of a premature stop codon, or because of the alteration of a splicing site.

***ASPM* gene** spans 62 kb in 1q31 (Bond *et al.,* 2002). It has a 10434 bp open reading frame (ORF), which includes 28 exons and codes for a protein of 3477 amino acid (figure I18a). However, two predominant mRNAs have been observed in humans as well as in mouse: the complete mRNA of 10434 bp and a smaller transcript of 5.6 kb (1892 amino acids), called variant 1, which lacks exon 18.

The full-length **ASPM protein** is predicted to contain the following domains (figure I18b) (Kouprina *et al.,* 2005): a) an amino terminal microtubuline-binding domain, which seems to be member of the novel family of ASH domains (Ponting, 2006), also present in other proteins related to cillia, flagela, centrosome or Golgi complex; b) a tandem of two calponin-homology (CH) domains, which are generally implicated in actin binding; c) a particularly large domain of 81 isoleucine-glutamine (IQ) motifs, which are normally related to calmodulin interaction; and finally, d) a carboxi-terminal region. The CH and IQ domains are normally found in motor proteins, such as non-conventional myosins: when calmodulin binds to the IQ repeats, a

conformational change in the myosin is induced, regulating actin union to CH domain (Bähler and Rhoads, 2002).

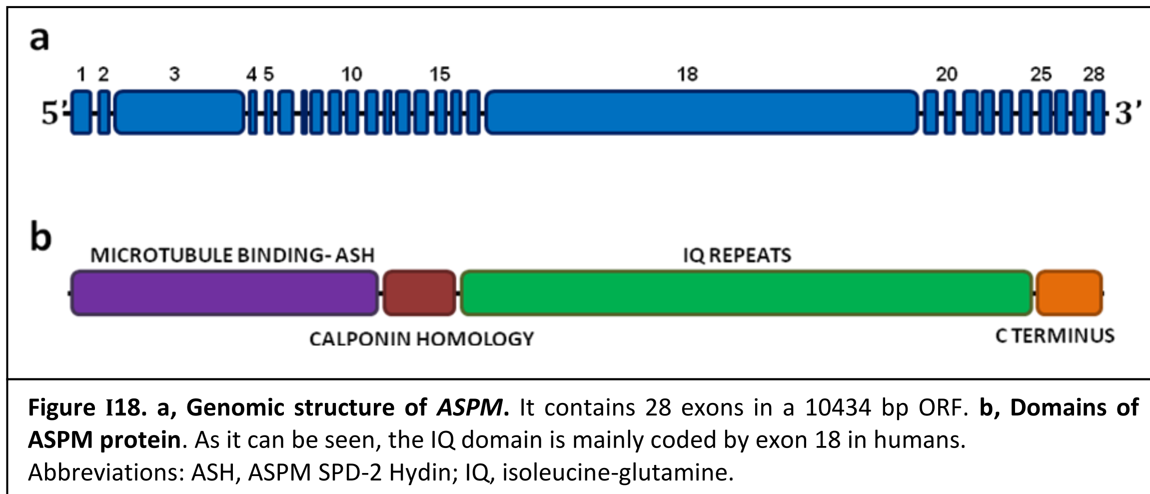


Figure I18. a, Genomic structure of *ASPM*. It contains 28 exons in a 10434 bp ORF. **b, Domains of ASPM protein**. As it can be seen, the IQ domain is mainly coded by exon 18 in humans. Abbreviations: ASH, ASPM SPD-2 Hydin; IQ, isoleucine-glutamine.

According to the investigations from Kouprina and colleagues (2005), the different IQ repeats have a variable length in humans. However, the central region (repeats 4-54) conforms a **high order repeat (HOR) structure**, with a highly regular pattern which comprises units of 1 long repeat (27 amino acids) + 2 short repeats (23 amino acids). Moreover, certain amino acids of this HOR are conserved among short repeats, whereas others are conserved among the long repeats. This conservation pattern can be observed in primates and rodents. These findings indicate that the HOR structure may have an important function, for example, during the interactions between ASPM and polymeric proteins such as actin and calmodulin.

Expression studies (Bond *et al.,* 2002) have shown that the murine aspm protein is highly expressed in the cerebral cortical ventricular areas, the proliferative region of the lateral and medial ganglionic eminence and the ventricular zone of the dorsal diencephalon. In coherence with these results, human *ASPM* is also expressed in the developing brain and in other tissues during development, but not in the adult brain (Kouprina *et al.,* 2005). Furthermore, ASPM protein can be detected in other proliferating tissues, including developing tissues as well as cancers. Interestingly, *ASPM* expression has been detected in spindles during mitosis, in coherence with a previous work with the homologue *Drosophila* asp protein, where it was also described that *asp* mutant cells suffered metaphase arrest (González *et al.,* 1990). These evidences point out the possibility that this gene plays an important role in cell division. Indeed, *ASPM* seems to be crucial in the control of the cleavage plane orientation that allows symmetric, proliferative divisions of embryonic neuroepithelial (NE) cells during mouse brain development (Fish *et al.,* 2006). As a result, it has been hypothesized that *ASPM* may control the **proliferative symmetry of progenitors that appears to be pivotal for the expansion of cerebral cortex**. Thus, the lack of *ASPM* could reduce the ability of neural stem cells to produce neurons and would also explain the reduction of brain volume observed in microcephalic patients. However, given that *ASPM* is also expressed in many other tissues, it is possible that *ASPM* codifies for different products, one that is necessary in brain and another necessary for the mitosis of other cells. According to this idea, Ponting (2006) suggested the possibility that

ASPM protein may have a ciliary function, since it contains an amino-terminal domain also present in other proteins, related to cillia. As we know, ASPM is associated to the centrosome and, although the centrosome has a key role in the regulation of cell divisions, it is also necessary for the basal bodies in the cilia. So, the possibility that ASPM has a **role in other functions related to cilia**, such as spermatogenesis, cannot be ruled out. Another conciliatory hypothesis would be that ASPM takes part in the cilia-mediated neuronal migration, rather than in neurogenesis (Ponting, 2006).

One of the most interesting features of *ASPM* gene are the evidences which support the existence of **accelerated evolution, presumably by positive selection**, particularly at the IQ domain, in the primate lineage leading to *Homo sapiens* (figure I19), especially in the 6 million year of hominid evolution, where *ASPM* accumulated one advantageous amino acid change every 350000 years (Zhang, 2003; Evans *et al.,* 2004; Kouprina *et al.,* 2004). A recent report from Ali and Meier (2008) also demonstrated that accelerated evolution of *ASPM* at 16 amino acid sites occurred in nine primate lineages with major changes in cerebral cortex size. Interestingly, this positive selection seems to be **still ongoing in humans**: Mekel-Bobrov and colleagues reported in 2005 the existence of a highly polymorphic variant (rs41310827 or A44871G, being the A allele the ancestral one) which arose in humans approximately 5800 years ago and increased its frequency by positive selection. An interesting fact which supports the existence of positive selection around this *ASPM* region is the existence of an extended LD in the region around A44871G (Mekel-Bobrov *et al.,* 2005).

These interesting findings suggest that *ASPM* gene could have had an outstanding role in the **progressive increasing of the relative brain size in the course of primate evolution**. Along with this idea, it is plausible to think that *ASPM* could be implicated in the evolution of cognition, as there is a strong correlation between brain size and mental abilities (Vernon *et al.,* 2000; Rushton and Ankney, 2007). Thus, it is also likely that *ASPM* could have also been related to psychiatric diseases which include cognitive symptoms, such as schizophrenia.

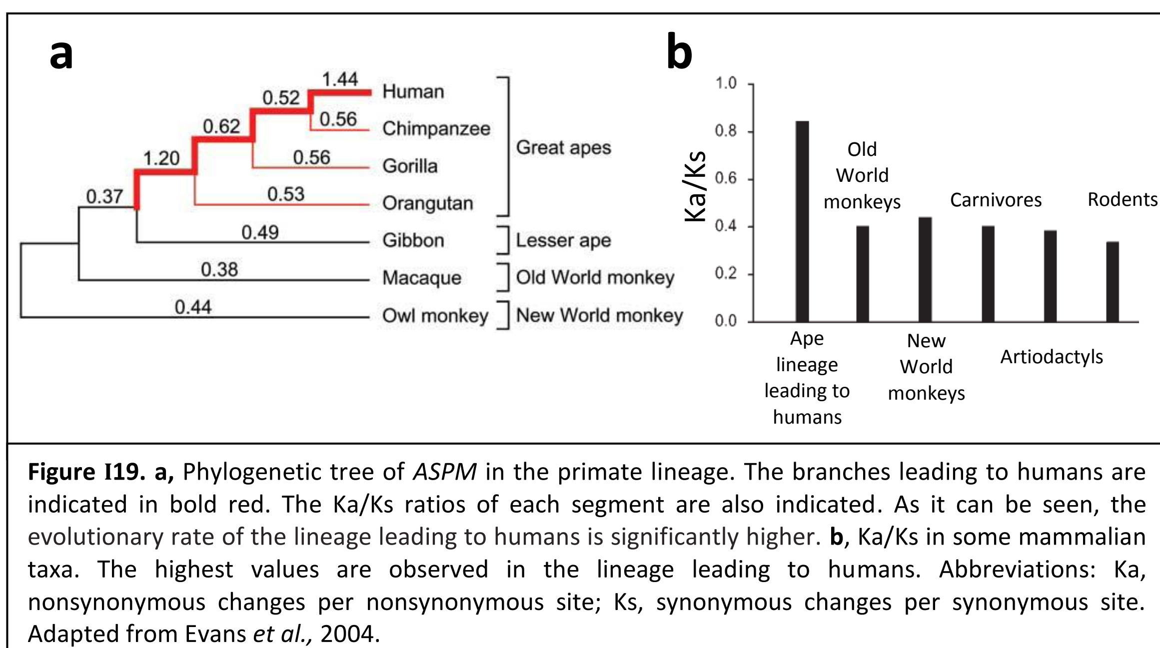


Figure I19. a, Phylogenetic tree of *ASPM* in the primate lineage. The branches leading to humans are indicated in bold red. The Ka/Ks ratios of each segment are also indicated. As it can be seen, the evolutionary rate of the lineage leading to humans is significantly higher. **b**, Ka/Ks in some mammalian taxa. The highest values are observed in the lineage leading to humans. Abbreviations: Ka, nonsynonymous changes per nonsynonymous site; Ks, synonymous changes per synonymous site. Adapted from Evans *et al.,* 2004.

Moreover, it has been recently argued that *ASPM* accelerated evolution could be related to **pressures on ciliary function**, rather than pressures on neurogenesis (Ponting, 2006). This fits with the idea that *ASPM* may have a role in sperm flagellar function (in fact, it is expressed in male germ cells) and it has been seen that proteins involved in male reproductive function have evolved adaptively.

However, despite the signs of positive selection and the firm evidences which relate *ASPM* mutations to a reduction in brain size, **the relationship between *ASPM* and human cognition still remains unclear.** None of the prior studies which have tried to relate this gene with global brain size or cranial volume (Woods *et al.,* 2006; Rushton *et al.,* 2007; Timpson *et al.,* 2007; Dobson-Stone *et al.,* 2007; Wang *et al.,* 2008) has obtained significant results. Furthermore, no relationship has been found between *ASPM* and intelligence or other related parameters, such as mental ability (Mekel-Bobrov *et al.,* 2007; Rushton *et al.,* 2007). However, these negative findings are not surprising, as some considerations should be taken into account. On one hand, we can presume that the effect of each individual gene on variables such as global brain size or intelligence may be small, as these variables are extraordinarily complex and multifactorial (Toga and Thompson, 2005). Moreover, until now, all these prior studies have only focused on one polymorphism of *ASPM* commented above, A44871G, but not in other variations along its sequence.

Therefore, a deeper study of *ASPM* variation is necessary to know the real role of this gene on brain evolution, neurodevelopment and vulnerability to schizophrenia and its cognitive deficits.

4.5. Other schizophrenia candidate genes.

The three last genes which had been considered for this PhD work were selected as a result of a previous exploratory study performed in the University of Würzburg under the supervision of Dr. Lesch (unpublished results). This study consisted in the search for rare copy number variations (CNVs) associating with ADHD in a sample of patients and control subjects. As a result, the region harboring the smallest isoform of the *PDE4D* gene (PDE4D6), as well as the region encoding *PLEKHB1* and *RAB6A* genes, appeared to be affected in ADHD patients. Since ADHD and schizophrenia share a common phenomenology, these results can be also considered to be of interest for schizophrenia research. Thus, these three genes were finally considered as schizophrenia candidate genes for this study.

4.5.1. *PDE4D* gene.

The intracellular **cyclic adenosine monophosphate** (cAMP) plays important roles as a second messenger molecule. It plays an important role in learning and memory formation (Dudai, 1986), through its role in long-term potentiation and synaptic plasticity, among others (Morimoto and Koshland, 1991). An important regulation point for cAMP is the **degradation of cAMP, a process mediated by phosphodiesterases (PDEs)**. These enzymes are distributed throughout the body and take part in a wide variety of roles in different organs, including the brain. PDEs are classified into 11 different families, named PDE1 to PDE11, which differ in molecular weight, charge, affinity for substrates and inhibitors, subcellular localization and

tissue distribution (Beavo, 1995; Soderling and Beavo, 2000). PDE heterogeneity is achieved through a large number of genes, which also suffer alternative splicing.

Four **cAMP-specific, rolipram-inhibited phosphodiesterases (PDE4s)** have been identified in mammals (Conti *et al.,* 2003); they are encoded by four different genes (PDE4A, B, C and D) which are homologs of *dunce*, a gene required for learning and memory in *Drosophila* (Byers *et al.,* 1981; Zhong and Wu, 1991). The four variants have specificity for cAMP as a substrate, a low enzyme-substrate affinity and Ca^{2+}-insensitivity. They interact with various scaffolding proteins through their unique amino-terminal sequence, the ultra conserved region 2 (UCR2) domain and the catalytic region (Wang *et al.,* 2003b). These unique N-terminal sequences of PDE4 isoforms may serve to direct the proteins to specific signaling pathways (Wang *et al.,* 2003b).

At least *PDE4A*, *PDE4B*, and *PDE4D* are expressed in the brain (Engels *et al.,* 1995; Iwahashi *et al.,* 1996). The distribution of these 3 subtypes occurs in nonoverlapping areas, suggesting that each of these enzymes may have different roles (Cherry and Davis, 1999). ***PDE4D* gene** maps on chromosome 5q11.2-5q12.1. It contains at least 27 exons (figure I20) over approximately 1.5 Mb overlapping with another gene (*PART1*) (Gretarsdottir *et al.,* 2003). It encodes at least nine protein isoforms and has at least seven promoters. All isoforms identified so far have an identical C-terminal catalytic domain but differ at the N-terminal regulatory domain. Miró *et al.* (2002) found that the different splice variants showed a distinct distribution pattern. The most relevant differences were in hippocampus, medial habenula, basal ganglia, and area postrema. Of special interest is PDE4D6; it is a supershort splicing variant that can only be detected in the brain, representing the most specifically expressed among the isoforms (Wang *et al.,* 2003b). The determinant for membrane insertion, localized in the helix-2 of the unique N-terminal region, is lacking in PDE4D6 and its intracellular localization remains to be determined.

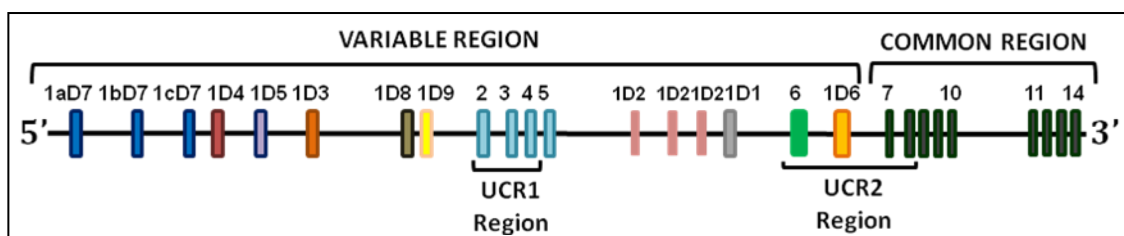


Figure I20. *PDE4D* exonic structure. The *PDE4D* gene encodes for at least 9 isoforms, which share the last nine exons (exons 7 to 14). However, the first exon used on each case (named 1D1 to 1D9) is different. Moreover, exons 2-5 are only common to long isoforms (PDE4D3, D4, D5, D7, D8 and D9). Isoform PDE4D6 also lacks exon 6 and part of the UCR2 region, and it is the shortest isoform. UCR1 and UCR2 regions are highly conserved among different species.

Interestingly, the PDE4 family, and particularly the PDE4D protein, appear to be particularly important in neuropsychopharmacology since they are **inhibited pharmacologically by the neuroactive drug rolipram** (Henkel-Tigges and Davis, 1990). The inhibition by rolipram and related drugs produces a characteristic symptomatology in rats (hypothermia, hypoactivity, forepaw shaking, grooming, and head twitches) and can be related to high levels of cAMP. Moreover, *PDE4D* expression is increased in mouse cerebral cortex by the repeated treatment

with desipramine, fluoxetine and rolipram and in the hippocampus by fluoxetine and rolipram (Shifman *et al.,* 2008b). In addition, rolipram and related drugs also produce antidepressant-like effects in behavioral models (Wachtel and Schneider, 1986; Zhang *et al.,* 2002) and can alleviate the symptoms of patients with major depression (Horowski and Sastre-y-Hernandez, 1985; Fleischhacker *et al.,* 1992). Finally, several regions in the human brain where *PDE4D* and other PDE4s are abundant could be possible sites in which mood-elevating effects of rolipram may be mediated (Cherry and Davis, 1999). All these findings suggest that the PDE4D subtype is an **essential mediator of the antidepressant effects of rolipram** and suggest a potential role for PDE4s in human behavior.

The implication of the *PDE4D* gene in depression and mood has been also evaluated through **association analyses**. Shifman *et al.* (2008b) performed the first genome wide association analysis for extreme scores of the personality trait neuroticism and found one significant SNP, rs702543, located in an intron of *PDE4D* gene, which was predicted to affect a putative cAMP response element (CRE). Subsequently, Heck and colleagues (2008) found nominal associations for 38 SNPs with Neuroticism and Harm Avoidance in healthy subjects. In addition, another intronic SNP was associated with some Harm Avoidance items in a group of patients with major depression. The case–control association analysis also revealed significant differences for two other polymorphisms. However, SNP rs702543 was not found to be associated with any response variable. To summarize, these observations indicate that the *PDE4D* gene is likely to be involved in susceptibility to neuroticism and associated psychiatric disorders (major depression and anxiety).

Sun *et al.* (2004) also found that chronic lithium treatment at a therapeutically relevant concentration decreased the expression of *PDE4D* in lymphoblasts from bipolar patients, all excellent responders to lithium prophylaxis. Interestingly, *PDE4D* was not regulated by lithium in healthy control subjects. Since lithium has multiple effects on cAMP signaling, it can be speculated that *PDE4D* is particularly relevant for the mechanism of action of lithium.

Another aspect which could be influenced and regulated by *PDE4*D is **memory formation**. Numerous studies have shown memory-enhancing effects of PDEs inhibitors, presumably via increasing intracellular cAMP levels, when performing different memory tasks (reviewed in Blokland *et al.,* 2006). In particular, it has been hypothesized and demonstrated that selective inhibition of the PDE4 protein has procognitive properties (reviewed in Blokland *et al.,* 2006). Moreover, rolipram can also facilitate LTP in the hippocampus (Barad *et al.,* 1998; Navakkode *et al.,* 2004) in addition to improving cognitive performance in several murine models (Barad *et al.,* 1998; Rutten *et al.,* 2006). PDE4 inhibitors have also been shown to reverse memory impairments in genetic mouse models of human disorders, including Rubinstein–Taybi syndrome and Alzheimer disease. Finally, *PDE4D -/-* mice present significant impairments in associative learning using a conditioned fear paradigm as well as alterations in synaptic plasticity, which may be explained by adaptive responses occurring throughout development (Rutten *et al.,* 2008).

The involvement of *PDE4D* in both memory and emotionality places this gene as **an interesting candidate for schizophrenia**. According to this idea, Benes and colleagues (2004) developed a 'partial' rodent model for schizophrenia to characterize the regulation of hippocampal genes in

response to amygdalar activation. Remarkably, they found that *PDE4D* expression was increased after the activation of the basolateral amygdala (BLA). They hypothesize that the synthesis and breakdown of cAMP could potentially have attained a steady-state condition. More recently, Murdoch and colleagues (2007) reported that the full length isoform of DISC1 can bind different members of the PDE4 family, including an isoform from PDE4D (PDE4D3). Moreover, an elevation of intracellular cAMP levels resulted in the release of PDE4D3 isoform from DISC1. This is an encouraging finding, since *DISC1* is one of the most promising schizophrenia candidate genes.

Curiously, *PDE4D* gene has also been related to **stroke** through several association studies. However, there are still doubts about the real implication of this locus on the vulnerability to stroke (for a revision, see Worrall and Mychaleckyj, 2006).

4.5.2. *PLEKHB1 and RAB6A genes*.

The last genes which had been included in this study are *PLEKHB1* and *RAB6A*. Both are adjacent genes located in chromosome 11 that appear in opposite orientations, thus their regulatory upstream regions probably overlap. However, both genes perform apparently unrelated functions.

4.5.2.1. *PLEKHB1* gene

PHR1, recently named with the official name of PLEKHB1, is an integral membrane protein that contains a pleckstrin homology (PH) domain. *PLEKHB1* is highly expressed in mature photoreceptor cells, cochlear and vestibular hair cells, and olfactory receptor neurons (Xu *et al.,* 1999; Xu *et al.,* 2004). In the retina, it can be found in the outer segments of the photoreceptors. *PLEKHB1* is also expressed in many other cells with a possible involvement in sensory function, such as peripheral retinal ganglion cells, cochlear interdental cells, and neurons of the circumventricular organ (Xu *et al.,* 2004). However, mice lacking *PLEKHB1* do not have apparent sensory deficits (Xu *et al.,* 2004).

As commented above, the **protein** encoded by the *PLEKHB1* gene contains an N-terminal pleckstrin homology domain (Krappa *et al.,* 1999; Xu *et al.,* 1999). PH domains are 100- to 120-amino acid modules characterized for their ability to bind to phosphoinositides (Lemmon *et al.,* 2002). PH domains are multifunctional motifs found in numerous and different proteins, many of which are involved in signal transduction pathways. All PH domains have a common tertiary structure (Ferguson *et al.,* 2000; Lemmon *et al.,* 2002). The amino-terminal sequence comprises seven antiparallel β-strands, with the first four and the last three arranged in two β-sheets and forming a β-sandwich structure. By contrast, the carboxi-terminal region of the PH domain is structured as a folded α-helix that “closes off” the wider end of the β-sandwich (Shaw, 1996; Ferguson *et al.,* 2000). In PLEKHB1, the PH domain comprises approximately half of the entire protein. However, unlike the great majority of PH domains, the one of PLEKHB1 lacks most of the eight conserved residues which coordinate binding to phosphoinositides (Xu *et al.,* 1999). In fact, using in vitro binding assays, Xu and colleagues (2004) showed that the PH domain of PLEKHB1 does not bind any of several inositol phosphates (IP), but it does exhibit specific binding to transducin βγ subunits, that suggests that *PLEKHB1* might play a role in **modifying signal transduction in the photoreceptors and probably in other cells**. Moreover,

Etournay and colleagues (2005) showed that PLEKHB1 protein interacts with the myosin 1c and myosin VIIa tails, and is able to dimerise. The **interaction with these myosin proteins** suggests that, as both myosins have been implicated in the mechanotransduction slow adaptation process occurring in the hair cells, PLEKHB1 protein could also be involved in this process.

The human *PLEKHB1* gene maps at chromosome 11 (11q13.5-q14.1). It spans around 28 kb and has 9 exons (figure I21). The use of two promoters and alternative splicing of exon 7 leads to four different *PLEKHB1* transcripts (Krappa *et al.,* 1999; Xu *et al.,* 1999), encoding isoforms of 243, 224, 208, and 189 amino acids, each one with an exposed pleckstrin homology domain at their N terminus and a transmembrane domain at their C terminus. Moreover, each isoform has different tissue or cell type-specificity. Both variants 1 and 2 result from the use of the more upstream promoter, which is photoreceptor-specific and has at least three Crx-like elements (Xu *et al.,* 1999), recognized by Crx, a photoreceptor-specific transcription factor (Chen *et al.,* 1997). These two isoforms contain a 19- amino acid amino-terminal sequence (NT19), which is absent from variants 3 and 4. In addition, variants 1 and 3 contain a region of 36 amino acids encoded by exon 7 that cannot be found in variants 2 and 4. To summarize, the PLEKHB1 proteic sequence can be subdivided in six different regions: NT19 sequence (only in variants 1 and 2), PH domain, a sequence corresponding to exon 7 (only in variants 1 and 3), a juxtamembrane domain (JMD), a transmembrane domain, and a short six amino acid carboxi-terminal peptide. All four transcripts are expressed in retina with transcripts 1 and 2 predominating. In brain, only transcripts 3 and 4 are expressed with transcript 4 predominating (Xu *et al.,* 1999).

Figure I21. Genomic structure of *PLEHKB1*. This gene has 9 exons and encodes for 4 different isoforms. Alternative exons are indicated in green and untranslated regions are shown in white. The variety of isoforms is achieved through the use of two different promoters (a photoreceptor-specific promoter located 5' upstream of exon 1/2, and an internal promoter located 5' upstream of exon Pre3), as well as the alternative splicing of exon 7.
Abbreviations: Pr, photoreceptor-specific promoter; Pi, internal promoter.

4.5.2.2. *RAB6A* gene

Rab proteins are small GTPases that are key regulators of intracellular membrane traffic through constant cycling between a membrane-associated GTP-bound active state and cytosolic GDP-bound inactive state (Wanschers *et al.,* 2008). The Rab family is huge and 60 proteins, including isoforms, are known (Stenmark and Olkkonen, 2001). Rab proteins exert their function by binding to a several effector proteins and taking part in different membrane trafficking events, such as fission, tethering, fusion of membranes, and transport along the actin or microtubule cytoskeleton (reviewed in Jordens *et al.,* 2005). With regard to the transport process, the small GTPase Rab6 is a key regulator in the retrograde transfer from endosomes via the Golgi to the endoplasmic reticulum (ER). It interacts with kinesins, such as Rabkinesin-6 (Echard *et al.,* 1998) or with the dynein/dynactin complex (Short *et al.,* 2002; Wanschers *et al.,* 2007).

Three different isoforms of the Rab6 protein have been identified until now: Rab6A (Goud *et al.,* 1990), encoded by the *RAB6A* gene; Rab6A', generated by alternative splicing of a duplicated exon within the *RAB6A* gene (Echard *et al.,* 2000); and Rab6B, a brain specific isoform (Opdam *et al.,* 2000) encoded by the *RAB6B* gene.

The *RAB6A* gene (figure I22) maps at chromosome 11q13.3 and has 9 exons, including two exons (4 and 5) resulting from a duplication, which are separated by a small 66 bp intron (Echard *et al.,* 2000). This exon duplication has occurred late during evolution as most organisms, including *Drosophila*, express only one copy of *Rab6*. Both Rab6A and Rab6A' isoforms have 208 amino acids, which give rise to proteins with the following domains: a GTP/Mg^{2+} binding domain; a putative guanine nucleotide exchange factor (GEF) interaction site; a putative guanine nucleotide dissociation inhibitor (GDI) interaction site; and two regions known as Switch1 and Switch2, which undergo conformational changes upon GTP-binding. Rab6A and Rab6A' only differ in three amino acids due to substitutions in regions flanking the GTP-binding domain (Echard *et al.,* 2000).

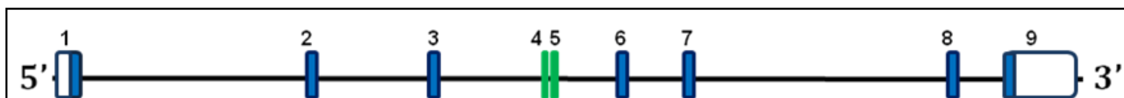


Figure I22. Exonic structure of the *RAB6A* gene. This gene has 9 exons. Alternative exons are indicated in green and untranslated regions appear in white. Alternative splicing of exons 4 and 5 gives rise to two different isoforms: Rab6A' and Rab6A.

Both Rab6A and Rab6A′ are ubiquitously expressed (Echard *et al.,* 2000). Until recently, both Rab6A and Rab6A' were thought to play a role in a Golgi to ER retrograde transport (White *et al.,* 1999; Young *et al.,* 2005) with an additional function for Rab6A' in specific endosome to Golgi transport (Mallard *et al.,* 2002). However, recent studies showed that **Rab6A is dispensable for Golgi to ER transport and that Rab6A' is the isoform that regulates the entire pathway from late endosomes to ER** (Del Nery *et al.,* 2006). Furthermore, Rab6A' has also been implicated in the transition from metaphase to anaphase during mitosis (Del Nery *et al.,* 2006; Miserey-Lenkei *et al.,* 2006). Very recently, Wanschers *et al.* (2008) showed for the first time and interaction of the Rab6 proteins with a member of the cytoplasmic dynein complex (DYNLRB1, one of the light chains of this complex) and co-localisation at the Golgi. Cytoplasmic dynein is the main motor protein complex for the retrograde transfer from endosomes via the Golgi to the ER.

As it can be shown, the relationship between schizophrenia and these two genes is not very clear, however, it cannot be discarded since both are novel genes which are expressed in the brain and which seem to be somehow affected in a neuropsychiatric disorder such as ADHD.

In summary, with the genetic analysis of these nine genes, this work has the main purpose to go further in the molecular bases of schizophrenia and its most characteristic symptoms.

HYPOTHESIS AND OBJECTIVES

This study is part of a multidisciplinary research project whose main objective is to further study the genetic bases of schizophrenia and its most important symptoms. According to this general framework, the general hypothesis and objectives of the present study are the following.

GENERAL WORKING HYPOTHESIS

- Variations in the genes previously presented modulate the vulnerability to schizophrenia or some of its most characteristic features, particularly auditory hallucinations, cognitive impairment, neuroanatomical alterations, as well as the emotional dysfunction linked to the pathological conditions described before.

GENERAL OBJECTIVES

- Analysis of several polymorphisms from the different candidate genes through case-control association analyses. For this purpose, different samples of schizophrenic patients and healthy controls will be included. This will allow the comparison among different clinical subtypes and populations of patients.

- Search of associations between single alleles or haplotypes and different quantitative variables and disease traits, including a) scores on general and hallucination schizophrenia scales, and b) performance on several cognitive tasks.

- Evaluation of the effect of several selected polymorphisms on certain magnetic resonance imaging (MRI) phenotypes which are known to be affected in schizophrenic patients.

MATERIALS AND METHODS

MATERIALS AND METHODS

1. Writing Guidelines

Genes are designated by their official symbol and name according to the HUGO Gene Nomenclature Committee (http://www.genenames.org/), which provides and approves names for human genes to avoid confusions when searching for information about genes.

With regard to the single nucleotide polymorphisms (SNPs), the rs number from the NCBI dbSNP database is preferred against other existing names. If the rs number is not available, then the most common name in the existing literature is used. Other polymorphic variants (VNTRs, indels) are also identified with the most common name in the previous literature.

Alleles from SNPs are designated with reference to the nucleotide located in the coding strand of the studied gene. However, there is an exception to this rule: if a genomic region with several genes and different coding strands is studied as a whole, then the alleles will be named with reference to the base located in the plus strand, according to the reference assembly from the NCBI dbSNP database.

All chemical and biochemical compounds are designated according to the recommendations from the IUPAC ("International Union of Pure and Applied Chemistry") and the IUBMB ("International Union of Biochemistry and Molecular Biology"), which can be consulted in http://www.chem.qmul.ac.uk/iupac/.

2. Clinical samples

Three different groups of patients and controls coming from three different countries (Spain, Germany and United States of America) have been used for this study.

General demographic information about both patients and controls can be found in table M2, whereas table M3 shows the most relevant clinical features of the three samples of patients.

Patients were evaluated for one or more of the following scales:

- BPRS (Brief psychiatric rating scale) from Overall and Gorham, 1962: It was originally developed as a tool to evaluate psychopathological characteristics and to measure treatment response in clinical psychopharmacology research. It allows the evaluation of 18 (originally 16) different psychiatric symptoms, which can be evaluated from 1 (not present) to 7 (extremely severe). The 18 items which can be assessed are: somatic concern, anxiety, emotional withdrawal, conceptual disorganization, guilt feelings, tension, mannerisms and posturing, grandiosity, depressive mood, hostility, suspiciousness, hallucinatory behavior, motor retardation, uncooperativeness, unusual thought content, blunted affect, excitation and disorientation and confusion.

- Psychiatric Assessment scale or KGV Symptom scale (Krawiecka *et al.,* 1977; Spanish version by Pérez-Fuster *et al.*, 1989): It is a 14-item scale to assess the patient's general mental state in chronic psychotic patients. Its main purpose is to provide a summary of the different symptoms suffered by these patients. It has 14 sub-scales which can be scored from 0 (absence of symptom) to 4 (severe manifestation of symptom). The 14 items are: anxiety, depression, suicidal thoughts and behaviors, elevated mood, hallucinations, delusions, flattened affect, incongruous affect, overactivity, psychomotor retardation, abnormal speech, poverty of speech, abnormal movements and accuracy of assessment.

- PANSS (Positive and negative syndrome scale) from Kay *et al.,* 1987 (Spanish version from Peralta and Cuesta, 1994): It is an exhaustive measurement which provides information about 30 different items, which can be grouped in three wide domains: Positive symptoms, Negative Symptoms and Cognitive or General Psychopathology. Moreover, it evaluates the schizophrenic syndrome from two different points of view: on the one hand, a dimensional perspective, which studies the severity of negative, positive and general symptoms; on the other hand, a categorical perspective, which classifies the disorder as negative, positive or mixed, depending of the scores for each domain. Table M1 shows a scheme of the items evaluated by PANSS.

More detailed information about the three samples of individuals included in this study can be also found below (tables M2 and M3).

Positive (p)	**Cognitive or general psychopathology (g)**	
P1. Delusions	G1. Somatic concern	
P2. Conceptual disorganization	G2. Anxiety	
P3. Hallucinatory behavior	G3. Guilt feelings	
P4. Excitement	G4. Tension	**SCORES**
P5. Grandiosity	G5. Mannerism and posturing	1. Absent
P6. Suspiciousness / persecution	G6. Depression	2. Minimal
P7. Hostility	G7. Motor retardation	3. Mild
	G8. Uncooperativeness	4. Moderate
Negative (n)	G9. Unusual thought content	5. Moderate severe
N1. Blunted affect	G10. Disorientation	6. Severe
N2. Emotional withdrawal	G11. Poor attention	7. Extreme
N3. Poor rapport	G12. Lack of judgment and insight	
N4. Passive / apathetic social withdrawal	G13. Disturbing of volition	
N5. Difficulty in abstract thinking	G14. Poor impulse control	
N6. Lack of spontaneity/flow of conversation	G15. Preoccupation	
N7. Stereotyped thinking	G16. Active social avoidance	

Table M1. List of items evaluated in PANSS scale.

Table M2. Demographic characteristics of the 3 samples analyzed in this study.

Sample origin	Geographical area of origin	N	Sex	Age at evaluation time (mean ± SE)	Ethnic group
University of Valencia	Valencia, Spain	328 patients	218 males, 110 females	39.7 ± 0.66	Caucasian
		348 controls	188 males, 117 females, 45 unknown	38.16 ± 0.80	
University of Würzburg	Lower Franconia, Germany	336 patients	156 males, 175 females, 6 unknown	41.63 ± 0.77	Caucasian
		540 controls	268 males, 264 females, 6 unknown	30.93 ± 0.43	
Clinical Brain Disorders Branch (NIMH)	United States of America (USA)	466 families	-	N/A	Caucasian
		498 patients	373 males, 125 females	37.16 ± 0.46	
		612 parents	270 males, 342 females	Unavailable	
		427 unaffected siblings	178 males, 249 females	36.32 ± 0.62	
		483 healthy controls	223 males, 260 females	33.01 ± 0.47	
TOTAL		1162 patients, 1371 controls, 612 parents, 427 unaffected siblings	1874 males, 1642 females, 57 unknown		

Data are displayed as mean ± SE. Abbreviations: NIMH, National Institute of Mental Health; SE, Standard Error; N/A, not applicable.

Table M3. Clinical characteristics of the patients' samples.

Sample	Diagnosis	Age of onset	Presence of AH	PANSS Total	BPRS	KGV scale	PSYRATS - AH
University of Valencia	244 SCZ; 18 schizoaffective; 2 brief psychosis; 14 delusional disorder; 34 BPD; 8 unspecified psychosis; 5 depressive psychosis; 3 atypical psychosis	25.9 ± 0.58	246 hallucinatory patients; 81 non-hallucinatory patients; 1 unknown	67.25 ± 7.11	51.96 ± 7.09	12.42 ± 3.37	20.25 ± 1.42
University of Würzburg	247 SCZ; 89 BPD	27.08 ± 0.63	N/A	N/A	39.33 ± 0.78	N/A	N/A
Clinical Brain Disorders Branch	SCZ or schizoaffective disorder	22.07 ± 0.25	N/A	58.59 ± 1.21	N/A	N/A	N/A

Data are displayed as mean ± SE.

Abbreviations: SCZ, schizophrenia; BPD, bipolar disorder; AH, auditory hallucinations; PANSS, Positive and Negative Syndrome Scale; BPRS, Brief Psychiatric Rating Scale; KGV, Krawiecka, Goldberg & Vaughan (psychiatric assessment scale) ; PSYRATS, Psychotic Symptom Rating Scale; N/A, not applicable.

2.1. Spanish sample

For this study, 328 unrelated psychiatric patients were included. They came from the Psychiatric In-patient and Out-patient Units of the Mental Health Service number 4 of the Clinical Hospital (University of Valencia, Spain). Age ranged from 18 to 77 years old. The retrospective clinical data collected from each patient were compared with the information provided from previous clinical reports and family members. All patients met DSM-IV criteria for different psychoses, mainly schizophrenia (76.25%). These diagnoses were confirmed for every patient by a consensus meeting with the treating psychiatrist and one of the psychiatrists of our research group. Patients also had a minimum one-year evolution of the illness and were on antipsychotic treatment at evaluation time. Exclusion criteria for this study included incoherence of speech and/or the incapacity for basic comprehension of the questions.

Symptom severity was assessed in some patients through BPRS, KGV and PANSS scales (136, 295 and 135 individuals, respectively). Furthermore, other scales were used to evaluate some specific symptoms:

- Auditory hallucinations were assessed in 314 patients using the Psychotic Symptom Rating Scale (PSYRATS) for auditory hallucinations (Haddock *et al.*, 1999; Spanish version by González *et al.*, 2003b) This standardized scale rates 11 parameters of AH on a five-point scale (0-4): frequency, duration, location, loudness, beliefs about the origin of voices, amount of negative content, degree of negative content, amount of distress, intensity of distress, disruption to life and controllability of voices.

- Delusions were also evaluated in a subsample of 166 individuals through the Psychotic Symptom Rating Scale (PSYRATS) for delusions (Haddock *et al.*, 1999). It is a six-item scale which assesses dimensions of delusions. The items are also rated on a five-point ordinal scale and are the following: amount of preoccupation, duration of preoccupation, conviction, amount of distress, intensity of distress and disruption.

348 healthy unrelated subjects with no history or familiar background of mental disorders were also selected for the analysis. The selection of control individuals with similar demographic characteristics to the psychotic group (Caucasian ethnic group, similar age…) was considered to be an important requisite to avoid sample stratification. These control subjects were between 18 and 91 years old and were also of Spanish origin. Drug abuse was also considered among the exclusion criteria.

All subjects gave their written informed consent to participate in this study, which was approved by the Ethical Committee of the Medicine Faculty, University of Valencia.

2.2. German sample

This sample was managed at the Department of Psychiatry and Psychotherapy, University of Würzburg. A total of 336 unrelated patients (mean age was 41.63 years old) took part in the study. They were hospitalized at the Department of Psychiatry at least once. Diagnoses were made by an extensive, semi structured interview performed by an experienced psychiatrist.

Clinical data obtained from the interview was contrasted with the information obtained from family members and previous clinical assessments. In short, 247 patients met ICD-10 criteria for chronic schizophrenia, while 89 individuals suffered from bipolar disorder (table M3). Symptom severity was assessed in some individuals through BPRS. Patients did not show other disorders (neurological disorders, epilepsy, mental retardation…) which could be the underlying cause of the psychiatric disorder. Substance-induced psychosis and affective disorders were also considered as exclusion criteria.

540 control subjects were also included in the study. They were healthy blood donors from the same catchment area as the patient group (Lower Franconia, Bavaria). To avoid ethnic stratification, they were also from Caucasian origin. Although these individuals were not assessed for psychiatric disorders, all of them were free of medication and the aim of the study was explained to them, so it is unlikely that the subjects from the control group are suffering from severe psychiatric disorders.

Only those patients and controls who gave their written informed consent were accepted for the study, which was approved by the Ethics Committee of the University of Würzburg.

2.3. North-American sample

All subjects were recruited for the Clinical Brain Disorders Branch (CBDB) "Sibling Study", a study of neurobiological abnormalities associated to genetic risk for schizophrenia. This study was developed at the National Institute of Mental Health (Bethesda, MD, USA). All study procedures were also approved by the Institutional Review Board of the National Institute of Mental Health.

In summary, the sample included 498 patients who fulfilled the DSM-IV criteria for schizophrenia-spectrum disorders (all of them suffered from schizophrenia or schizoaffective disorder), 427 unaffected siblings, 612 parents and 483 unrelated healthy controls. All participants were between 18 and 60 years of age, had a premorbid IQ above 70 and were able to give a written informed consent. Significant medical problems, history of head trauma and alcohol or drug abuse within the last 6 months were considered as exclusion criteria. In order to avoid ethnic stratification, only Caucasian subjects of European ancestry were included in this study. Patients were also evaluated through PANSS (see table M3).

Cognitive measures were obtained for some subgroups. Control subjects and schizophrenic patients performed an extensive battery of neuropsychological tests previously found to be abnormal in healthy siblings of patients with schizophrenia (Elvevag and Goldberg 2000; Egan *et al.,* 2001b). The test battery included the Weschler Adult Intelligence Scale, revised edition (WAIS-R) as a measure of general intelligence; selected subtests of the Wechsler Memory Scale, revised version (WMS-R), to assess episodic memory; Trail Making Test Parts A and B, to measure psychomotor speed and also as an oculomotor scanning; Verbal and Category Fluency tests; a measure of attention (Gordon's Continuous Performance Task or CPT); two measures of working memory: N-Back (Zero-back, One-back, Two-back, and Three-back conditions) and the Wisconsin Card Sorting Test (WCST); the California Verbal Learning Test (CVLT), which tests episodic memory; and finally, the Judgment of Line Orientation Test as a measure of

visuospatial ability. To avoid the increase of type I error related to multiple testing, effect of genotype was examined only in seven measures derived from these cognitive tests: Verbal Memory, Working Memory-N back, Visual Memory, IQ-Processing Speed-Fluency, Executive Function-WCST, Attention and Digit Span (Genderson *et al.,* 2007).

3. Genomic DNA extraction methods

Genomic DNA from the Spanish individuals described above was obtained from different methods and cellular types:

- DNA extraction from blood leukocytes: blood was the preferable source of genomic DNA, since the amount of DNA which can be obtained from blood cells is generally higher than the DNA obtained from other tissues.

- DNA extraction from saliva: Although DNA extraction from blood samples is more desirable and preferable because of its higher yield, sometimes it was necessary to use other types of cellular tissues, such as buccal epithelial cells, which can be obtained from saliva with a less invasive method than blood extraction.

3.1. DNA extraction from peripheral blood leukocytes

Three different methods were used.

3.1.1. Phenol-chloroform DNA extraction method (Sambrook and Russell, 2001)

This methodology is mainly based on the use of an organic solvent (phenol-chloroform solution). After destroying the leukocytes' cellular and nuclear membranes with the use of detergents (nonidet and SDS) and proteinase K, proteins can be separated from nucleic acids since they are captured by the organic solvents, while nucleic acids remain in the aqueous phase. After phenolisation, it is also necessary to wash the DNA phase with chloroform-isoamilic solution to eliminate phenol residues which can remain in the aqueous DNA phase. Finally, DNA is precipitated with absolute ethanol in the presence of NaCl.

REAGENTS AND SOLUTIONS

- Saline solution (0.9% NaCl):
  - NaCl 9 g
  - Distilled water 1 L
- Erythrocytes lysis solution:
  - Tris 2M pH 7.5 5 mL
  - MgCl2 1M 2.5 mL
  - Distilled water up to 500 mL

* Nonidet P-40 0.1%:

  - Nonidet™ P-40 (FLUKA, catalog no. 21-3277) — 1 mL
  - Distilled water — 999 mL

* Solution A:

  - NaCl 5M — 1 mL
  - EDTA 0.25M pH 8 — 5 mL
  - SDS 10% — 2.5 mL
  - Distilled water — up to 50 mL

* Proteinase K (Sigma-Aldrich, catalog no. P2308) 10 mg/mL
* Phenol-chloroform-isoamilic solution 25:24:1
* Chloroform-isoamilic solution 24:1
* NaCl 5M
* Cold absolute ethanol
* 70% Ethanol solution
* TE buffer pH 7.5:

  - Tris-HCl 10mM pH 7.5 — 5 mL
  - EDTA 1mM pH 8 — 1 mL
  - Distilled water — up to 500 mL

PROCEDURE

a) Obtaining of blood cells

  * Mix peripheral blood (from 10 to 20 mL, collected in tubes with EDTA-K_3 as a coagulant) with saline solution, up to a final volume of 15-50 mL.
  * Centrifuge for 20 minutes at 4^oC and 3000 rpm.
  * Aspirate and discard the supernatant. The process can be stopped at this point and the pellet, which corresponds to the cellular layer, can be frozen at -20^oC.

b) Lysis of erythrocytes

  * Add the sterile erythrocytes lysis solution to the pellet obtained in the previous step, up to a final volume of 50 mL. Shake carefully by inversion to homogenize the mix.
  * Leave at 4^oC for 30 minutes, shaking by inversion from time to time.
  * Centrifuge for 15 minutes at 4^oC and 3000 rpm.
  * Discard and decant the supernatant.
  * To dry the pellet, leave the tube in an inverted position on a filter paper sheet for some minutes.

COMMENT: if the pellet is still red (a sign of the presence of undesirable blood cells), this process can be repeated again by adjusting volumes and lysis times. The final pellet must be whitish or slightly pink.

c) Lysis of leukocytes

* Add 15 mL of Nonidet P-40 0.1% to the pellet. Mix gently.
* Centrifuge for 20 minutes at 4°C and 3500 rpm. During the centrifugation time, prepare the solution A, which must be fresh.
* Discard the supernatant and break up the pellet vortexing it.
* Add 10 mL of solution A and vortex the tube until it is completely homogenized.
* Add 200 µL of proteinase K.
* Incubate at 56°C overnight.

d) DNA purification

* Add an equal volume (v/v) of the phenol-chloroform-isoamilic solution. Shake vigorously.
* Centrifuge for 10 minutes at 4°C and 3000 rpm.
* Discard the lower phase by aspiration with a pasteur pipette and transfer the upper DNA phase to a new tube.
* Repeat the three previous steps.
* Add an equal volume of the chloroform-isoamilic solution to remove any trace of phenol and shake vigorously.
* Centrifuge for 10 minutes at 4°C and 3000 rpm.
* Transfer the upper phase to a new tube.
* Repeat the three previous steps.

e) DNA precipitation and wash

* Add NaCl 5M (400 µL per 5 mL of solution) and shake vigorously.
* Precipitate the DNA with two volumes of absolute ethanol.
* Homogenize by gentle inversion until the DNA precipitate appears. The precipitate will look as a white fibrous medusa.
* Catch the DNA precipitate and wash it by immersion in 70% ethanol.
* Let the medusa dry and transfer it with a sterile microbiology loop (or something similar) to a Nunc CryoTube™ which contains 1mL of TE buffer pH 7.5.

3.1.2. DNA extraction by saline precipitation

The saline precipitation method was selected to substitute the phenol-chloroform method, since it avoids the use of organic solvents, such as phenol, which are highly contaminant and toxic. It is based on the use of a moderated concentration of a monovalent cation such as Na^+, followed by the addition of absolute cold ethanol to obtain the precipitation of DNA. Since the

first three steps (obtaining of blood cells, erythrocytes lysis and leukocytes lysis) are common to the phenol-chloroform extraction method, we will focus on the DNA purification step.

d) DNA purification

* Add 1mL of saturated NaCl 5M per 3 mL of the resulting solution from step c).
* Shake vigorously for 2 minutes.
* Centrifuge for 15 minutes at room temperature and 13000 rpm.
* Transfer the supernatant to a fresh tube and repeat the two previous steps.
* Add one volume of cold absolute ethanol. Homogenize by gentle inversion until the DNA precipitate appears.
* Catch the DNA precipitate and wash it by immersion in 70% ethanol.
* Redisolve the DNA medusa in 1mL of TE buffer pH 7.5.

3.1.3. DNA extraction by Puregene Blood Kit

The Gentra® Puregene® Blood kit (Qiagen®, catalog no. 158389) allows the extraction of high quality DNA from whole blood with a high yield and without the use of organic solvents. Cells are lysed with an anionic detergent in the presence of a DNA stabilizer, which reduces the activity of DNases. Proteins and other contaminants are removed by salt precipitation and finally DNA is precipitated with alcohol and redisolved in a buffered solution. Thus, this method fulfills the best features of the two previous methods and for this reason it was considered to be the best alternative to DNA extraction from blood cells.

The protocol used in this study presents some variations with respect to the original manufacturer's protocol.

REAGENTS AND SOLUTIONS

* PBS solution 1X pH 7.4 (prepared at the laboratory):
  - NaCl 8 g
  - KCl 0.2 g
  - KH_2PO_4 0.24 g
  - Na_2HPO_4 1.44 g
  - Distilled water up to 1 L
* Erythrocytes lysis solution (prepared at the laboratory):
  - NH_4Cl 7.007 g
  - NH_4CO_3 0.071 g
  - Distilled water up to 1 L
* Cell Lysis Solution (from the commercial kit)
* Protein Precipitation Solution (from the commercial kit)
* 100% Isopropanol

* <u>70% Ethanol</u>

* <u>DNA Hydration Solution</u> (from the commercial kit)

PROCEDURE

a) Obtaining of leukocytes

* Centrifuge 10 mL of whole peripheral blood (collected in EDTA-K_3 tubes) for 15 minutes at 2500 rpm and room temperature (rt).
* Aspirate and discard the upper liquid layer (which corresponds to the plasma).
* Add up to 10 mL of PBS solution to the buffy coat, which contains the red and white cells, and mix by inversion.
* Centrifuge for 15 minutes at 2500 rpm and rt.
* Discard the supernatant and repeat the two previous steps, to ensure the complete wash of the buffy coat.
* Aspirate the white layer which contains the leukocytes with a transfer or pasteur pipette. Transfer these cells to a fresh 10 mL tube.
* Add 7 mL of the erythrocytes lysis solution and mix by inversion.
* Incubate 20 minutes at room temperature, mixing by inversion from time to time. This step will lyse those red blood cells which were transferred together with the white blood cells.
* Centrifuge for 15 minutes at 2500 rpm and rt to pellet the leukocytes.
* Carefully discard the supernatant and wash the borders of the pellet with 1 mL of erythrocytes lysis solution.
* Resuspend the pellet in 1-2 mL of erythrocytes lysis solution and add more lysis solution up to a volume of 10 mL.
* Centrifuge for 15 minutes at 2500 rpm and rt to pellet the leukocytes.
* Discard the supernatant and wash the borders of the pellet again with erythrocytes lysis solution.
* Drain the tube by inverting on a clean piece of absorbent paper, taking care that the pellet remains in the tube.
* At this point, it is possible to store the leukocytes for a later DNA extraction. In this case, resuspend the pellet in 0.9 mL of PBS solution, transfer it to a cryotube and store at -80°C for an optimal cell conservation. For a prompt DNA extraction, then resuspend the leukocytes pellet with 0.4 mL of PBS, transfer it to a fresh 13 mL tube and add 10 mL of Cell Lysis Solution.
* Incubate the tube at room temperature for at least 7 days (however, samples are stable in Cell Lysis Solution for at least 2 years at rt).

b) Protein precipitation

* Add 3.33 mL of Protein Precipitation Solution and vortex the tube vigorously for 15 seconds to homogenize the sample.
* Incubate on ice for 30 minutes.

* Centrifuge for 20 minutes at 4000 rpm and rt. The precipitated proteins should form a tight, dark brown pellet.

c) DNA precipitation

* Aspirate the supernatant (which contains the DNA) with a transfer pipette and transfer it to a tube with 5 mL of cold 100% Isopropanol.
* Mix gently by inversion until the DNA is visible as threads or a clump.
* Centrifuge for 6 minutes at 4000 rpm. The DNA will be visible as a small white pellet.
* Carefully discard the supernatant and drain the tube by inverting on a clean piece of absorbent paper.
* Add 10 mL of cold 70% ethanol and invert several times to wash the DNA pellet.
* Centrifuge for 8 minutes at 4000 rpm.
* Carefully discard the supernatant and drain the tube again on a piece of absorbent paper for 30-40 minutes at room temperature to dry the DNA pellet.

d) DNA hydration

* Add 600 uL of DNA Hydration Solution to the tube which contains the DNA pellet.
* Incubate at room temperature for 3-4 days with gentle shaking. This step is necessary to completely rehydrate the DNA.
* Store the DNA sample in a cryotube at -80°C.

3.2. DNA extraction from saliva samples

Two different methods were used.

3.2.1. DNA extraction from saliva with SSS DNA extraction kit.

The SSS DNA extraction kit (Real®, catalog no. RBME02) is an easy and scalable method with allows the DNA extraction from different sources, including saliva. It is also based on the saline precipitation method and the protocol used for this study includes few modifications from the original manufacturer’s protocol.

REAGENTS AND SOLUTIONS

* Lysis Solution (from the commercial kit)
* Protein Precipitation Solution (from the commercial kit)
* RNase 10mg/mL
* 100% Isopropanol

* 70% Ethanol
* TE buffer pH 7.5

PROCEDURE

a) Saliva collection

* Ask the subject to rinse mouth with cold water just before collecting saliva. It is also desirable not to eat, drink or brush teeth before collection.
* Ask the subject to deliver saliva in a sterile tube, up to a minimum volume of 1-2 mL.
* Take the saliva tube and prepare 800 µL aliquots.
* Store the samples at -20°C (or 4°C for a prompt extraction).

b) Cellular lysis

* Centrifuge for 90 seconds at 13000 rpm.
* Discard the supernatant with a pipette, leaving a residual volume of 10-20 µL.
* Vortex the tube to resuspend the pellet and to optimize the cellular lysis in the following step.
* Add 800 µL of Lysis Solution and mix with the pipette to lyse the buccal cells.
* Incubate at 37°C for 5 minutes.
* Add 20 µL of RNase solution and mix by inversion at least 25 times to ensure the complete homogenization of the sample.
* Incubate at 37°C for 45 minutes.

c) Protein precipitation

* Let the sample to temper at rt.
* Add 480 µL of Protein Precipitation Solution and vortex it vigorously for 30 seconds.
* Centrifuge for 4 minutes at 14000 rpm. A dark brown precipitate (which contains the protein fragments) will appear.

d) DNA precipitation

* Transfer the supernatant (which contains the DNA) to a fresh tube containing 800 µL of Isopropanol.
* Mix by inversion at least 50 times.
* Centrifuge for 2 minutes at 14000 rpm. The DNA precipitate appears as a small white medusa.
* Discard the supernatant and drain the tube briefly by inverting on a clean sheet of absorbent paper.
* Add 800 µL of 70% Ethanol solution to wash the DNA.
* Centrifuge for 1 minute at 14000 rpm.
* Carefully discard the supernatant without disturbing the DNA pellet.

* Centrifuge again for 1 minute at 14000 rpm to fully eliminate the residual ethanol.
* Dry the tube by inverting it on a clean sheet of absorbent paper for 15 minutes.

e) DNA hydration

* Add between 70 and 100 µL of TE buffer to the tube which contains the DNA pellet. Resuspend the DNA with a pipette.
* Incubate at rt overnight with gentle shaking. This step will ensure the complete rehydration of the DNA.
* Transfer the DNA sample to a cryotube and store at -80°C.

3.2.2. DNA extraction from saliva with Oragene.

The Oragene™ DNA self-collection kit (DNA Genotek Inc., catalog no. OG-250) is based on the use of an innovative recollection tube (the Oragene vial, see figure M1) containing a stabilizer which preserves saliva samples for long term storage at room temperature without DNA degradation. For this reason, this methodology (also based on the saline precipitation method) is clearly superior to other DNA extraction methods from saliva, such as the SSS DNA extraction kit, and allows the obtaining of high DNA yields, which are comparable to the yields when using whole blood. Moreover, the purity and quality of the DNA is also very high and similar or even better than DNA from blood.

Figure M1. Oragene™ vial used to collect saliva for DNA extraction.

REAGENTS AND SOLUTIONS

* Oragene vial (from the commercial kit)
* Oragene Purifier (from the commercial kit)
* 95% Ethanol
* TE buffer pH 7.5

PROCEDURE

a) Saliva collection and stabilization

* Ask the subject to deliver saliva in the Oragene vial. Once the vial is capped, the DNA-preserving fluid is mixed with saliva. Saliva is now stable for long-term storage at room temperature.
* Incubate the saliva sample in the Oragene vial at 50°C in a water bath overnight.

b) Cell lysis and protein precipitation

* Transfer 500 µL of the saliva sample to a fresh tube (the remaining sample can be stored at room temperature for further use).
* Add 20 µL of the Oragene Purifier and mix gently by inversion. With the Purifier, all impurities (including proteins) will precipitate.
* Incubate on ice for 10 minutes.
* Centrifuge for 3 minutes at 13000 rpm at room temperature.

c) DNA precipitation

* Carefully transfer the supernatant with the DNA to a fresh tube and discard the pellet.
* Add 500 µL of 95% ethanol and mix gently by inversion at least 5 times or until the DNA medusa appears.
* Let the solution stand for 10 minutes at room temperature to ensure the full precipitation of DNA.
* Centrifuge for 1 minute at 13000 rpm at room temperature.
* Discard the supernatant and centrifuge again to remove the residual ethanol.

d) DNA hydration

* Once all of the ethanol has been completely eliminated, dissolve the DNA pellet in 100 µL of TE buffer. Vigorous vortexing is also useful.
* Incubate at room temperature overnight to dissolve the DNA.
* Store the sample at -80°C.

3.3. DNA quantification

DNA samples were quantified by absorbance. Two different spectrophotometers, a BioPhotometer from Eppendorf and a NanoDrop® ND-1000 were used. The Nanodrop was especially useful in those cases when the DNA yield was very low (for example, for DNA samples from saliva), as this spectrophotometer allows measurements of small volumes (1 µL) without any dilution of the sample.

The absorbances at the following wavelengths were measured:

- A_{260}: It measures amounts of DNA. To ensure an accurate measurement of DNA concentration with the Biophotomether, this value should fall between 0.1 and 1, thus it is necessary to prepare a 1:20 dilution of the original sample. With regard to the Nanodrop, its high sensitivity allows to make the measurement directly on the original sample.
  It is considered that 1 unit of optic density (DO) at 260 nm corresponds to 50 ng/µL of double-stranded DNA.

- A_{280}: It is a measure of the protein concentration.

- A_{320}: It measures the light scattering and it also gives an estimation of the background turbidity. If the DNA extraction protocol has been followed successfully, this value is expected to be low.

The calculation of concentration and purity was as it follows:

- **DNA concentration in ng/µL** = (A_{260} – A_{320}) x dilution factor x 50 ng/µL (conversion factor)

- **DNA purity** (measured with the A_{260}-A_{280} ratio) = (A_{260} – A_{320}) / (A_{280} – A_{320}). For a good DNA performance, this value should fall between 1.6 and 2.

4. Genotyping

4.1. Selection of polymorphisms

Several polymorphisms located in the 9 candidate genes were selected for this study. Almost all the polymorphisms are Single Nucleotide Polymorphisms (SNPs), with the exception of some VNTRs (variable number of tandem repeats) which were selected due to their functional implications.

In short, the polymorphism selection process could be divided in two steps:

* Selection of informative SNPs, known as tag SNPs, to capture all the variability present in the regions of interest. This is applicable to all genes except *TPH2, HTR2A* and *NOS1*, because only a few markers of interest were studied on those genes.

* Selection of other additional polymorphisms.

Table M4 shows an overview of the Internet resources used to select the genetic markers.

Table M4. Internet databases and utilities used for the selection of polymorphisms.

Name	Web page	Description
NCBI-dbSNP	http://www.ncbi.nlm.nih.gov/sites/entrez/	Database from the National Center for Biotechnology Information which contains information about millions of polymorphisms from *Homo sapiens* and other species.
Ensembl	http://www.ensembl.org/	It provides access to all the databases from the Ensembl Project, a joint project between EMBL - EBI and the Sanger Institute.
UCSC Genome Browser	http://genome.ucsc.edu/	Group of interrelated databases and utilities from the Genome Bioinformatics Group at the University of California Santa Cruz.
HapMap	http://www.hapmap.org/	International approach to the study of the genetic differences and similiarities of human beings, with special attention to SNPs.
Pupa Suite	http://pupasuite.bioinfo.cipf.es/	Online utility which performs a high-throughput search of SNPs with a potential phenotypic effect.
Polyphen	http://genetics.bwh.harvard.edu/pph/	Tool which evaluates the possible impact of a non synonymous change on the structure and function of human proteins.

4.1.1. Selection of tag SNPs.

Although nowadays it is possible to genotype all the SNPs located in a gene of interest, this approach is in fact unnecessary, since many SNPs could provide redundant information, depending on the linkage disequilibrium (LD) structure of the area. An alternative is to select a group of polymorphisms which ensure that we are capturing an acceptable level of variability for our study. These polymorphisms are called tag SNPs, since they give indirect information about nearby variants (also called proxies) which are in high LD with the selected tags. Therefore, the selection of tags is based on the LD pattern between the different polymorphisms.

However, it is also important to consider which source of genotypic data we will use to analyze the LD pattern and to select the tags. These genotype data can be provided by the HapMap Project, a big collaborative effort whose main objective is to determine the common patterns of DNA sequence variation in the human genome and to make this information freely available in the public domain (International HapMap Consortium, 2003). Currently (HapMap data release #23a, March 2008, on NCBI B36 assembly, dbSNP b126), almost four millions of SNPs have been genotyped in four different populations, representing a huge volume of information.

The procedure followed to select the tags for our studies was the following:

* Download the genotype data for all the SNPs located in the region of interest. The HapMap population used as a reference for tag selection is CEPH (Utah residents with ancestry from northern and western Europe). These DNA samples come from the Centre d'Etude du Polymorphisme Humain and are also known as CEU. Although it is unclear how accurately these samples reflect the patterns of genetic variation in European people, we decided to use these individuals as our reference because we assume that CEU samples present the highest similarity with the samples analyzed in this study. Moreover, we considered a chromosomic region which spans the genomic region of our candidate gene plus some kilobases (Kb) downstream and upstream the gene.

* Selection of tag SNPs with the software Tagger, implemented in Haploview program version 4.0 (Barrett *et al.,* 2005). Generally, only those SNPs with a minor allele frequency (MAF) equal or higher than 0.1 were considered. Moreover, the threshold value for the correlation coefficient (r^2) was set at 0.8, which means that each tag SNP has at least an LD value of 0.8 with respect to its proxies. Finally, we used an aggressive multimarker test, where single tags as well as haplotypes of tags are tested against other single SNPs.

4.1.2. Selection of additional polymorphisms.

Other polymorphisms were also selected attending to the following criteria:

* Location in the coding region. Non synonymous changes are of special interest given its possible effect on the protein's structure and function. To test the effect of a non synonymous SNP, the Polyphen utility was used.

* Polymorphisms which had been previously associated with schizophrenia or other mental or neurological disorders. Examples of this category are SNP rs6311, from *HTR2A* gene, rs4570625 from *TPH2* and the VNTR from the *NOS1* gene.

* Polymorphisms with a demonstrated effect on gene function, like 5-HTTLPR and STin2, from the serotonin transporter gene.

* Polymorphisms with a potential phenotypic effect, estimated through the software PupaSuite (Conde *et al.,* 2006), an online tool which evaluates the effect of SNPs located in conserved regions that the cellular machinery uses for the correct processing of genes (intron/exon boundaries, exonic splicing enhancers) as well as predicted transcription factor binding sites. As an example, PupaSuite (Conde *et al.*, 2006) identified that SNP rs1042173 (from the serotonin transporter gene) was altering a predicted exonic splicing enhancer.

4.1.3. Polymorphisms selected for this study.

The tables M5 to M12 show all the polymorphisms selected and analyzed in this doctoral thesis work. The chromosomic positions are in base pairs (bp) and refer to the NCBI build 36. Moreover, a figure with the structure of each gene is also included and the position of each polymorphism is also indicated with an orange arrow (figures M2 to M9).

Table M5. *SLC6A4* polymorphisms.

rs code (or other name)	Type	Position (dbSNP Build 127)	MARKER ORDER	DISTANCE FROM M1	ALLELE CHANGE	CEU MAF	LOCATION/ FUNCTION
rs3813034	SNP	25548930	M1	0	G/T	0.440 (G)	3' near gene
rs1042173	SNP	25549137	M2	207	T/G	0.433 (G)	3'UTR
rs140700	SNP	25567515	M3	18585	A/G	0.108 (A)	INTRON 5
rs2228673	SNP	25569357	M4	20427	C(Asn)/ G(Lys)	NO DATA	EXON 4
rs2020942	SNP	25571040	M5	22110	A/G	0.383 (A)	INTRON 2
STin2	VNTR	25572717-25572518	M6	23787	9rep 10rep 12rep	NO DATA	INTRON 2/ transcriptional enhancer
rs2020939	SNP	25574858	M7	25928	C/T	0.400 (T)	INTRON 1A
rs2020936	SNP	25574940	M8	26010	T/C	0.200 (C)	INTRON 1A
rs2066713	SNP	25575791	M9	26861	C/T	0.383 (T)	INTRON 1A
rs4251417	SNP	25575984	M10	27054	A/G	0.117 (A)	INTRON 1A
5-HTTLPR (including rs25531)	VNTR + SNP	25588600-25588250	M11	39670	LA/LG/ SA/SG	NO DATA	Promoter/ regulator of expression
rs12945042	SNP	25596054	M12	47124	G/A	0.300 (A)	5' Upstream

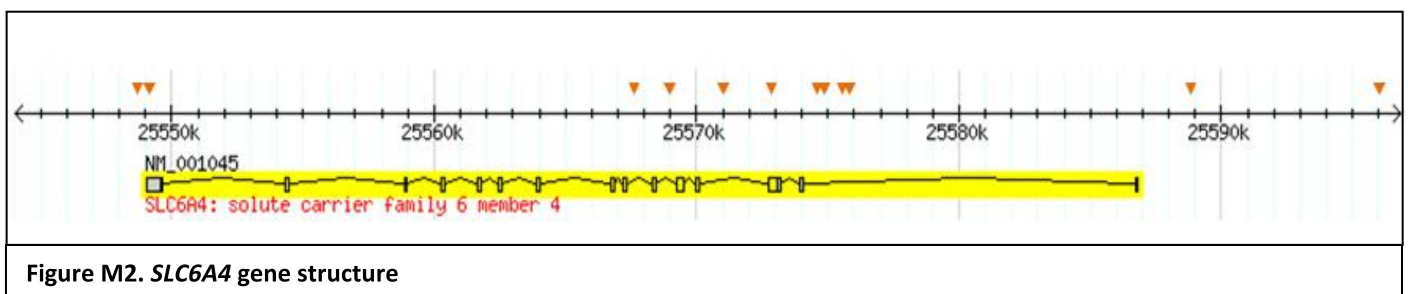


Figure M2. *SLC6A4* gene structure

Table M6. ***HTR2A*** **polymorphisms.**

rs code (or other name)	Type	Position (dbSNP Build 127)	MARKER ORDER	DISTANCE FROM M1	ALLELE CHANGE	CEU MAF	LOCATION/ FUNCTION
rs6313	SNP	46367941	M1	0	T(Ser)/ C(Ser)	0.450 (T)	EXON 1
rs6311	SNP	46369479	M2	1538	G/A	0.450 (A)	PROMOTER/ regulator of expression

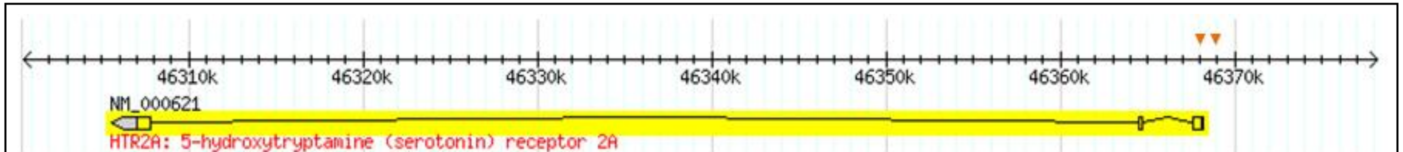


Figure M3. ***HTR2A*** **gene structure**

Table M7. ***TPH2*** **polymorphisms.**

rs code (or other name)	Type	Position (dbSNP Build 127)	ALLELE CHANGE	CEU MAF	LOCATION/FUNCTION
rs4570625	SNP	70618190	T/G	0.207 (T)	5' Upstream/possible regulator of *TPH2* expression

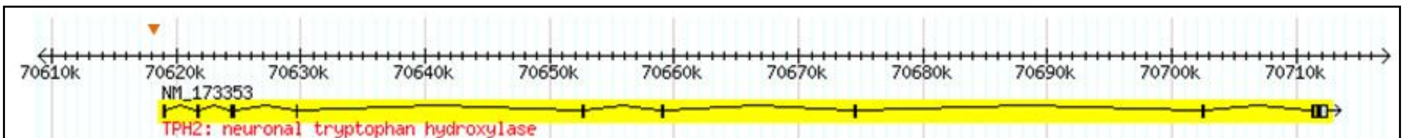


Figure M4. ***TPH2*** **gene structure**

Table M8. ***NOS1*** **polymorphisms.**

rs code (or other name)	Type	Position (dbSNP Build 127)	ALLELE CHANGE	CEU MAF	LOCATION/FUNCTION
NOS1 Ex1f-VNTR	VNTR	116284130-116284026	from 180 to 210 CA repeats	NO DATA	Exon 1f/regulation of gene expression

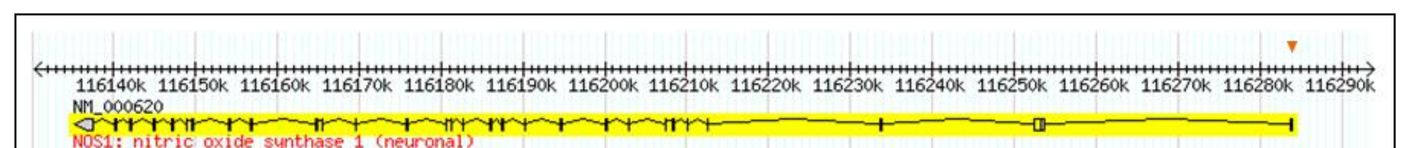


Figure M5. ***NOS1*** **gene structure**

Table M9. *STMN1* polymorphisms.

rs code (or other name)	Type	Position (dbSNP Build 127)	MARKER ORDER	DISTANCE FROM M1	ALLELE CHANGE	CEU MAF	LOCATION/ FUNCTION
rs12037513	SNP	26097220	M1	0	C/T	0.325 (C)	3' near gene
rs182455	SNP	26107570	M2	10350	T/C	0.408 (T)	5' Upstream

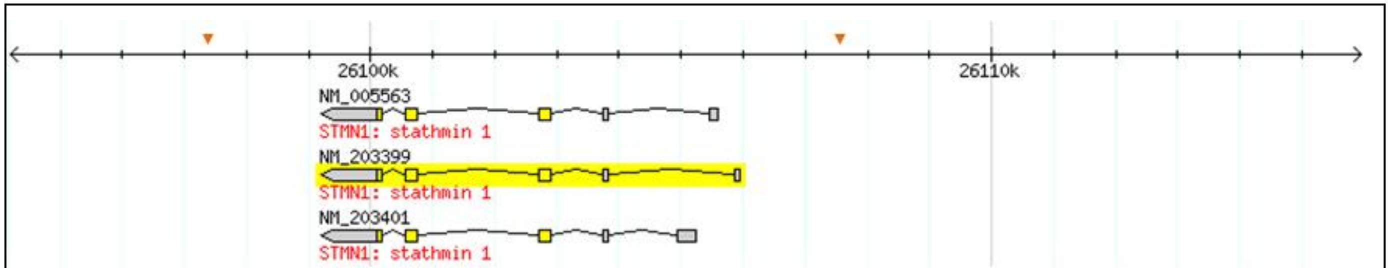


Figure M6. *STMN1* gene structure

Table M10. *ASPM* polymorphisms.

rs code (or other name)	Type	Position (dbSNP Build 127)	MARKER ORDER	DISTANCE FROM M1	ALLELE CHANGE	CEU MAF	LOCATION/ FUNCTION
rs6700180	SNP	195331119	M1	0	T/C	0.492 (C)	INTRON 19
rs3762271	SNP	195337065	M2	5946	C(Leu)/A(Ile)	0.43 (A)	EXON 18
rs12138336	SNP	195337144	M3	6025	G(Gln)/C(His)	0.078 (C)	EXON 18
rs41310927	SNP	195337320	M4	6201	A(Ser)/G(Gly)	0.470 (A)	EXON 18
rs964201	SNP	195337524	M5	6405	T(Tyr)/C(His)	0.020 (T)	EXON 18
rs10922163	SNP	195344014	M6	12895	G/A	0.490 (A)	INTRON 17
rs12116571	SNP	195355616	M7	24497	G/A	0.120 (A)	INTRON 16
rs4915337	SNP	195358160	M8	27041	A(Ser)/T(Ser)	0.068 (T)	EXON 14
rs12025066	SNP	195379068	M9	47949	A(Ile)/G(Val)	0.009 (G)	EXON 3
rs6677082	SNP	195379156	M10	48037	T(Ser)/C(Ser)	0.080 (C)	EXON 3
rs9726778	SNP	195383377	M11	52258	C/G	NO DATA	PROMOTER

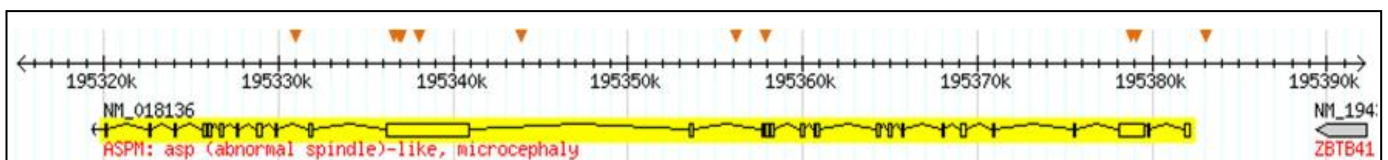


Figure M7. *ASPM* gene structure

Table M11. *PDE4D* polymorphisms.

rs code (or other name)	Type	Position (dbSNP Build 127)	MARKER ORDER	DISTANCE FROM M1	ALLELE CHANGE	CEU MAF	LOCATION/ FUNCTION
rs17291089	SNP	58301362	M1	0	C/A	0.103 (C)	3´near gene
rs829259	SNP	58303733	M2	2371	A/T	0.45 (A)	3 ´UTR
rs1058458	SNP	58306373	M3	5011	C(Arg)/ T(Cys)	0.0 (C)	EXON 15
rs17719378	SNP	58309205	M4	7843	G/A	0.325 (G)	INTRON 11
rs10055954	SNP	58309342	M5	7980	G/C	0.164 (C)	INTRON 11
rs10461656	SNP	58312107	M6	10745	G/A	0.356 (A)	INTRON 11
rs7713345	SNP	58314588	M7	13226	G/C	0.186 (G)	INTRON 11
rs12656462	SNP	58316381	M8	15019	T/A	0.110 (A)	INTRON 11
rs17853590	SNP	58320100	M9	18738	T(Phe)/ C(Ser)	NO DATA	EXON 11
rs10056492	SNP	58327740	M10	26378	T/C	0.119 (T)	INTRON 6
rs4700316	SNP	58330448	M11	29086	G/C	0.153 (G)	INTRON 6
rs7714708	SNP	58330771	M12	29409	T/C	0.333 (T)	INTRON 6

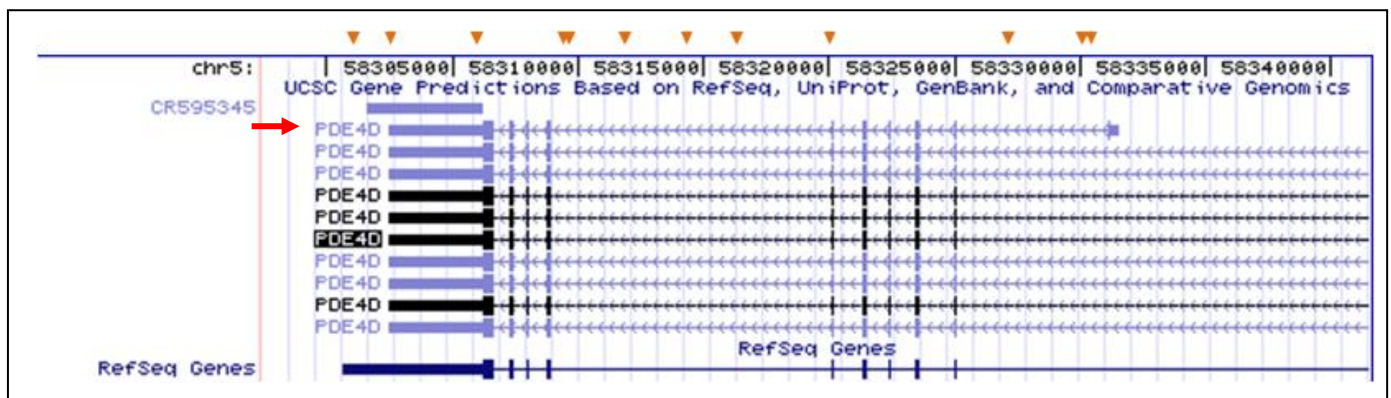


Figure M8. Genomic structure of the *PDE4D* small isoform (PDE4D6). Image taken from UCSC genome browser. The isoform of interest for this study is indicated by a red arrow. All the selected SNPs are located in the PDE4D6 region.

Table M12. *PLEKHB1* and *RAB6A* polymorphisms.

GENE	rs code (or other name)	Type	Position (dbSNP Build 127)	MARKER ORDER	DISTANCE FROM M1	ALLELE CHANGE	CEU MAF	LOCATION/ FUNCTION
-	rs663303	SNP	73029494	M1	0	C/T	0.208 (T)	N/A
PLEKHB1	rs4944850	SNP	73033744	M2	4250	A/C	0.183 (C)	5´NEAR GENE
PLEKHB1	rs11538627	SNP	73040539	M3	11045	A(Glu)/ T(Asp)	NO DATA	EXON 4
PLEKHB1	rs591804	SNP	73040858	M4	11364	A/G	0.367 (G)	INTRON 4
PLEKHB1	rs6592527	SNP	73042016	M5	12522	C/G	0.288 (C)	INTRON 5
PLEKHB1	rs940828	SNP	73043807	M6	14313	G/T	0.267 (G)	INTRON 5
PLEKHB1	rs3741147	SNP	73044399	M7	14905	G/T	0.142 (G)	INTRON 5

Table M12 (continuation). *PLEKHB1* and *RAB6A* polymorphisms.

GENE	rs code (or other name)	Type	Position (dbSNP Build 127)	MARKER ORDER	DISTANCE FROM M1	ALLELE CHANGE	CEU MAF	LOCATION/ FUNCTION
-	rs12274970	SNP	73055790	M8	26296	C/T	0.258 (C)	N/A
RAB6A	rs3182788	SNP	73066620	M9	37126	T (Thr)/ G (Pro)	NO DATA	EXON 8
RAB6A	rs10736793	SNP	73080224	M10	50730	A/C	0.186 (A)	INTRON 6
RAB6A	rs3203705	SNP	73107544	M11	78050	G (Leu)/ A (Phe)	NO DATA	INTRON 3 (variant 2)/ EXON 4 (variant 1)
RAB6A	rs11235876	SNP	73108249	M12	78755	A/G	0.450 (A)	INTRON 3
RAB6A	rs11235880	SNP	73112944	M13	83450	A/C	0.200 (A)	INTRON 2
RAB6A	rs7127066	SNP	73136522	M14	107028	C/G	0.250 (C)	INTRON 1

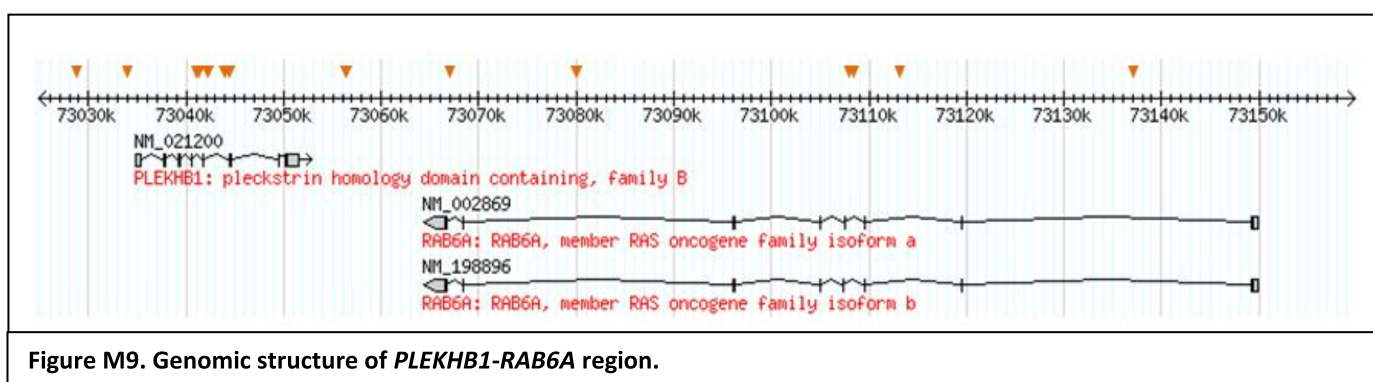


Figure M9. Genomic structure of *PLEKHB1-RAB6A* region.

4.2. Genotyping Methods

Different genotyping methodologies were used to carry out this project. A brief description of these techniques can be found here, although they will be explained in detail later.

a. Agarose gel-based methods: Based on polymerase chain reaction (PCR) amplification and genotype discrimination in agarose gels. For some polymorphisms, the genotypes could be determined directly by electrophoresis of the PCR products. However, in almost all cases it was necessary to digest the products which an appropriate restriction enzyme, which only recognizes and cuts one of the alleles. This type of variation is called Restriction Fragment Length Polymorphism (RFLP).

b. Genotyping by capillary electrophoresis: It is especially useful when the resolution of agarose electrophoresis is insufficient.

c. Taqman® SNP genotyping assays: This methodology is based on the use of allele-specific fluorescent probes.

d. MassArray® genotyping: It is based on the generation of multiplex PCR and extension products with different size and weight depending on the allele and the SNP. These products can be then analyzed by MALDI-TOF (Matrix-Assisted Laser Desorption/Ionization - Time-Of-Flight) mass spectrometry (MS) to determine the size and subsequently the genotype of each individual for every SNP.

Table M13 shows the methodology used to genotype each SNP.

Table M13. Genotyping methods used for each polymorphism.

GENE	Polymorphism	Marker number	Genotyping method (sample)
SLC6A4	rs3813034	M1	RFLP (1)
	rs1042173	M2	RFLP (1)
	rs140700	M3	MassArray (1)
	rs2228673	M4	RFLP (1)
	rs2020942	M5	RFLP (1)
	STin2	M6	Product size determination in agarose (1)
	rs2020939	M7	MassArray (1)
	rs2020936	M8	RFLP (1)
	rs2066713	M9	RFLP (1)
	rs4251417	M10	MassArray (1)
	5-HTTLPR (+ rs25531)	M11	RFLP (1)
	rs12945042	M12	MassArray (1)
TPH2	rs4570625	-	RFLP (1)
HTR2A	all	M1-M2	RFLP (1)
NOS1	VNTR1	-	Product size determination in capillary EF (1)
PLEKHB1-RAB6A	all	M1-M14	MassArray (1, 2)
STMN1	rs12037513	M1	MassArray (1)
	rs182455	M2	RFLP (1)
PDE4D	all	M1-M12	MassArray (1, 2)
ASPM	rs6700180	M1	MassArray (1)
	rs3762271	M2	TaqMan assay (3); RFLP (1)
	rs12138336	M3	TaqMan assay (3); RFLP (1)
	rs41310927	M4	TaqMan assay (3); RFLP (1)
	rs964201	M5	RFLP (3)
	rs10922163	M6	TaqMan assay (3); MassArray™ (1)
	rs12116571	M7	TaqMan assay (3)
	rs4915337	M8	RFLP (1)
	rs12025066	M9	RFLP (1)
	rs6677082	M10	TaqMan assay (3); MassArray (1)
	rs9726778	M11	RFLP (1)

1: It indicates that this is the methodology used for the Spanish samples.
2: It indicates that this methodology was used for the German samples.
3: it indicates that this methodology was used to genotype the American samples.
Abbreviation: EF, electrophoresis; RFLP, Restriction Fragment Length Polymorphism.

It is important to mention that several research centers with different resources took part in the genotyping process:

* Department of Genetics, University of Valencia, Spain: RFLP genotyping of *SLC6A4*, *HTR2A* and *ASPM* genes.

* Department of Psychiatry and Psychotherapy, University of Würzburg, Germany: during my short stay at this center, the genotyping by MassARRAY technology of *PLEKHB1*, *RAB6A* and *PDE4D* genes was performed, as well as the genotyping of the *NOS1* VNTR and *STMN1* and *TPH2* RFLPs.

* Clinical Brain Disorders Branch (CBDB), National Institute of Mental Health, USA: during my short internship at the CBDB, *ASPM* genetic variation was genotyped in the CBDB sample via Taqman assays.

* Spanish National Genotyping Centre (CeGen): it provides many services, being the most important a high throughput genotyping platform. The SNPs from *SLC6A4*, *ASPM* and *STMN1* genes genotyped through MassArray technology were genotyped at this center, as an external service.

4.2.1. Gel-based genotyping methods.

4.2.1.1. Design of primer sequences

Several primer sequences were used to amplify the regions around the polymorphisms of interest. Some of them were from the literature, whereas others were designed manually according to the following criteria:

* Oligonucleotide size between 18-22 base pairs (generally 20-21 bp).

* Balanced content of adenine, guanine, thymine and cytosine.

* Absence of sequences which could potentially generate secondary structures.

* When possible, avoid primer sequences beginning and ending in T or A, since this will make the primer annealing and extension by the polymerase more difficult.

* Avoid more than three consecutive G/C or A/T.

* Absence of other polymorphisms located in the primer sequences.

* Similar melting temperatures for each pair of reverse and direct primer.

* The PCR product must have an adequate size according to the genotyping conditions.

Oligonucleotides were synthesized by TIB MOLBIOL (Berlin, Germany), Metabion International (Martinsried, Germany) or Operon Biotechnologies (Cologne, Germany).

4.2.1.2. Amplification conditions

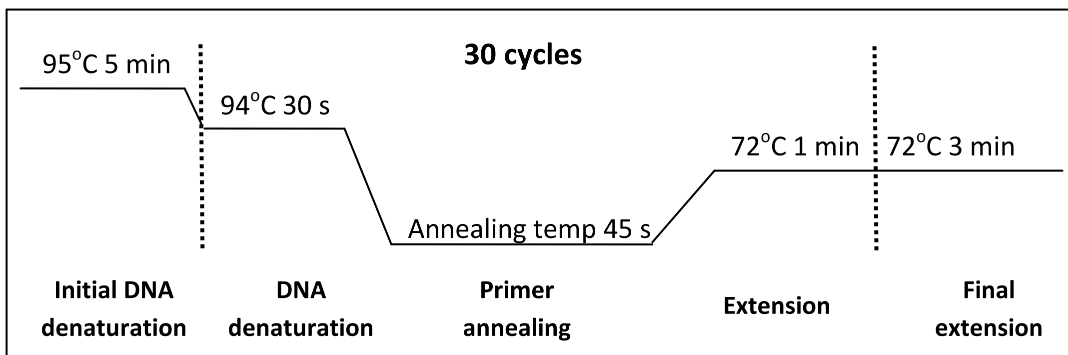
The adjustment of PCR conditions was made with test DNA samples, which were at the same concentration than the working dilutions of the DNA samples from our study (25 ng/µL). Conditions were adjusted depending on the primers' melting temperatures.

The standard reaction mix was the following:

DNA (25 ng/µL)	1-2 µL
Nucleotides (dNTPs) 10 mM (Roche, catalog no. 1969064)	0.5 µL
Forward primer (10 pmol/µL)	0.5 µL
Reverse primer (10 pmol/µL)	0.5 µL
PCR Buffer 10X (with Mg^{2+} 25mM)	2.5 µL
Polymerase Netzyme™ 1U/µL (NEED, catalog no. NE-002)	0.5 µL
dH_2O	up to 25 µL

In some cases, to improve PCR efficiency, it was necessary the addition of 5µL of Dimethyl Sulfoxide (DMSO) 50% (Sigma, catalog no. D8418) or Glycerol 50% (Sigma, catalog no. G5516) to the reaction mix.

Once the PCR conditions were adjusted, reactions took place in 96-well PCR plates. Reactions were made on Eppendorf Mastercycler and MJ Research PTC 200 thermocyclers. The general amplification program was:



Tables M14 to M18 show the primer sequences and amplification conditions (annealing temperature, product sizes) as well as the restriction enzymes used in the RFLP analysis for all those polymorphisms genotyped through a gel-based methodology. In some cases, when applicable, a modification of the standard reaction mix or PCR program is indicated.

Table M14. Genotyping conditions for *SLC6A4* polymorphisms (gel-based technology).

Marker	Primer sequences 5' → 3'	Temp	PCR cond.	PCR product (bp)	Restriction enzyme	Alleles sizes after digestion
rs3813034	TGCTGGAATCTACTAGAACCTTC TCCAATAATAACCTCCATACACA (Melke *et al.*, 2003)	58 °C	Standard	244	*Tru*1I	G: 244 T: 192+52
rs1042173	CATGGTAGACTGTGACACAGC CTCACAAGCTTGCATGGACAC	61 °C	Standard	377	*Acs*I/*Xap* I	T: 377 G: 262+115
rs2228673*	GGCCTGGAGTCCTTGGAATGG GTTGCCAGTGTTCCAGGAG**C**T	58 °C	Standard	295	*Sac*I	C: 295 G: 278 + 17
rs2020942	ATCTCCTGCTCCAGAGGACTC TGGTGTCTCCTGGATCTTGTG	60 °C	Standard	366	*Hpa*II	A: 366 G: 209+157
STin2	GTCAGTATCACAGGCTGCGAG TGTTCCTAGTCTTACGCCAGTG (Wendland *et al.*, 2006)	65.5 °C	-35 cycles -Final extension 7 min	2.9: 250 2.10: 267 2.12:300	-	-
rs2020936	ACCACTGACTACCAAGTTCAG CACCAGGCAATGTCAGTCAC	59 °C	Standard	503	*Eco*31I	T: 503 C: 242+261
rs2066713	CTCCTGAGAACACACGTTGCC CACAGGTGTGAGACACCATGC	59.5 °C	-DMSO -35 cycles	365	*Bfm*I	C: 61+304 T:61+147+157
5-HTTLPR (including rs25531)	TCCTCCGCTTTGGCGCCTCTTCC TGGGGGTTGCAGGGGAGATCCTG (Wendland *et al.*, 2006)	61 °C	- glicerol -Annealing 1 min -Initial denat. 15 min -Final extension 10 min -35 cycles	L: 512 S: 469	*Hpa*II (for rs25531)	L_A: 512 L_G: 402+110 S_A:469 S_G: 402+67

* One of the primers used to amplify the region around rs2228673 includes a mutation site which introduces a recognition site for *Sac*I in one of the alleles. This site is indicated in bold.

Abbreviations: Temp, annealing temperature; denat, denaturation; bp, base pairs;

Table M15. Genotyping conditions for *TPH2* polymorphism.

Marker	Primer sequences 5' → 3'	Temp	PCR cond.	PCR product (bp)	Restriction enzyme	Alleles sizes after digestion
rs4570625*	TTTCCATGATTTCCAGTAGAGAG AAGCTTTTTCTGACTTGACA**A**AT	55 °C	-40 cycles	309	*Apo*I/*Xap*I	T: 309 G: 285 + 24

* One of the primers used to amplify the region around rs2228673 includes a mutation site which introduces a recognition site for *Apo*I and X*ap*I in one of the alleles. This site is indicated in bold.
Abbreviations: Temp, annealing temperature; bp, base pairs.

Table M16. Genotyping conditions for *HTR2A* polymorphisms.

Marker	Primer sequences 5' → 3'	Temp	PCR cond.	PCR product (bp)	Restriction enzyme	Alleles sizes after digestion
rs6313	TCTGCTACAAGTTCTGGCTT CTGCAGCTTTTTCTCTAGGG	54 °C	-Annealing 1 min -35 cycles	342	*Msp*I/*Hpa*II	T: 342 C: 125 + 217
rs6311	AAGCTGCAAGGTAGCAACAGC AACCAACTTATTTCCTACCAC	56 °C	Standard	469	*Msp*I/*Hpa*II	A: 471 G: 243 + 226

Abbreviations: Temp, annealing temperature; bp, base pairs.

Table M17. Genotyping conditions for *STMN1* polymorphism.

Marker	Primer sequences 5' → 3'	Temp	PCR cond.	PCR product (bp)	Restriction enzyme	Alleles sizes after digestion
rs182455	CCCTCTCAGTCCTCAAGCAG CCCCCACATTTTCTTTCTCC	60 °C	-32 cycles	200	*Sac*I	T: 200 C: 168 + 32

Abbreviations: Temp, annealing temperature; bp, base pairs.

Table M18. Genotyping conditions for *ASPM* polymorphisms.

Marker	Primer sequences 5' → 3'	Temp	PCR cond.	PCR product (bp)	Restriction enzyme	Alleles sizes after digestion
rs3762271	TCAGACAATGGCATTCTGCTG TAACTGCTTGGGTACGCA	58 °C	Standard	426	*Dde*I	A: 426 C: 357+69
rs12138336	TCAGACAATGGCATTCTGCTG TAACTGCTTGGGTACGCA	58 °C	Standard	426	*Hinf*I	C: 426 G: 278+148
rs41310927	GAAGAGTATGCATTCCTCTGC ACCTGCCTGAACACAAGTCTC	59 °C	Standard	622	*Bsh*NI	A: 622 G: 473 149
rs964201	GAAGAGTATGCATTCCTCTGC ACCTGCCTGAACACAAGTCTC	59 °C	Standard	622	*Csp*6I/ *Rsa* I	C: 143+479 T:143+126+353
rs4915337	TTACAGTGTCTTTCTCAGACG TCTAGGAGCTCTTTGTATAGC	55 °C	Standard	318	*Alw*26I	A: 318 T: 182+136

Table M18 (continuation). Genotyping conditions for *ASPM* polymorphisms.

Marker	Primer sequences 5' → 3'	Temp	PCR cond.	PCR product (bp)	Restriction enzyme	Alleles sizes after digestion
rs12025066	TACCTACTCATCTCTTCATGC CATGTTTGCTGAGATGTACAC	55 °C	Standard	498	*Csp6I*/ *Rsa I*	A: 498 G:48+156+294
rs9726778	ATGACCTGTATTCCTCATCTG GGACTCAGTACATTTACATGC	55 °C	Standard	624	*Msp*A1I	C:624 G:136+488

Abbreviations: Temp, annealing temperature; bp, base pairs.

4.2.1.3. Verification of the PCR

Electrophoreses on agarose gels were performed to verify the existence and size of each PCR product. The concentration of agarose gels varied between 1% and 4% depending on the product's size. For the VNTR STin2, from the serotonin transporter gene, genotypes could be determined directly by electrophoresis of the PCR products in a 4% agarose gel.

The protocol of electrophoresis in agarose gels is the following:

- **Solutions and additives:**

  Agarose (Roche, catalog no. 1685678)

  TBE buffer solution 1x:

Tris	89 mM
Boric Acid	89mM
EDTA	2mM

  Ethidium bromide solution 10 mg/mL (Sigma, catalog no. E-8751)

  Gel loading solution 6x:

Bromophenol blue	0.05%
Xylene cyanol blue	0.05%
EDTA 0.5M pH 8	100 mM
Glycerol	50%

  DNA molecular weight marker XIV (Roche, catalog no. 1721933)

  DNA molecular weight marker XIII (Roche, catalog no. 1721925)

  Lambda DNA/*Hind*III Marker 2 (Fermentas, catalog no. SM0101)

- **Procedure:** The necessary amount of agarose is dissolved in TBE 1x by boiling the solution twice in a microwave oven. Afterwards, the solution is cooled down to 55°C and then the ethidium bromide is added up to a final concentration of

0.5µg/mL. After homogenizing the solution, it is poured into a casting tray, which also contains a comb necessary to form the wells in the gel. Generally after one hour the gel has solidified and can be placed in a horizontal tank which contains TBE 1x (with ethidium bromide 0.5µg/mL) as the electrophoresis buffer.

The gel loading solution is added to the DNA samples, which are then loaded on the gel wells and run together with an appropriate molecular weight marker. Electrophoreses are performed at 90-100 V and a variable running time. Finally, DNA fragments can be visualized in a UV transilluminator at 312 nm.

4.2.1.4. RFLP genotyping

The PCR-RFLP genotyping technique is based on the idea that SNPs can modify the target sequence for restriction endonucleases. Thus, this fact can be used to discriminate between the different alleles of a SNP through the use of an appropriate restriction enzyme. Figure M10 shows an overview of the entire process.

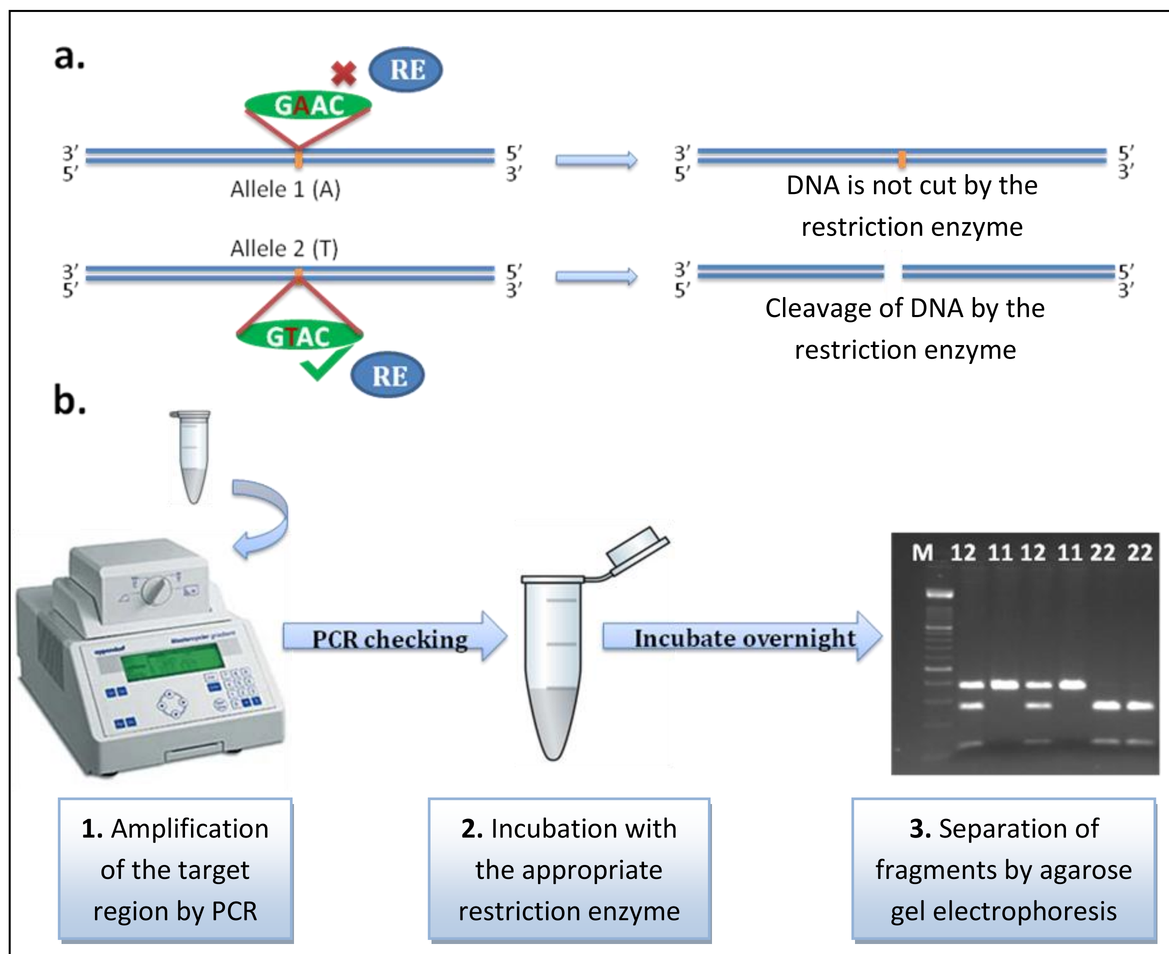


Figure M10. The RFLP-based genotyping process. a. Description of the basis of RFLP-based genotyping technique. If one of the alleles changes the recognition sequence for a restriction endonuclease, this restriction enzyme will not be able to cleave the DNA fragment. **b.** The genotyping process step by step. Abbreviations: RE, restriction endonuclease. M, molecular weight marker; 1, allele 1; 2, allele 2.

Moreover, if the polymorphic change is not altering the recognition sequence for any commercial restriction endonuclease, it is possible to create a mutagenic primer which creates a restriction site for one of the alleles. This approach was used for SNPs rs2228673 and rs4570625, as it is indicated in tables M15 and M16.

The online utility Webcutter 2.0 (http://rna.lundberg.gu.se/cutter2/) was used to check all the restriction enzymes affected by the polymorphic site. This program identifies all the restriction enzymes in a given sequence and it also indicates the length of the restriction fragments, thus it is especially useful to decide the most suitable enzyme in each case. Restriction endonucleases used in this study can be found in table M19.

Table M19. List of the restriction endonucleases used for the RFLP genotyping.

Name	Manufacturer/Cat. number	Recognition sequence	Buffer	Digestion temperature
*Acs*I	NEB/no longer available	5'...R↓AATTY...3' 3'...YTTAA↑R...5'	---	50 °C
*Alw26*I	Fermentas/ER0031	5'...GTCTC(N)1↓...3' 3'...CAGAG(N)5↑...5'	Tango™	37 °C
*Apo*I	NEB/R0566L	5'...R↓AATTY...3' 3'...YTTAA↑R...5'	NEBuffer 3 (+ BSA*)	50 °C
*Bfm*I	Fermentas/ER1162	5'...C↓TRYAG...3' 3'...GAYRT↑C...5'	Tango™	37 °C
*Bsh*NI	Fermentas/ER1001	5'...G↓GYRCC...3' 3'...CCRYG↑G...5'	O	37 °C
*Csp*6I	Fermentas/ER0211	5'...G↓T A C...3' 3'...C A T↑G...5'	B	37 °C
*Dde*I	NEB/R0175L	5’...C↓TNAG...3’ 3’...GANT↑C...5’	NEBuffer 3	37 °C
*Eco*31I	Fermentas/ER0291	5'...GGTCTC(N)1↓...3' 3'...CCAGAG(N)5↑...5'	G	37 °C
*Hinf*I	Fermentas/ER0801	5'...G↓ANTC...3' 3'...CTNA↑G...5'	R	37 °C
*Hpa*II	Fermentas/ER0511	5'...C↓CGG...3' 3'...GGC↑C...5'	Tango™	37 °C
*Msp*A1I	NEB/R0577L	5'...CMG↓CKG...3' 3'...GKC↑GMC...5'	NEBuffer 4 (+ BSA*)	37 °C
*Msp*I	Fermentas/ER0541	5'...C↓CGG...3' 3'...GGC↑C...5'	Tango™	37 °C
*Rsa*I	Fermentas/ER1121	5'...GT↓AC...3' 3'...CA↑TG...5'	Tango™	37 °C

Table M19 (continuation). List of the restriction endonucleases used for the RFLP genotyping.

Name	Manufacturer/Cat. number	Recognition sequence	Buffer	Digestion temperature
*Sac*I	NEB/R0156S	5'...GAGCT↓C...3' 3'...C↑TCGAG...5'	NEBuffer 1 (+ BSA*)	37 °C
*Tru1*I	Fermentas/ER0982	5'...T↓TAA...3' 3'...AAT↑T...5'	R	65 °C
*Xap*I	Fermentas/ER1382	5'…R↓AATTY...3' 3'…YTTAA↑R...5'	Tango™	37 °C

*Some NEB restriction enzymes required the separate addition of Bovine Serum Albumin (BSA) to prevent adhesion of the enzyme to the reaction tubes and pipette tips and to stabilize some proteins during incubation. Its use will guarantee the complete digestion of products.

The general digestion mix was:

PCR assay	10-16 µL
Digestion buffer 10x	2 µL
Restriction enzyme	5 U
dH_2O	up to 20 µL

Digestion assays were incubated at the manufacturer's recommended temperature in a water bath overnight. Exceptionally, for digestions at 65°C performed at 96-well plates, assays were incubated on a thermal cycler at a constant temperature of 65°C for 8 hours, since the adhesive foils used to close the plates are not very resistant to wet heat. After incubation, samples were loaded into a gel of variable agarose percentage depending on the size of DNA fragments. Electrophoreses were run at a constant voltage (100-150 V, depending on the tank's length) and time enough to ensure the separation of digested DNA fragments. Digestion conditions for each SNP can be consulted at tables M14 to M19.

4.2.1.5. Purification of PCR products

During the adjustment of genotyping conditions, each PCR product was sequenced to a) verify that the target region was really amplified and b) to check that the genotype resulting from RFLP genotyping was correct.

For this purpose, first it was necessary to purify PCR products to eliminate all the nucleotides, salts and remaining primers which could interfere with the sequencing reaction.

Two different methods were used:

* **JETQUICK PCR Product Purification Spin Kit (Genomed, Catalog no. 410250):** With this kit, the PCR products are extracted using micro spin cups. The binding matrix is a surface modified silica membrane which binds DNA but not nucleotides or

oligonucleotides. The DNA is then eluted from the membrane in sterile distilled water.

Solutions (from the commercial kit):

Solution H1 (binding solution): it contains guanidine hydrochloride and isopropanol.
Solution H2 (washing solution): it contains ethanol, NaCl, EDTA and Tris/HCl.

Procedure:

1. Add 400 µL of solution H1 to a 20 µL PCR assay and mix thoroughly.
2. Place a JETQUICK spin column inside a 2 mL receiver tube (also included with the kit). Load the mixture from step 1 into the prepared spin column.
3. Centrifuge at 14000 rpm for 1 min and discard the flowthrough.
4. Reinsert the spin column into the empty receiver tube and add 500 µL of solution H2.
5. Centrifuge at 14000 rpm for 1 minute, discard the flowthrough and place again the column into the same receiver tube.
6. Centrifuge again at 14000 rpm for 1 minute.
7. Place the spin column into a fresh 1.5 mL tube and add 50 µL of sterile water directly onto the center of the column matrix.
8. Centrifuge at 14000 rpm for 2 minutes.

* **DNA precipitation with ammonium acetate:** With this method, the PCR products are precipitated with ammonium acetate 4M. This method is recommended when primer concentrations in the PCR reaction are below 10 pmol.

Solutions:

Ammonium acetate 4M
100% ethanol
70% ethanol
Sterile water

Procedure:

1. Mix 15 µL-20 µL of PCR assay with one volume (15 µL) of ammonium acetate and 6 volumes (90 µL) of absolute ethanol. Mix thoroughly.
2. Centrifuge for 15 minutes at 13000 rpm at rt. Discard the supernatant.
3. Add 400 µL of 70% ethanol solution and mix well with a vortex mixer.
4. Centrifuge for 5 minutes at 13000 rpm and room temperature. Discard the supernatant.
5. Dry the DNA pellet at 37°C for approximately 5 minutes.
6. Resupend the pellet in 20 µL of sterile water.

4.2.1.6. Purification of DNA from agarose gel bands

Sometimes the purification from PCR products is not useful because of the presence of unspecific products (seen as additional undesired bands in an agarose gel). Therefore, in these cases, the best option is to purify the DNA directly from the band of interest and use this extracted DNA for the sequencing reaction. The GFX™ PCR DNA and Gel Band Purification Kit (GE Healthcare, Catalog no. 27-9602-01) was used for this purpose. It is based on the use of a chaotropic agent which denatures protein, dissolves agarose and facilitates the binding of double-stranded DNA to a glass fiber matrix. Then, the DNA can be eluted with sterile water.

Solutions:

Capture buffer: it contains acetate and chaotrope.
Wash buffer: tris EDTA pH 8.0, 80% ethanol.
Sterile water.

Procedure:

1. Weigh an empty 1.5 mL microcentrifuge tube to the nearest 10 mg and record the weight.
2. Using a scalpel, cut the agarose fragment containing the DNA band of interest. It is very important to cut as close to the band as possible.
3. Transfer the agarose fragment to the tube weighted in step 1. Weigh again the tube to determine the weight of the agarose slice.
4. For each 10 mg of agarose, add 10 µL of capture buffer to the agarose fragment. Close the tube and mix by vortexing thoroughly.
5. Incubate at 60°C until the agarose is fully dissolved. During the incubation, place a GFX column in a collection tube.
6. Once the agarose is fully dissolved, centrifuge briefly to collect the sample at the bottom of the tube.
7. Transfer the sample to the GFX column and incubate at room temperature for 1 minute.
8. Centrifuge at 13000 rpm for 30 seconds.
9. Discard the flow-through in the collection tube and place again the GFX column back inside the collection tube.
10. Add 500 µL of wash buffer and centrifuge at 13000 rpm for 30 seconds twice.
11. Transfer the GFX column to a new 1.5 mL microcentrifuge tube and add from 10 to 50 µL of elution buffer directly to the center of the column matrix.
12. Incubate at room temperature for 1 minute and centrifuge at 13000 rpm for one minute to recover the DNA.

4.2.1.7. DNA Sequencing

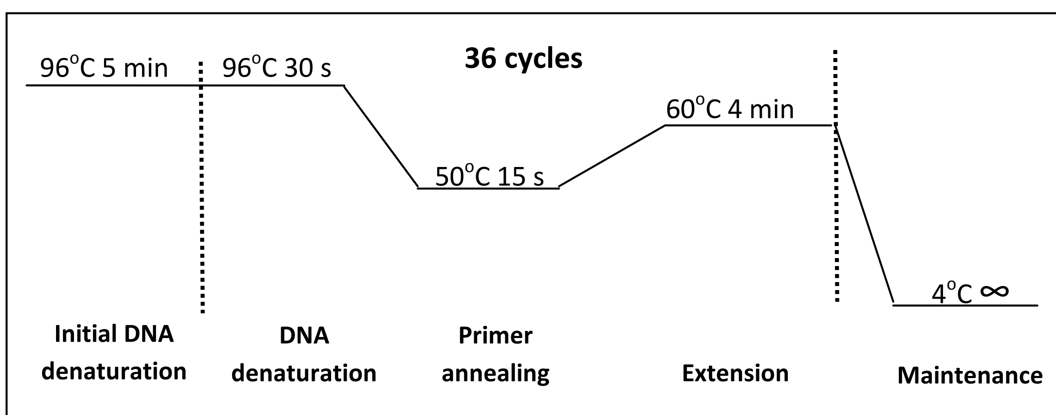
The BigDye® terminator v3.1 Cycle sequencing kit (Applied Biosystems, catalog no. 4336911) was used for the sequencing reactions of the purified DNA fragments. This kit

is based on the dideoxynucleotide termination method (Sanger *et al.*, 1977). It contains a ready-to-use reaction mix which includes all the components we need for the reaction excepting the primer: dNTPs, polymerase, reaction buffer and the four terminator dideoxynucleotides labeled with four different fluorochromes. At the end of the sequencing reaction, we will have a mix of fragments of different size, each of them ending in a fluorescent nucleotide.

The following reaction mix was used:

DNA template (purified PCR product)	50 ng (between 1 and 7 µL)
Forward or reverse primer (10 pmol/µL)	1 µL
BigDye®	2 µL
dH_2O	up to 10 µL

Reactions were made on Eppendorf Mastercycler and MJ Research PTC 200 thermocyclers. The general amplification program was:



Reaction products were purified and separated by capillary electrophoresis in a 48-capillary sequencer (Applied Biosystems 3730 DNA Analyzer) at the Sequencing Service from the University of Valencia. The sequences obtained from the Sequencing Service were analyzed with the Sequencher software v.4.0.5 (Gene Codes Corporation). With this program, it is possible to check the quality of chromatograms and also to edit those base calls which have been wrongly assigned by the sequencer. Moreover, it is also possible to verify the identity of the sequences by aligning them with the reference sequence in the NCBI database. Finally, we also checked that the genotype for each polymorphism in study agreed with the genotype assigned by RFLP methodology (see figure M11).

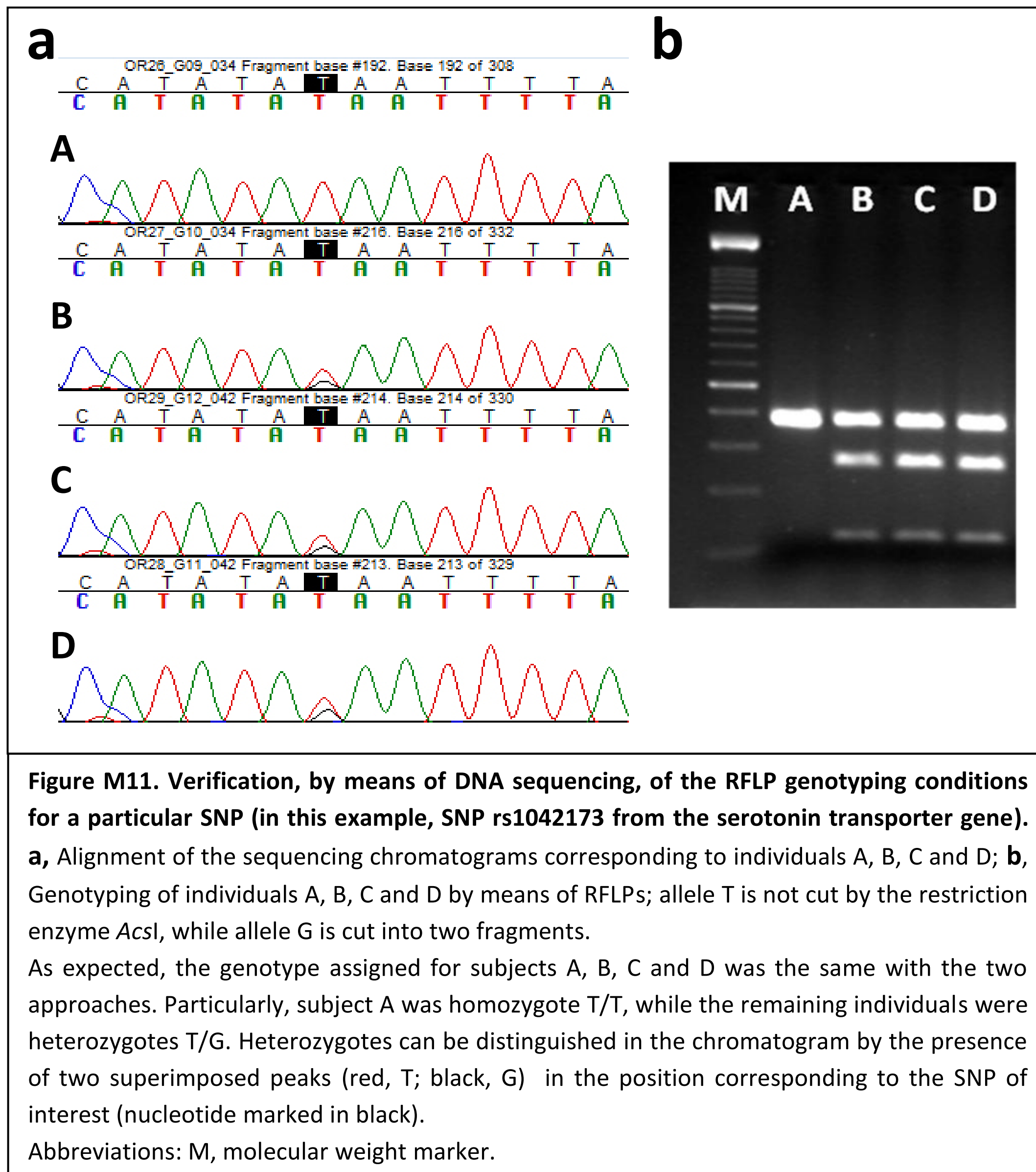


Figure M11. Verification, by means of DNA sequencing, of the RFLP genotyping conditions for a particular SNP (in this example, SNP rs1042173 from the serotonin transporter gene). **a,** Alignment of the sequencing chromatograms corresponding to individuals A, B, C and D; **b**, Genotyping of individuals A, B, C and D by means of RFLPs; allele T is not cut by the restriction enzyme *Acs*I, while allele G is cut into two fragments.

As expected, the genotype assigned for subjects A, B, C and D was the same with the two approaches. Particularly, subject A was homozygote T/T, while the remaining individuals were heterozygotes T/G. Heterozygotes can be distinguished in the chromatogram by the presence of two superimposed peaks (red, T; black, G) in the position corresponding to the SNP of interest (nucleotide marked in black).

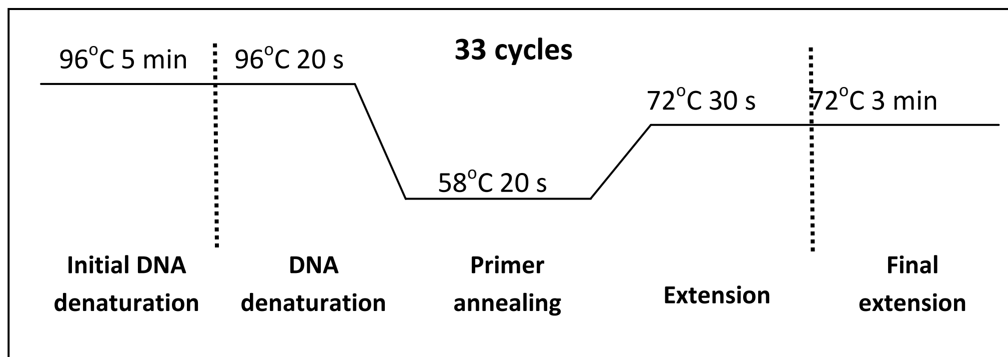
Abbreviations: M, molecular weight marker.

4.2.2. Genotyping by product size determination in capillary electrophoresis.

Genotypes for Ex1f-VNTR polymorphism from the *NOS1* gene were determined by PCR amplification and product size determination by capillary electrophoresis in a DNA sequencer. For this purpose, the forward primer was labeled with cy-5 fluorescent dye (TIB MolBiol, Berlin), enabling detection of the resulting PCR product. The reaction mix included:

DNA (25 ng/µL)	50 ng
dNTPs	200 µM
Forward primer (TGCGTGGCGACTACATTCAGC)	50 nM
Reverse primer (GGAGACGTCGCAACCCTCAT)	50 nM
PCR Buffer 10X (with Mg^{2+} 15mM)	5 µL
Taq polymerase	0.4 U
dH_2O	up to 50 µL

The amplification program was the following:



Electrophoretic separation of the PCR products was performed using a CEQ8000 DNA-sequencer from Beckman-Coulter. The use of an external standard allows size determination of the PCR products. Moreover, internal standards containing previously sequenced DNA fragments of known alleles were also used. *NOS1* Ex1f-VNTR genotyping was accomplished by comparing the allele sizes of each sample with the sizes of these known alleles from the STR markers D2S2186, D3S1314, D4S2950 and D22S420.

4.2.3. Taqman SNP genotyping assays.

4.2.3.1. Basis of the methodology

ASPM polymorphisms were genotyped in the American samples through the Taqman® SNP genotyping technology. Taqman® SNP genotyping assays from Applied Biosystems (ABI) provide an efficient and optimized method to genotype SNPs. Each assay allows the genotyping of one particular SNP and contains the following components in a single tube:

* Two primers used to amplify the sequence of interest.

* Two Taqman® probes, each one specific for one allele. Each probe contains:
    - A reporter dye linked to the 5' end (VIC for allele 1 and FAM™ for allele 2).
    - A minor groove binder (MGB): this modification allows designing shorter probes, since it increases the melting temperature (T_m) without increasing the probe length. As a result, the difference in T_m between matched and mismatched probes is higher, which produces a better allelic discrimination.
    - A nonfluorescent quencher (NFQ) at the 3' end of the probe. If the probe is intact, the presence of the NFQ suppresses the reporter fluorescence by Förster-type energy transfer.

During the PCR reaction (see figure M12), each probe binds specifically to its complementary sequence in the genomic DNA, between the forward and reverse primers. When the DNA polymerase arrives to the position where the probe is hybridized to the target, the polymerase cleaves the probe with its exonuclease activity, which results in the separation of the quencher and the dye and a production of

fluorescence by the reporter. It is remarkable that only when the probe matches the target genomic sequence perfectly, the polymerase will cleave it and the fluorescence will increase. Furthermore, the DNA polymerase is more likely to displace a mismatched probe without cleaving it, which does not produce a fluorescent signal, giving a high specificity to the method.

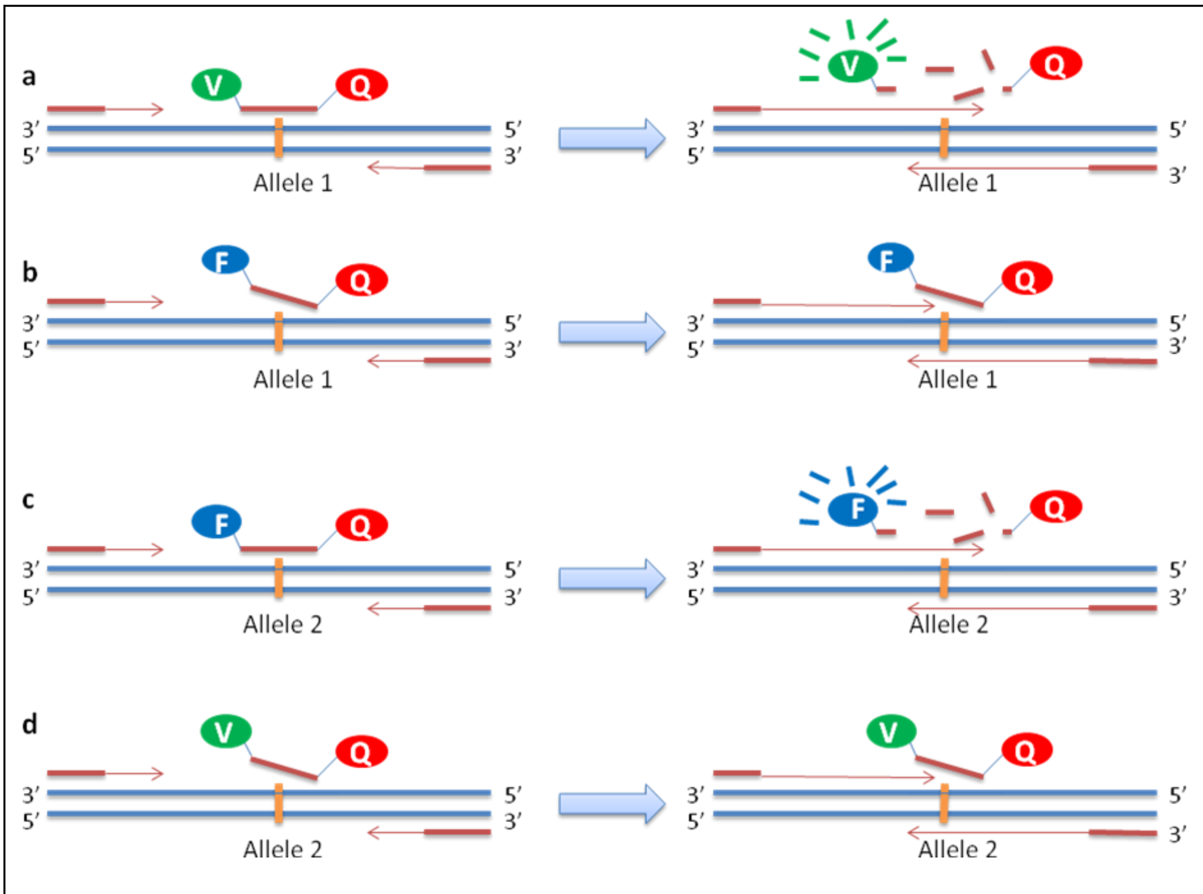


Figure M12. Scheme of the TaqMan® SNP genotyping methodology. In situations **a** and **c,** the VIC and FAM™ probes match and hybridize perfectly with allele 1 and allele 2, respectively, while in situations **b** and **d** there is a mismatch between the probe and the genomic sequence. Only when there is a perfect match, the polymerase cleaves the probes, which are hybridized to the target. The cleavage separates the fluorescent reporter dye from the quencher dye, resulting in a fluorescence emission from the reporter. This fluorescence emission is specific for each allele. (Adapted from Livak *et al.,* 1995a)

In summary, the presence of a high increase in VIC fluorescence indicates that the individual is homozygote for allele 1, while a substantial increase in FAM fluorescence will happen if the individual is homozygote for allele 2. Finally, an increase in both fluorescence signals indicates that the individual is heterozygote.

4.2.3.2. PCR reaction

The following table (M20) shows some features of the Taqman SNP genotyping assays used in this study.

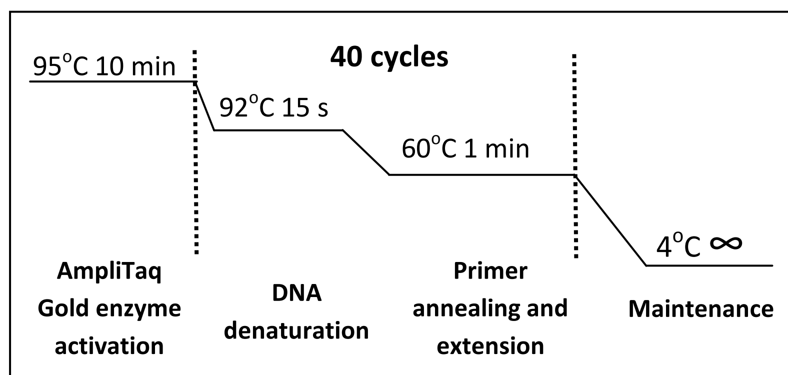
Table M20. Conditions of the TaqMan® genotyping assays.

GENE	SNP code	Assay code number	Allele detected with VIC® dye	Allele detected with FAM™ dye
ASPM	rs6677082	C___2759679_10	T	C
ASPM	rs12116571	C___2759692_10	G	A
ASPM	rs10922163	C___2759697_10	G	A
ASPM	rs41310927	C__27830876_10	G	A
ASPM	rs12138336	C__31565480_10	G	C
ASPM	rs3762271	C__27478941_10	C	A

The following reaction mix was used:

DNA (predelivered and dried in the bottom of the well)	10 ng
20x SNP genotyping assay mix	0.06 µL (final concentration: 0.25x)
2x Taqman Universal PCR Mastermix (Applied Biosystems, catalog no. 4304437)	2.5 µL
TE buffer	2.44 µL

Reactions were prepared in ABI Prism™ 384-Well Clear Optical Reaction Plates (Applied Biosystems, catalog no. 4309849) and run on ABI GeneAmp® PCR System 9700 thermal cyclers. The reaction program (standard for all assays) was:



4.2.3.3. Plate read

After PCR amplification, plates were read on an ABI PRISM® 7900HT Sequence Detection System (Applied Biosystems) to measure the end-point fluorescence levels for each sample in the plate. The software SDS v. 2.1 allowed us to calculate the measurements and to make a plot based on the fluorescence levels from each well. A template file with all the information regarding the assay (type of plate, dyes…) was also created to read the fluorescence for the FAM and VIC dyes from all the plates that were genotyped.

Once the plate was read and the plot was created, genotypes were assigned manually. Figure M13 shows how a plot looks after genotype calling. Finally, after genotype assignment, data was exported as a text file.

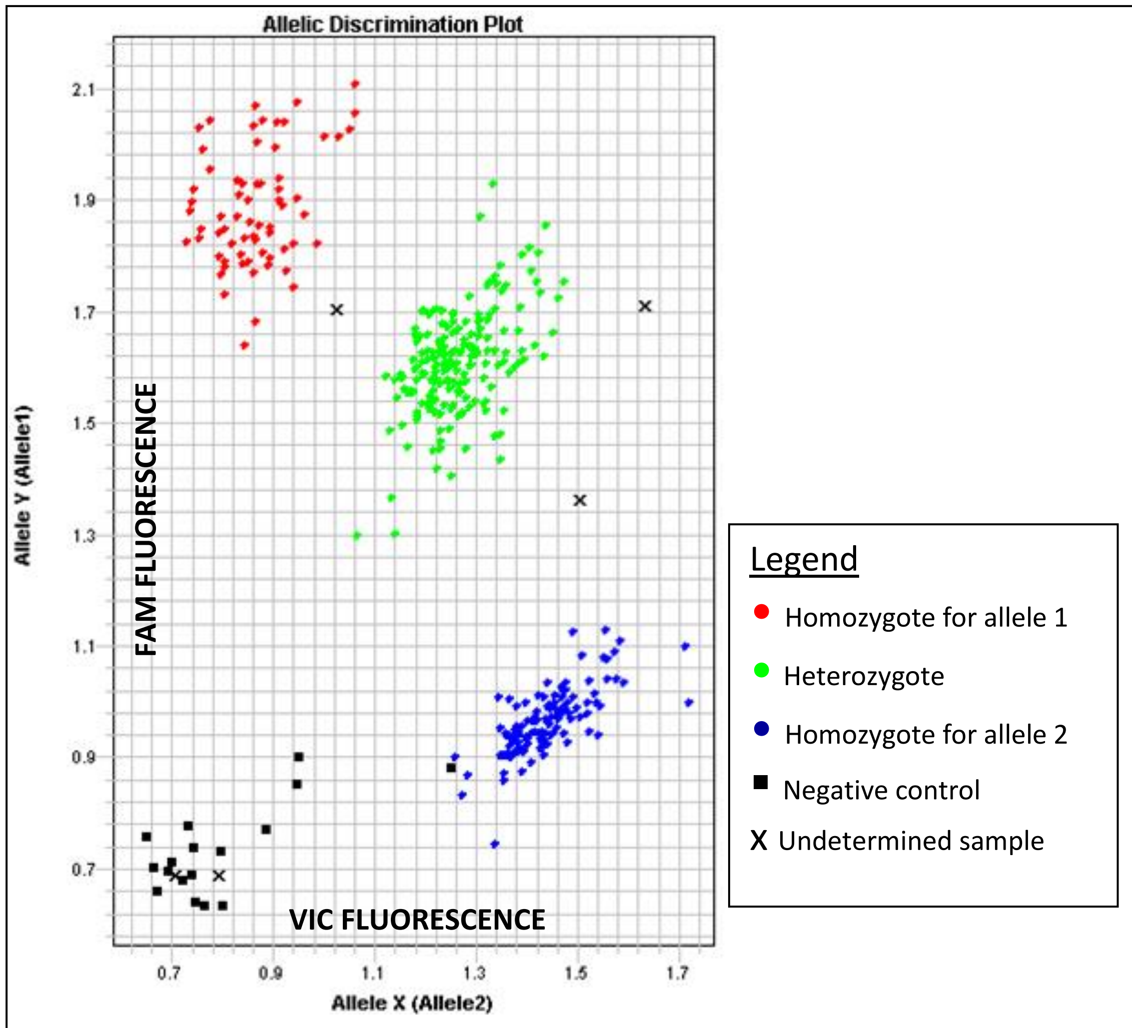


Figure M13. Plot from a plate read for one SNP genotyping assay. x-axis corresponds to VIC dye fluorescence, while FAM dye fluorescence levels can be found at y-axis. In this case, although there is some dispersion on each cluster, the separation between clusters is good and there are only a few samples of undetermined genotype. Moreover, negative controls only present some residual fluorescence, as expected.

4.2.4. SNP Genotyping through MassARRAY® System.

4.2.4.1. Basis of the methodology

iPLEX™ genotyping assays, together with the MassARRAY® platform (Sequenom®), were used to genotype all the SNPs from *PDE4D*, *PLEKHB1* and *RAB6A* genes, as well as some SNPs from *SLC6A4*, *ASPM* and *STMN1*. The iPLEX assays allow the genotyping of up to 29

SNPs in the same reaction (Oeth *et al.*, 2005). This method has the following basic steps (figure M14):

- Amplification of the target regions by multiplex PCR. In order to improve the reaction performance, PCR primers have a non-templated tag on the 5' ends, to increase the masses of unused primers so they fall outside the mass range of analytical peaks.
- iPLEX reaction: it is a post-PCR primer extension reaction, with the particularity that all reactions are terminated after a single base extension (SBE), because only terminator dideoxynucleotides (ddNTPs), but not dNTPs, are added to the reaction mix. This extension reaction generates fragments with a specific mass for each allele of each SNP. Moreover, the use of mass-modified terminators avoids the overlap between different SBE products.
- SBE products are loaded onto a chip and analyzed through MALDI-TOF mass spectroscopy. Finally, masses are converted to genotypes by specific software.

The next sections will explain in detail all the steps of this genotyping technique.

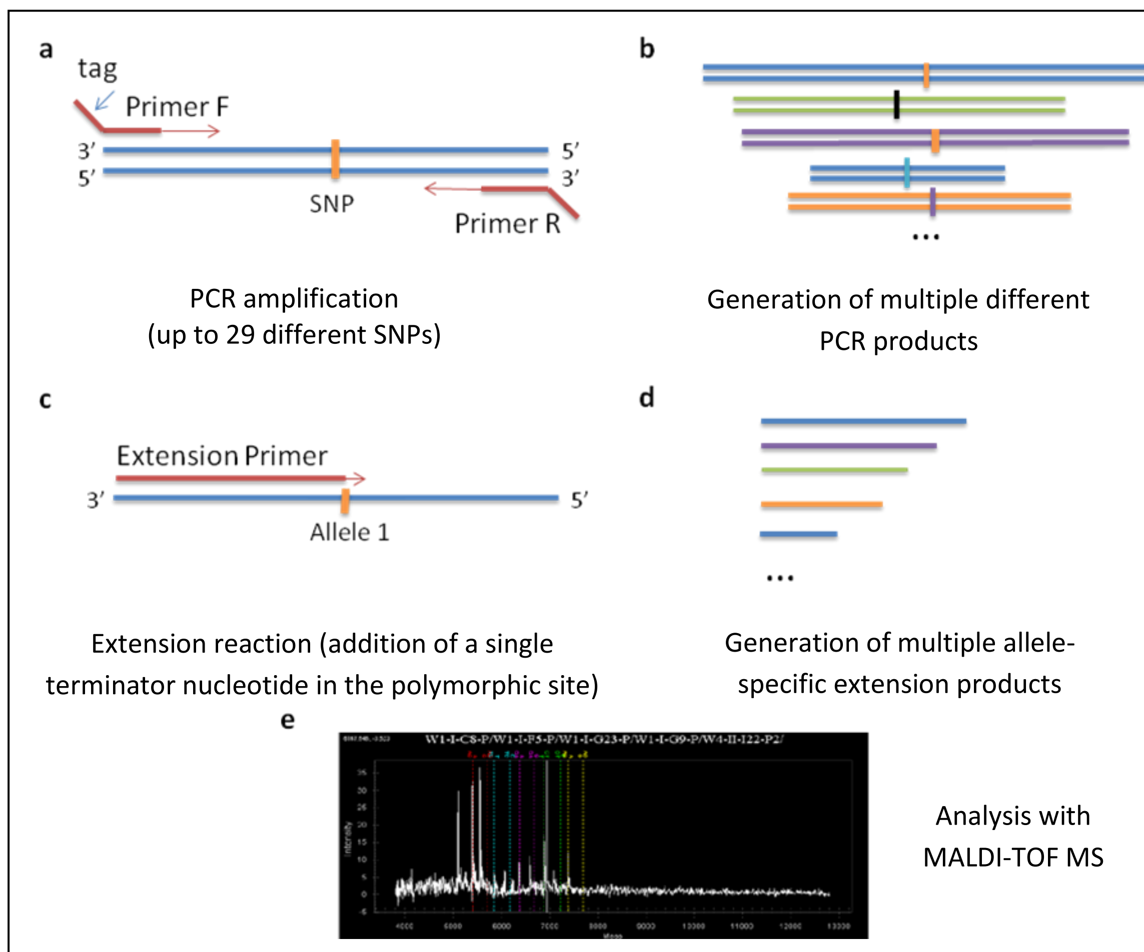


Figure M14. Overview of the MassARRAY™ genotyping technology. The process can be divided into three main steps. First, a PCR reaction of the regions which contain the SNPs of interest (**a** and **b**). Second, an extension process (**c** and **d**), which generates allele-specific extension products, each of them having a unique molecular mass. Finally, the masses of the extension products are analyzed by MALDI-TOF Mass Spectroscopy and genotypes are automatically assigned.
Abbreviations: MS, Mass Spectroscopy.

4.2.4.2. Assay design

The primer design for iPLEX assays is especially sensitive, since these assays require the use of multiplex primers with marked mass differences. Therefore, the use of specific software is essential.

A sequence of 200-400 bp around the SNPs of interest was pre-edited through ProxSNP and PreXTEND online utilities from the webpage www.realsnp.com (Sequenom). ProxSNP is used to search for additional registered SNPs that are within a given proximity of the SNP of interest and prevents primers for being designed over those locations. By contrast, PreXTEND is used to pre-design PCR primers with respect to the whole genome and also re-formats the sequences so that subsequent assay design will employ these primers. This program avoids the amplification of undesired regions from the genome with each pair of primers.

Afterwards, Mass-ARRAY Assay design 3.0 software from Sequenom was used to design the extend primers for each assay. These primers will ensure that the generated SBE products have different masses.

Finally, the PleXTEND application, also from www.realsnp.com, was used to validate multiplexed assay designs with respect to the genome. The purpose of this application is also to check for undesired amplification products resulting from random couplings of all primers present in a multiplex of assays.

All oligonucletides were synthesized by Metabion International (Martinsried, Germany). Concentrations from extend primers were adjusted to avoid high levels of signal-to-noise ratio. Thus, the concentration of those oligonucleotides which produced the largest SBE products was doubled relative to the remaining assays in the multiplex reaction. Table M21 shows the conditions for each iPLEX assay, with the exception of the genotyping of *ASPM*, *STMN1* and *SLC6A4* SNPs, which were genotyped by CeGen as a private service and therefore this information was not provided.

Table M21. Assay conditions for *PDE4D*, *PLEKHB1* and *RAB6A* SNPs genotyped through MassArray® platform.

GENE	Marker	Plex no.	PCR primer sequences 5' → 3'	Extension primers 5' → 3'	Mass of extension products
PDE4D	rs17291089	1	ACGTTGGATGCTTAATTGAATATTCTGGG ACGTTGGATGGAGTAGGTAACCAAAAATA	taTGGGATATTGAAAATTATTCAGAT	C: 8294.5 A: 8318.5
PDE4D	rs829259	1	ACGTTGGATGCCAAAACATTCTCATTCCTC ACGTTGGATGAGATTCTAGGCACTCTGTGG	ATTCCTCTCCTCCCTAC	T: 5263.5 A: 5319.4
PDE4D	rs1058458	2	ACGTTGGATGACACTAGCTGCAGTGACTCC ACGTTGGATGTTCAACCTGTTCATCAAGGG	ccGCTGCAGTGACTCCAAGACTCTT	C: 7825.1 T: 7905.0
PDE4D	rs17719378	1	ACGTTGGATGGCTCATGGCAACTCTAGAGG ACGTTGGATGCTCTGTAGGTCTCAGCTTTC	AACTCTAGAGGAGAGGTT	A: 5858.9 G: 5874.9
PDE4D	rs10055954	1	ACGTTGGATGGTACCCTCTTTGAGTACAAAC ACGTTGGATGCTGTGAATTACCTACCCTGC	ctgACAAACAATCCTGTAACACA	C: 7216.8 G: 7256.8

Table M21 (continuation). **Assay conditions for *PDE4D*, *PLEKHB1* and *RAB6A* SNPs genotyped through MassArray®.**

GENE	Marker	Plex no.	PCR primer sequences 5' → 3'	Extension primers 5' → 3'	Mass of extension products
PDE4D	rs10461656	1	ACGTTGGATGCAAATGAGCACAATTGATCTA ACGTTGGATGTTTTTATTGAGAGGCCATGC	ATGATGGCTTTGAGTTTGTA	A: 6464.2 G: 6480.2
PDE4D	rs7713345	1	ACGTTGGATGTTCTTTCTCACCAGTTTTCC ACGTTGGATGGGGCCGTAGCCAATGATTTG	ggcATCATATTCCTTCTAAGTCCCT	G: 7790.1 C: 7830.1
PDE4D	rs12656462	1	ACGTTGGATGAAAAAGTATCAAAAGTACAG ACGTTGGATGAGGCATCTTTGCTATTAAC	GTATCAAAAGTACAGAATATAGCA	A: 7673.1 T: 7729.0
PDE4D	rs17853590	2	ACGTTGGATGTGCAGCAATTTTTGCCAGTG ACGTTGGATGACTTACTTGTATTGATCAG	TGTAGATCATCCTGGTGTGT	C: 6401.2 T: 6481.1
PDE4D	rs10056492	2	ACGTTGGATGCTACATTAGGTATTTTGCC ACGTTGGATGCATCACACACCAGGGCCTAT	gatGTATTTTGCCTAATGCTGT	C: 6993.6 T: 7073.5
PDE4D	rs4700316	1	ACGTTGGATGTTGTGTCCATGCTTGGCAGG ACGTTGGATGCCTCCTTCTATTGTGGAGAC	TGCTTGGCAGGCTTTTT	C: 5445.6 G: 5485.6
PDE4D	rs7714708	1	ACGTTGGATGCATACTAACTGGAATCCAT ACGTTGGATGGGTAATTAAATACATTCATTGC	AATCAGTATAAGTAATTTGGCCC	T: 7317.8 C: 7333.8
-	rs663303	1	ACGTTGGATGCTCTTGCTCCAATAATGACC ACGTTGGATGGTGACTGAACTTTGTCAAGA	cctACAGATCATTTCTCAAGAGACG	T: 7881.2 C: 7897.2
PLEKHB1	rs4944850	2	ACGTTGGATGGGAAATAGTGGACTGGCTC ACGTTGGATGTCTCATTATATTATACTCTC	cCTGGCTCATCAAATTCAAAT	C: 6636.4 A: 6676.3
PLEKHB1	rs11538627	2	ACGTTGGATGATGGCCTGCTGACTGTGAAC ACGTTGGATGACAGGGCATCATCCTTGGTC	ACTGTGAACCTACGGGA	A: 5490.6 T: 5546.5
PLEKHB1	rs591804	2	ACGTTGGATGTGCCTCAGGTATCTGGTTCC ACGTTGGATGTGCTGCTTTGCATCACCCTC	CTGGTTCCTGGTCTTTAGC	A: 6048.0 G: 6064.0
PLEKHB1	rs6592527	2	ACGTTGGATGGTGTGGTGCAGGTATCATTC ACGTTGGATGCAGAGAGAGAACAATAACCC	GTATCATTCCAGCTGCT	C: 5383.5 G: 5423.6
PLEKHB1	rs940828	1	ACGTTGGATGAGGCCGCTTCATAGAGTCAC ACGTTGGATGAGTTCCACCAAAGAGAGGCG	gggTCATAGAGTCACAGAGTG	G: 6782.4 T: 6806.5
PLEKHB1	rs3741147	1	ACGTTGGATGAGAGACTCAAGAGCTCTTTC ACGTTGGATGAGAAAGAGGATGCTCCCTGC	aAGCTCTTTCCAGACCCT	G: 5641.7 T: 5665.7
-	rs12274970	1	ACGTTGGATGTTTTCCTTTGTTACTCCACC ACGTTGGATGTCTCCATGTCATCACCAGCC	TTTGTTACTCCACCATTTCT	T: 6255.1 C: 6271.1
RAB6A	rs3182788	1	ACGTTGGATGCATAAAACTGGAAAAGCCTC ACGTTGGATGGAAGATGACATGGGAGATTA	ccttAAAAGCCTCAGGAGCAA	G: 6655.4 T: 6679.4
RAB6A	rs10736793	2	ACGTTGGATGCTGCACTGATTGTAATAGCC ACGTTGGATGATGAGAGCCTTGCTCTCCAG	TAATAGCCTATTTTTACTTCTCT	C: 7176.7 A: 7200.7
RAB6A	rs3203705	1	ACGTTGGATGCACGGATGTAACTGGGAATG ACGTTGGATGCATCTCACAGATCAGGCTTC	gAACTGGGAATGAGGCTACGGA	A: 7144.7 G: 7160.7
RAB6A	rs11235876	1	ACGTTGGATGCGACTTTATCATGATGCAAG ACGTTGGATGCTTTACGTACTATCCATATC	GATACTTGTTTTCAGGCTT	G: 6047.0 A: 6126.9
RAB6A	rs11235880	2	ACGTTGGATGGTACTTTGATGTTCGAACT ACGTTGGATGTATCCCACTGAACACTTCAT	ATATATTCTCAAGTGAATGTTTTT	C: 7634.0 A: 7673.9
RAB6A	rs7127066	1	ACGTTGGATGTTGTCTGATGTTTTCTCACG ACGTTGGATGGCACATCAGTTCTGTGGTAG	ttcTGTTTTCTCACGATTAGACAGAG	C: 8198.4 G: 8238.4

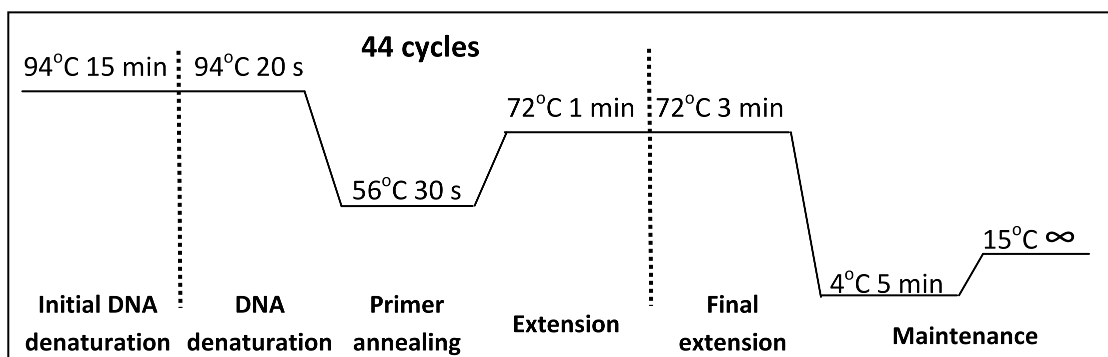
Note: all these SNPs were genotyped as a part of the same assay.

4.2.4.3. PCR amplification

With the help of the workstation epMotion 5075 (Eppendorf AG), the following reaction mix was prepared for each assay in 384-well PCR microplates (the volumes refer to one single reaction):

DNA (8-25 ng/µL)	1 µL
dNTPs 25mM (Qiagen, cat. no. 201912)	0.1 µL
Primer mix (0.5 µM)	1 µL
HotStar PCR Buffer 10X	0.625 µL
$MgCl_2$ 25mM	0.325 µL
HotStar Taq DNA polymerase (5U/µL) (Qiagen, cat. no. 203205)	0.1 µL
dH_2O	1.85 µL

Reactions were run on Biometra TGradient thermocyclers with the following standard program:



4.2.4.4. SAP treatment

Before the iPLEX reaction, it is necessary to dephosphorylate unincorporated dNTPs with shrimp alkaline phosphatase (SAP). If this step is omitted, unincorporated dNTPs could extend in the primer extension reactions, giving as a result contaminant products which could be detected by the mass spectrometer. SAP treatment consists in the addition of 0.17 µL of 10x SAP buffer, 0.3 µL of SAP enzyme (1U/µL) and 1.53 µL of sterile distilled water to each well. Then, the plate is incubated in a thermocycler with the following program: 37°C for 20 minutes (dephosphorylation step), 85°C for 5 minutes (SAP inactivation) and 4°C forever.

4.2.4.5. iPLEX reaction

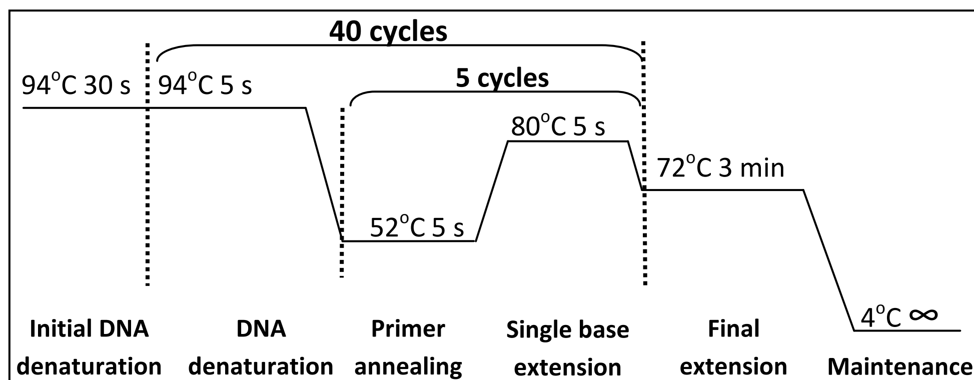
The iPLEX reaction mix contained:

iPLEX termination mix*	0.2 µL
iPLEX Buffer 10X *	0.2 µL
iPLEX enzyme*	0.1 µL
dH_2O	0.755 µL
Primer mix**	0.804 µL
TOTAL VOLUME (for one single reaction)	**2 µL**

* from the iPLEX reaction kit (SEQUENOM, catalog no. 10116).

** the concentrations of each primer pair vary according to the mass of each SBE product.

The mix was added to each well of the PCR plates and the plates were thermocycled with the following 200-cycle program with two cycling loops:



4.2.4.6. MALDI-TOF MS analysis

Samples were firstly desalted to optimize MS analysis. For this purpose, samples were diluted with 16 µL of water and 6 mg of resin (SEQUENOM, catalog no. 10053) were added to each well. Then the 384-well was rotated for 20 minutes to allow the resin to trap all interfering ions in the sample. Finally, the plate was centrifuged at 3000 rpm for 5 minutes to deposit the resin together with the salts at the bottom of each well.

After resin treatment, a small volume (around 15 nL) of each sample of the PCR plate was transferred onto a 384-well SpectroCHIP bioarray (SEQUENOM, catalog no. 00601) with the MassARRAY® Nanodispenser S instrument (SEQUENOM AG). Moreover, a calibrant solution was also dispensed onto the chip.

Once the chip was ready to be read, it was placed on the Scout plate of the MALDI-TOF mass spectrometer, which was controlled by the MassARRAY Typer Workstation version 3.4 software, where the position of the chip and the assay definitions had been previously set. Spectra were then acquired and automatically sent to the MassARRAY Typer software. Genotypes were also automatically assigned by this software.

4.2.4.7. Post-read quality checking

Genotype calls and spectra for each assay were subsequently checked with the MassARRAY Typer 3.4 software to detect wrong calls and low-quality peaks. Genotype data were edited when necessary.

5. Statistical methods

Many computer programs have been used for the statistical analysis of the genotypic data. Table M22 shows a summary of their uses.

Table M22. Software used for the statistical analyses.

Program	Uses
Quanto version 1.2.3	Statistical power calculation
Haploview version 4	Hardy-Weinberg Equilibrium Linkage disequilibrium analysis
Merlin version 0.10.2	Mendelian inheritance checking
SNPstats http://bioinfo.iconcologia.net/snpstats/start.htm	Case control association analysis (genotypic frequencies) Association analysis of quantitative variables Gene-gene interaction analysis
STATA version 8.2	Case-control association analysis Association analysis of quantitative variables
FBAT version 2.0.2	Family-based association analysis
Unphased version 3.0.12	Association analysis of allelic frequencies Case-control haplotype analysis
Haplostats	Case-control haplotype analysis
PLINK version 1.0.2	Pooled association analysis
SeqBon	Correction for multiple testing
SPM	Neuroimaging analysis

5.1. Genotyping verification

All genotype data were checked through different methods to ensure that there were no genotyping errors.

5.1.1. Hardy-Weinberg Equilibrium.

All polymorphisms were assessed for Hardy-Weinberg Equilibrium (HWE) in both patient and control samples by applying a χ^2 test implemented in Haploview Program version 4 (Barrett *et al.,* 2005).

The deviation from HWE in the control sample could be indicating a genotyping error, since control subjects are not suffering from any psychiatric disorder which could be acting as a selective pressure, thus explaining the deviation from equilibrium. Therefore, in those cases, the best solution is to re-genotype the samples to verify the initial finding. However, although a genotyping error is the most plausible explanation for the Hardy-Weinberg disequilibrium, other possibilities cannot be ruled out.

By contrast, when the control sample is in HWE but the patients' sample is not, this finding could suggest a manifestation of the association of genotype with the disease.

Finally, we cannot forget that the existence of HWE is not a guarantee of absence of genotyping errors.

5.1.2. Mendelian inheritance errors.

For the American family samples from CBDB, the presence of Mendelian inconsistencies on each pedigree was checked through Merlin software. Those pedigrees with Mendelian inheritance errors were discarded before the association analyses, since they are a clear sign of genotyping error.

5.2. Linkage disequilibrium analysis

The Haploview program version 4.0 (Barrett *et al.,* 2005) was used to estimate, in both cases and controls separately, the statistical values Delta prime (D') and squared correlation coefficient (r^2) as measures of pair wise linkage disequilibrium LD. Haplotype block structure was determined according to the criteria shown in Gabriel *et al* (2002).

5.3. Measurement of the statistical power.

Prior to any association analysis, it is important and highly recommendable to make a priori calculation to know the statistical power of the study. This power varies attending to different parameters, such as the sample size, the risk allele frequency, the incidence of the disease and the risk (odds ratio) assumed for each polymorphism, among others.

For this study, QUANTO software version 1.2.3 (Gauderman, 2002; Gauderman and Morrison, 2006) was used to calculate the statistical power to find associations between genetic polymorphisms and the different study variables that include case-control, family-based and quantitative trait analyses. For case-control and family-based association studies we assumed an odds ratio between 1.3 and 2, while for quantitative traits we considered that the proportion of variance explained by each polymorphism could vary between 1% and 20%. We also set the incidence of schizophrenia at 1% and the inheritance model as additive.

5.4. Case-control analyses

These analyses are based on the comparison of the allelic, genotypic or haplotype frequencies between two groups of individuals, generally patients versus healthy subjects, but also comparisons between two subsets of patients. The different comparisons performed in this study are the following.

SPANISH SAMPLE

1. Controls versus all patients.
2. Controls versus hallucinatory patients.
3. Controls versus patients with no AH.
4. Hallucinatory patients vs patients with no AH.
5. Controls versus schizophrenic patients.
6. Controls versus schizophrenic patients with AH.
7. Controls versus patients with chronic AH.
8. Patients with chronic AH versus patients with no AH.

GERMAN SAMPLE

1. Controls versus all patients.
2. Controls versus schizophrenic patients.
3. Controls versus patients with bipolar disorder.

AMERICAN SAMPLE

1. Controls versus all patients.

It should be noted that the comparisons performed on each sample depended on the nature of each patient group and the clinical information available on each case.

In all cases, statistical significance threshold was set at 0.05.

5.4.1. Allelic frequencies.

Allelic differences between patients and control subjects were assessed with a χ^2 test via Unphased program version 3.0.12 (Dudbridge, 2003, 2006).

5.4.2. Genotypic frequencies.

Different programs and approaches were used depending on the sample:

* Spanish and German samples: The web-based application SNPStats (Solé *et al.,* 2006) was used to evaluate the existence of differences in the genotypic frequencies between groups. This software uses a logistic regression approach for the case-control comparisons and calculates the odds ratios (OR) and 95% confidence intervals as a sign of the risk associated to each genotype. Different inheritance models (codominant, dominant, recessive, and additive) were considered and tested during the analysis. To decide the best inheritance model, a score associated to each model based on minimizing the expected entropy (the Akaike's information criterion or AIC) is also calculated. According to this value, the model with the less AIC value would be the most probable model.

  Moreover, it should be noted that, when differences in the allelic, genotypic or haplotype distribution between sexes were detected, sex was used as a covariable.

* American sample: we used STATA software for single SNP analysis by using two approaches: a Fisher's exact test (performing a comparison of the three genotypic frequencies) and a logistic regression analysis with two different contrasts, where the homozygotes 1/1 (where 1 refers to the major allele) were compared against the other two genotypes (1/2 or 2/2) separately.

5.4.3. Haplotype analysis.

Haplotype frequencies were estimated in the German and the Spanish sample through a retrospective likelihood algorithm and compared between patients and controls with the Unphased package version 3.0.12 (Dudbridge 2003, 2006). Sliding windows of two, three and four-marker haplotypes were tested. Moreover, those haplotypes with a frequency below 0.03 were considered as rare and therefore discarded from the statistical analysis. A 1000-permutation run was performed in each case to better estimate the significance of the positive associations.

By contrast, the haplotype analysis in the American sample was performed via HAPLOSTATS program. This program uses the expectation-maximization algorithm, which is based on the determination of those haplotype frequencies which maximize the probability to obtain the

observed genotypes. Analysis was made by considering a sliding window of three-marker haplotypes.

5.4.4. Pooled association analysis.

When genotype data for more than one case or control sample was available, we performed a pooled analysis by combining the affected individuals and healthy subjects with a different country of origin. Moreover, to ensure certain level of homogeneity between all the affected individuals included in the pooled analysis, only those patients diagnosed as schizophrenics (according to DSM-IV criteria) were included in the analysis.

The linkage disequilibrium patterns between SNPs were determined to assess the validity of such type of analysis. Moreover, these analyses were only stratified according to the region of origin of each individual (Germany vs. Spain; Spain vs. USA) since there were no differences in other possible stratification variables, for example ethnicity.

Particularly, we analyzed pooled data for the following genes, SNPs and samples (see table M23).

Table M23. List of the polymorphisms which were analyzed in the pooled analyses.

Pooled samples	Gene	SNPs included in the pooled analysis
USA + Spain	*ASPM*	rs6677082 rs12116571 rs10922163 rs41310927 rs12138336 rs3762271
Germany + Spain	*PLEKHB1-RAB6A*	rs663303 rs940828 rs3741147 rs12274970 rs3182788 rs3203705 rs11235876 rs7127066
Germany + Spain	*PDE4D*	rs17291089 rs829259 rs17719378 rs10055954 rs10461656 rs7713345 rs12656462 rs4700316 rs7714708

PLINK software version 1.0.2 (Purcell *et al.*, 2007) was used for the analyses of the pooled samples. This package is especially designed for GWA studies, although it can work on a small scale, as this is case. The selected statistical tests were the Cochran-Mantel-Haenszel (CMH) tests, which are valid with a small number of large clusters. These tests are very useful when the presence of possible stratifying variables is suspected, since they are based on an "average" odds ratio that controls for the potential confounding due to the cluster variable.

Two variants of the CMH tests were performed:

* CMH test for 2x2xK stratified tables: it performs the case-control association test but controlling the effect of the cluster variable. In our particular case, the cluster variable was the country of origin (Spain, Germany or USA).

* CMH test for IxJxK stratified tables: this test is useful to see if a SNP varies between clusters and can be extraordinarily helpful to interpret the results from the CMH test for 2x2xK tables and also to understand the results of the different clusters separately.

5.5. Family-based association analysis.

This type of analysis was performed with the family American samples. The association study was performed on each SNP as well as in haplotypes of SNPs. We used FBAT program to test for association of single SNPs or haplotypes (with a sliding window of three-marker haplotypes) with schizophrenia. FBAT (acronym for Family-Based Association Tests) compares the genotypic distribution observed in the patients to its expected distribution under the null hypothesis ("no linkage and no association with the disease"). The expected distribution is based on Mendelian segregation.

The obtained *P* values are based on a run of at least 500 permutations.

5.6. Association analysis with disease traits.

Information about different clinical variables was available for individuals of the following samples:

* Spanish sample: different scales and measures were assessed in some patients and were available for its study: BPRS general score, PANSS scores, KGV scale and PSYRATS scales for auditory hallucinations and delusions. It should be noted that the scores corresponding to the PSYRATS subscale for AH are only available for hallucinatory patients. Moreover, we also studied the effect of genetic variation on other dichotomic clinical variable: age at onset of the disease. In this case, patients were divided in two categories: patients with early-onset psychosis (age at onset of up to 20 years old) and patients with late-onset psychosis (when the age at onset is more than 20 years old). The online utility SNPStats (Solé *et al.*, 2006) was used to study the likely relationship between genetic polymorphisms and all the response variables. The program applies a linear regression analysis to test the existence of associations between polymorphisms and quantitative variables, also considering different inheritance models (codominant, dominant, recessive, and additive). The analysis is summarized by the mean value for each genotype and mean differences

with respect to a reference category, as well as the Bayesian Information Criterion (BIC) score (similar to the AIC score), a score which indicates the most probable inheritance model in the linear regression analyses. By contrast, for the variable “Age of onset”, a logistic regression analysis was performed. This approach, similar to the case-control analysis, compares the genotypic frequencies in both groups of patients.

* American sample: scores for seven measures derived from different cognitive tests were available in both unrelated controls and schizophrenic patients. These variables are Verbal Memory, Working Memory-N back, Visual Memory, IQ-Processing Speed-Fluency, Executive Function-WCST, Attention and Digit Span. A linear regression analysis via STATA software was used to examine the effect of genotype, both in controls and patients, on the performance of the seven cognitive factors. Particularly, two types of comparisons were assessed: on one hand, 1/1 genotype (where 1 refers to the major allele) was compared to the heterozygote genotype, whereas, on the other hand, the two types of homozygotes (1/1 and 2/2) were compared to each other.

5.7. Correction for multiple testing.

The Bonferroni sequential test for multiple comparisons (Rice*,* 1989) was applied to correct all the reported *P* values. We considered that this correction was more appropriate than the Bonferroni correction because it has an increased statistical power in detecting a false negative result and the rejection criteria are less stringent.

5.8. Gene-gene interaction analysis.

Gene-gene interaction analyses were performed when we considered that two polymorphic variants from different genes could be interacting with each other and thus modifying the effect each single variant has on certain phenotype (e.g. the disease risk or another disease trait). PLINK software version 1.0.2 (Purcell *et al.*, 2007) was used for this purpose. This program performs either a linear or logistic regression test, depending on whether the phenotype is a quantitative (e.g. clinical scales) or a binary trait (for example a case-control variable). PLINK makes a model based on the allele dosage for each SNP separately, as well as for the SNP epistasis. As a result, the program gives an estimation of the effect of the epistasis (an Odds Ratio value for binary traits, a β value for quantitative traits) and an asymptotic p-value.

5.9. Correlation with neuroimaging variables.

We used VBM and fMRI to assess the impact of certain polymorphisms on brain structure and function of both normal controls and schizophrenic patients. This study was made with the collaboration of the neuroimaging group of the Clinical Brain Disorders Branch at NIMH, as well as the neuroimaging group of Dr. Luis Martí Bonmatí from the Departments of Radiology of the Dr. Peset University Hospital and Quirón Hospital (Valencia).

5.9.1. Structural Magnetic Resonance – Voxel-Based Morphometry (VBM).

We analyzed how different polymorphisms affected human brain volumes. The effect of genotype on total intracranial volume from patients or controls was examined using a multiple regression test. Total brain volume, gender and age were used as covariables. Moreover, the effect on regional intracranial volumes was also studied. All results were thresholded at $p<0.05$ and corrected for multiple comparisons using the false discovery rate criteria (Genovese *et al.,* 2002).

The genes analyzed through this approach were:

* Serotonin transporter gene (*SLC6A4*): the effect of 5-HTTLPR polymorphism on the gray matter (GM) volume of limbic areas was studied in a group of 24 patients and 30 controls from the Spanish sample. Both groups where matched by sex (all males), ethnic group (all Caucasian), laterality (all right handed) and educational level (all had a secondary school qualification) to avoid stratification in the sample. The structural image methodology chosen to extract features for genetic analysis was based on a VBM optimized protocol. The quantification and processing analysis was performed with Statistical Parametric Mapping (SPM5, Wellcome Institute, London, United Kingdom). A region of interest (ROI) around the limbic system was defined (figure M15). This region included the cingulate cortex, fornix, hippocampus, parahippocampal gyrus and amygdale. ROI masks were overlayed with the gray matter maps to define the areas to study.

  Statistical measurements were performed following a non-parametric approach, which provides a robust variance estimation method based on permutation test. Statistical non-Parametric Mapping (SnPM) tool was used to examine the structural associates of genetic features. Three separate analyses were designed in order to measure the relationship between image and genotype. First, a model to check the effects of the 5-HTTLPR polymorphism on the variability of the GM in limbic areas in both the healthy control subjects and schizophrenic patients separately was performed. Statistical models were estimated by performing an independent voxel by voxel Student-*t* test with analysis of the covariance (ANCOVA).

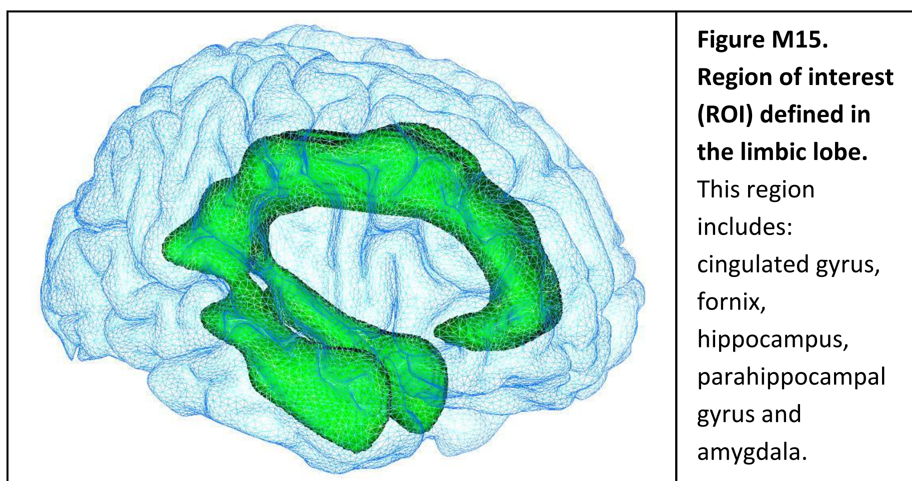


Figure M15. Region of interest (ROI) defined in the limbic lobe. This region includes: cingulated gyrus, fornix, hippocampus, parahippocampal gyrus and amygdala.

* *ASPM* gene: MRI scans for VBM of 169 patients and 153 normal controls from the American sample were available. We analyzed how *ASPM* variation affected regional intracranial volumes in the two separated samples. However, in this case, we decided not to use a hypothesis-driven region of interest (ROI) approach, given that there are no previous studies which can throw light about the brain regions which could be more affected by a variation in *ASPM* gene. Thus, all reported loci for genotype effects show uncorrected *P* values at $p<0.001$ and are reported with reference to the MNI (Montreal Neurological Institute) standard space. Statistical measurements were performed with Statistical Parametric Mapping (SPM5, Wellcome Institute, London, United Kingdom).

5.9.2. Functional Magnetic Resonance Imaging (fMRI).

* *SLC6A4* gene: the working hypothesis was that the 5-HTTLPR polymorphism influenced the emotional response of psychotic patients. According to this hypothesis, the main objective was to study the differences in the amygdala activation to neutral and emotional words depending on the 5-HTTLPR genotype. For this purpose, an emotional-auditory paradigm was designed to replicate those words and emotions related to hallucinatory experiences (Sanjuán *et al.*, 2007): during the fMRI procedure, two sessions were randomly presented to each subject, one session with high emotional content words and the other with neutral emotional content words. For each session (see figure M16), the stimuli consisted of four blocks of activation interleaved with four blocks of rest. Each activation block was composed of 13 Spanish words. fMRI images were obtained by means of BOLD (Bold Oxygenation Level Dependent) and data were preprocessed and transformed into a standard space (MNI150, Montreal Neurological Institute). A region of interest (ROI) including the left and the right amygdala was considered. The statistical analysis was performed with SPM2 software, to evaluate the existence of differences in the amygdala activation depending on the genotype. Bonferroni correction for multiple testing was also applied with a $p<0.05$ threshold.

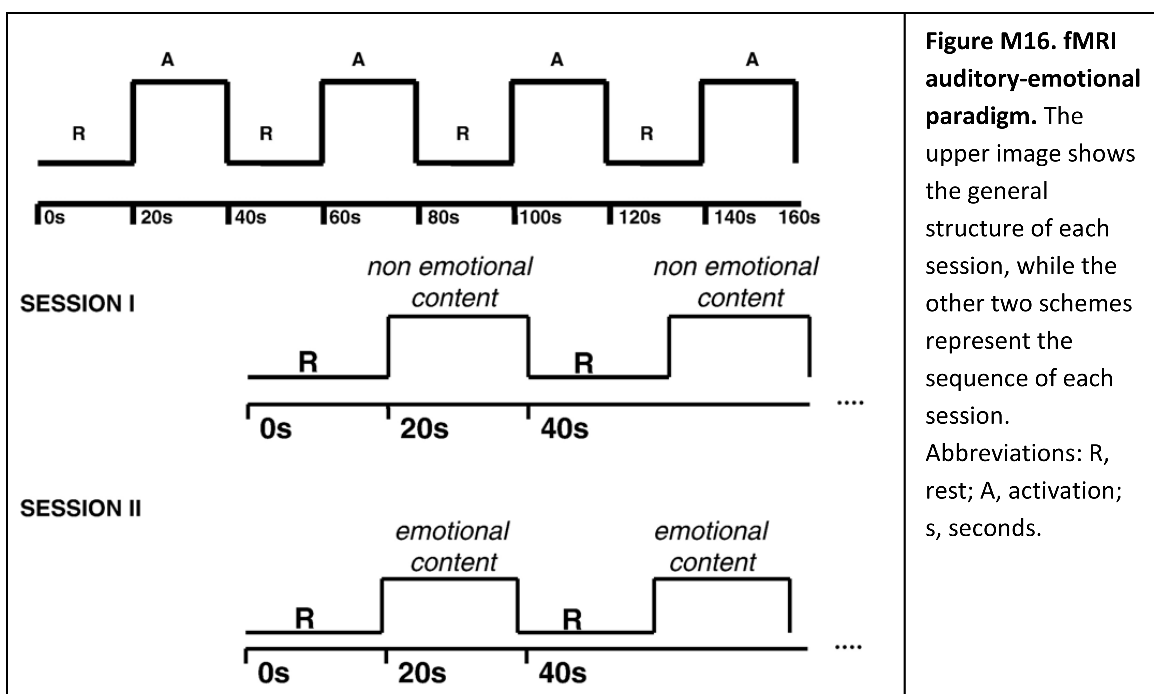


Figure M16. fMRI auditory-emotional paradigm. The upper image shows the general structure of each session, while the other two schemes represent the sequence of each session. Abbreviations: R, rest; A, activation; s, seconds.

* *ASPM* gene: To test the hypothesis that *ASPM* has an impact on brain function, we used fMRI to measure BOLD activation during the N back task, a measure of short-term working memory, in 79 normal subjects from the American sample. Subjects performed the task as it was previously described (Callicott *et al.* 1999). Briefly, subjects were scanned while performing a simple N back block design (see figure M17) alternating between a two back (2B) working memory condition and a no back (0B) control condition. Subjects were instructed to recall the stimuli (numbers 1-4 shown randomly) seen "n" previously (two prior for the 2B condition and the currently presented digit for 0B).

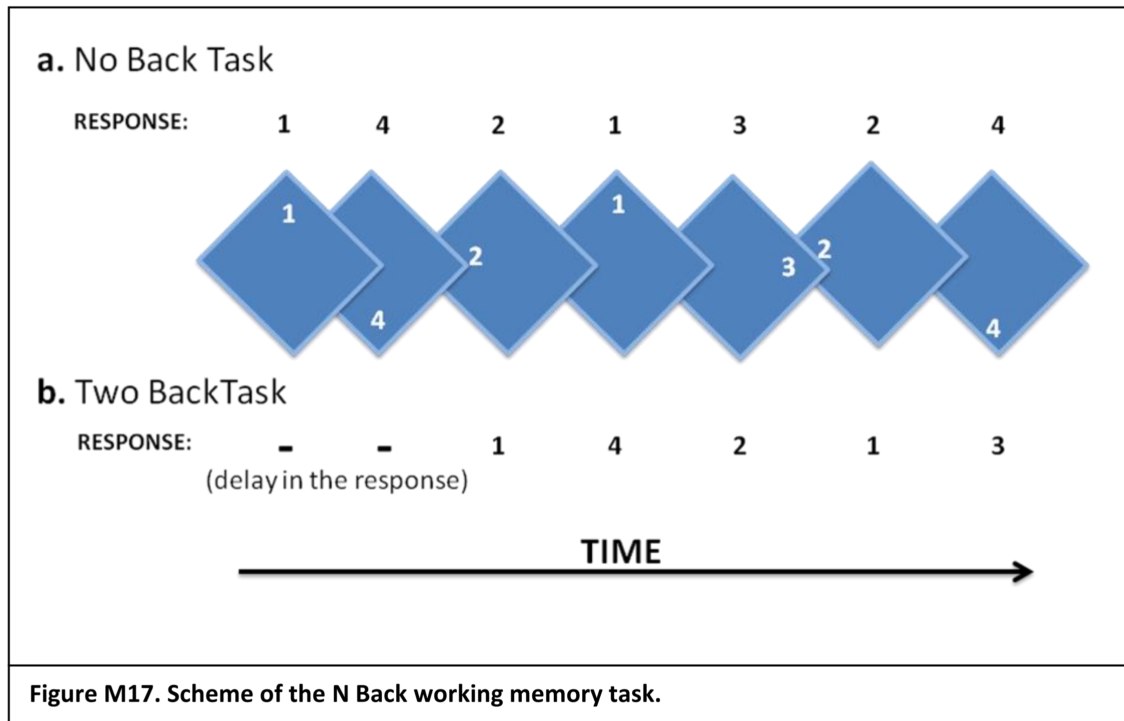


Figure M17. Scheme of the N Back working memory task.

All normal subjects were matched for N back performance and reaction time, handedness, age, gender and IQ. Whole brain BOLD fMRI data were collected on a 3-T Signa scanner by using GE-EPI-RT pulse sequence (GE Medical Systems). All fMRI data were processed and spatially normalized to a MNI standard space and analyzed via SPM2 software as described previously. Reported loci for genotype effects on task-related activation exceed significance levels at $p<0.001$ (uncorrected *P* value).

RESULTS

RESULTS

In this doctoral thesis work, several schizophrenia candidate genes have been studied and many different statistical analyses have been performed depending on the gene and the clinical information available for each sample. Thus, the Results section has been structured to facilitate the access to all data: first, there will be a section which will include the tests for Hardy-Weinberg Equilibrium (HWE) and Mendelization. Afterwards, the analyses of the linkage disequilibrium (LD) patterns for each gene will be presented. Then, the results for the case-control and family-based analyses will be shown. The fourth section will include the significant associations with quantitative variables and disease traits. Following these results, the positive findings from the gene-gene interaction analyses will be presented. Finally, the last section will show the association analysis with neuroimaging variables (fMRI, sMRI-VBM and MRS).

It is important to remark that, prior to the analysis, 32 patients and 6 control subjects from the Spanish sample were excluded from the study for several reasons:

- Impossibility to obtain DNA samples (33 individuals).
- Existence of a relationship with other subjects (2 individuals).
- Doubts about the ethnicity (3 subjects).

Moreover, depending on the genotyping technique and the quality of each DNA sample, there was a variable number of fails during the genotyping procedure.

1. Genotyping verification

The existence of HWE and the absence of Mendelian inconsistencies (when applicable) were verified for all samples and polymorphisms. The results can be found below.

1.1. *SLC6A4* gene

Nine markers located along *SLC6A4* gene were included in the study. These markers were genotyped in the Spanish sample and subsequently analyzed.

Table R1 shows information about the minor allele frequencies for both healthy subjects and the whole group of psychotic patients, as well as the *P* values corresponding to the χ^2 tests performed to verify the existence of Hardy-Weinberg Equilibrium (HWE). As it can be seen, one SNP (rs2228673, located in exon 4) was found to be monomorphic for allele G in our sample; therefore it was excluded of the analyses. Furthermore, one SNP (rs2020936, located in intron 1A) deviated from HWE in controls. However, the significance is very slight (p = 0.0434) and did not resist Bonferroni sequential correction, and therefore we decided not to exclude this polymorphism from the analyses. Moreover, the low frequency of the nine repeat allele of STin2 (0.009 in control subjects) led us to exclude all genotypes including this allele from all the analyses and therefore the 10-repeat allele was considered as the minor allele.

Table R1. Frequency and HWE data for *SLC6A4* polymorphisms.

Polymorphism	Marker order	MAF (controls)[a]	HWE *P* value (controls)	MAF (patients)[b]	HWE *P* value (patients)
rs3813034	M1	0.471 (G)	0.529	0.47 (G)	0.888
rs1042173	M2	0.474 (G)	0.537	0.48 (G)	1.0
rs140700	M3	0.083 (A)	1.0	0.05 (A)	1.0
rs2228673	M4	Monomorphic (G)	N/A	Monomorphic (G)	N/A
rs2020942	M5	0.328 (A)	0.693	0.365 (A)	0.868
STin2	M6	0.322 (10rep)	0.694	0.356 (10rep)	0.916
rs2020939	M7	0.443 (T)	0.545	0.458 (T)	1.0
rs2020936	M8	0.213 (C)	**0.043** (0.473 corrected)	0.167 (C)	0.196
rs2066713	M9	0.328 (T)	0.693	0.353 (T)	0.426
rs4251417	M10	0.074 (A)	0.319	0.064 (A)	0.622
5-HTTLPR (including rs25531)	M11	0.006 (S_G)	0.259	0.011 (S_G)	0.975
rs12945042	M12	0.279 (A)	0.186	0.279 (A)	0.628

Significant P values (P < 0.05) are indicated in bold.
a. The minor allele in control subjects is indicated in brackets.
b. The minor allele in patients is indicated in brackets.
Abbreviation: MAF, minor allele frequency; HWE, Hardy-Weinberg Equilibrium; N/A, not applicable; rep, repeat.

1.2. *HTR2A* gene

As it is shown in table M6, from the Materials and Methods section, two markers from the serotonin receptor 2A gene, rs6313 (located in exon 1) and rs6311 (located in the promoter region), were selected for this study. These markers were genotyped in the individuals corresponding to the Spanish sample.

According to the information shown in table R2, both SNPs were found to be in HWE in both controls and patients.

Table R2. Frequency and HWE data for *HTR2A* polymorphisms.

Polymorphism	Marker order	MAF (controls)[a]	HWE *P* value (controls)	MAF (patients)[b]	HWE *P* value (patients)
rs6313	M1	0.439 (T)	0.6344	0.435 (T)	1.0
rs6311	M2	0.449 (A)	0.1114	0.463 (A)	0.1428

a. The minor allele in control subjects is indicated in brackets.
b. The minor allele in patients is indicated in brackets.
Abbreviation: MAF, minor allele frequency; HWE, Hardy-Weinberg Equilibrium.

1.3. *TPH2* gene

One marker, SNP rs4570625, located in the 5' regulatory region and with probable functional implications, was genotyped in the Spanish sample and statistically analyzed.

The minor allele frequencies (MAF) of rs4570625 in both control subjects and psychotic patients, as well as the results for the HWE tests can be found in table R3. The polymorphism was in HWE in both subsets.

Table R3. Frequency and HWE data for the *TPH2* polymorphism.

Polymorphism	MAF (controls)[a]	HWE *P* value (controls)	MAF (patients)[b]	HWE *P* value (patients)
rs4570625	0.183 (T)	0.5725	0.153 (T)	1.0

a. The minor allele in control subjects is indicated in brackets.
b. The minor allele in patients is indicated in brackets.
Abbreviation: MAF, minor allele frequency; HWE, Hardy-Weinberg Equilibrium.

1.4. *NOS1* gene

A VNTR located in exon 1f of the *NOS1* gene, (*NOS1* Ex1f-VNTR) was genotyped in all the Spanish subjects and then analyzed in order to find associations with psychosis. Table R4 shows information about the minor allele frequencies (MAF) of the *NOS1* VNTR in control subjects and psychotic patients, as well as the results for the HWE test. This polymorphism was in HWE in both groups of individuals.

Table R4. Frequency and HWE data for the *NOS1* VNTR.

Polymorphism	MAF (controls)[a]	HWE *P* value (controls)	MAF (patients)[b]	HWE *P* value (patients)
***NOS1* Ex1f-VNTR**	0.464 (S)	0.4546	0.472 (S)	0.8143

a. The minor allele in control subjects is indicated in brackets.
b. The minor allele in patients is indicated in brackets.
Abbreviation: MAF, minor allele frequency; HWE, Hardy-Weinberg Equilibrium; S, Short alleles.

1.5. *STMN1* gene

As it is shown in table M9, from the Materials and Methods section, two tags from the *Stathmin* gene, rs12037513 (located downstream the gene) and rs182455 (located in the putative 5' regulatory region), were selected for the study. These two markers were genotyped in the Spanish sample. Table R5 shows information about the minor allele frequencies in both samples of cases and controls, as well as the results from the HWE tests. Both SNPs were found to be in HWE.

Table R5. Frequency and HWE data for *STMN1* polymorphisms.

Polymorphism	Marker order	MAF (controls)[a]	HWE *P* value (controls)	MAF (patients)[b]	HWE *P* value (patients)
rs12037513	M1	0.308 (C)	0.8807	0.266 (C)	0.8472
rs182455	M2	0.447 (T)	0.0751	0.419 (T)	0.5383

a. The minor allele in control subjects is indicated in brackets.
b. The minor allele in patients is indicated in brackets.
Abbreviation: MAF, minor allele frequency; HWE, Hardy-Weinberg Equilibrium.

1.6. *ASPM* gene

The *ASPM* gene, located in chromosome 1q, was genotyped in both the Spanish and the American samples. Specifically, 10 different polymorphisms were genotyped in the Spanish sample, while 6 SNPs were genotyped in the North-American sample. Of those SNPs, five of them were genotyped in both samples: rs3762271, rs12138336 and rs41310927, located in exon 18; rs10922163, located in intron 17; and rs6677082, situated in exon 3.

Minor allele frequencies for all the polymorphisms, as well as the results from the test to verify the existence of HWE can be found at tables R6 (Spanish sample) and R7 (American sample). Regarding the Spanish sample (table R6), two SNPs (rs964201 and rs12025066), located in the coding region, were found to be monomorphic. Moreover, two other SNPs (rs6700180 and rs10922163, located in introns 19 and 17, respectively) were out of HWE in both patients and controls. The deviation from HWE in the control group could be indicating a genotyping error. However, the significance is low, thus we cannot discard other possibilities. In any case, this deviation from HWE should be taken into account in the subsequent association analyses, especially the haplotype analyses. Finally, 8 SNPs were included in the association analysis, although the results from SNPs which deviated from HWE were considered cautiously.

Table R6. Frequency and HWE data for *ASPM* SNPs in the Spanish sample.

Polymorphism	Marker order	MAF (controls)[a]	HWE *P* value (controls)	MAF (patients)[b]	HWE *P* value (patients)
rs6700180	M1	0.457 (T)	**0.0211** (0.168 corrected)	0.495 (T)	**0.0424** (0.297 corrected)
rs3762271	M2	0.429 (A)	0.3633	0.396 (A)	0.6481
rs12138336	M3	0.067 (C)	0.3643	0.086 (C)	1.0
rs41310927	M4	0.414 (G)	0.3342	0.396 (G)	0.6481
rs964201	M5	Monomorphic (C)	N/A	Monomorphic (C)	N/A
rs10922163	M6	0.447 (G)	**0.0238** (0.168 corrected)	0.494 (G)	**0.0308** (0.246 corrected)
rs4915337	M7	0.1 (T)	1.0	0.111 (T)	0.521
rs12025066	M8	Monomorphic (A)	N/A	Monomorphic (A)	N/A
rs6677082	M9	0.111 (C)	0.4388	0.105 (C)	0.3529
rs9726778	M10	0.112 (C)	0.7207	0.122 (C)	0.6327

Significant P values ($P < 0.05$) are indicated in bold.
a. The minor allele in control subjects is indicated in brackets.
b. The minor allele in patients is indicated in brackets.
Abbreviation: MAF, minor allele frequency; HWE, Hardy-Weinberg Equilibrium; N/A, not applicable.

With regard to the American sample (table R7), all the polymorphisms were in HWE, with the only exception of rs12116571, which deviated from HWE in the patients group, but not in the control group. Therefore, all the SNPs were included in the association analysis. Moreover, thirteen families showed Mendelian inconsistencies for some SNPs (data not shown). Therefore, all the unlikely genotypes were discarded for the analysis.

Table R7. Frequency and HWE data for the *ASPM* SNPs in the North-American sample.

Polymorphism	Marker order	MAF (controls)[a]	HWE *P* value (controls)	MAF (patients)[b]	HWE *P* value (patients)
rs3762271	M1	0.407 (A)	1.00	0.400 (A)	0.6735
rs12138336	M2	0.076 (C)	1.00	0.082 (C)	0.3389
rs41310927	M3	0.418 (G)	1.00	0.403 (G)	0.7516
rs10922163	M4	0.473 (G)	0.6992	0.494 (A)	0.1963
rs12116571	M5	0.130 (A)	0.2031	0.107 (A)	**0.0006 (0.0036 corrected)**
rs6677082	M6	0.104 (C)	0.2978	0.080 (C)	0.0942

Significant P values ($P < 0.05$) are indicated in bold.
a. The minor allele in control subjects is indicated in brackets.
b. The minor allele in patients is indicated in brackets.
Abbreviation: MAF, minor allele frequency; HWE, Hardy-Weinberg Equilibrium; N/A, not applicable.

1.7. *PDE4D* gene

This gene was studied in both the Spanish and the German samples with the genotyping of several tags. Particularly, nine markers were genotyped in the Spanish sample, whereas twelve SNPs were genotyped in the German sample (the nine genotyped in the Spanish individuals plus three additional SNPs).

The information about minor allele frequencies and tests of Hardy-Weinberg Equilibrium can be found below. Table R8 shows the results for the Spanish sample, while table R9 contains the information about the German sample.

In both samples, all markers were found to be in HWE in control subjects and patients. Moreover, two of the SNPs genotyped in the German individuals (rs1058458 and rs17853590) were monomorphic in this sample for alleles T and C, respectively.

Table R8. Frequency and HWE data for *PDE4D* polymorphisms in the Spanish sample.

Polymorphism	Marker order	MAF (controls)[a]	HWE *P* value (controls)	MAF (patients)[b]	HWE *P* value (patients)
rs17291089	M1	0.059 (C)	0.4782	0.072 (C)	1.0
rs829259	M2	0.353(A)	0.2576	0.376 (A)	0.9784
rs17719378	M3	0.38 (G)	0.8013	0.37 (G)	0.824
rs10055954	M4	0.149 (C)	0.1702	0.148 (C)	0.4534
rs10461656	M5	0.296 (A)	0.1109	0.3 (A)	0.983
rs7713345	M6	0.131(G)	0.328	0.151 (G)	0.465
rs12656462	M7	0.047 (A)	1.0	0.074 (A)	0.536
rs4700316	M8	0.208 (G)	0.5724	0.18 (G)	0.9445
rs7714708	M9	0.347 (T)	0.6822	0.328 (T)	0.9634

a. The minor allele in control subjects is indicated in brackets.
b. The minor allele in patients is indicated in brackets.
Abbreviation: MAF, minor allele frequency; HWE, Hardy-Weinberg Equilibrium.

Table R9. Frequency and HWE data for *PDE4D* polymorphisms in the German sample.

Polymorphism	Marker order	MAF (controls)[a]	HWE *P* value (controls)	MAF (patients)[b]	HWE *P* value (patients)
rs17291089	M1	0.065	0.3433	0.07	0.9667
rs829259	M2	0.404	0.3934	0.406	0.6833
rs1058458	M3	Monomorphic (T)	N/A	Monomorphic (T)	N/A
rs17719378	M4	0.351	0.423	0.331	1.0
rs10055954	M5	0.171	0.7506	0.169	0.7428
rs10461656	M6	0.337	0.6916	0.329	0.5718
rs7713345	M7	0.166	0.761	0.159	0.1746
rs12656462	M8	0.089	0.8259	0.1	1.0
rs17853590	M9	Monomorphic (C)	N/A	Monomorphic (C)	N/A
rs10056492	M10	0.131	0.8354	0.106	0.2816
rs4700316	M11	0.2	0.6543	0.195	0.4819
rs7714708	M12	0.363	0.3856	0.353	0.0815

a. The minor allele in control subjects is indicated in brackets.
b. The minor allele in patients is indicated in brackets.
Abbreviation: MAF, minor allele frequency; HWE, Hardy-Weinberg Equilibrium; N/A, not applicable.

Finally, nine markers were analyzed in the Spanish sample, while ten SNPs were studied in the German subjects (the same nine SNPs plus an additional one, rs10056492, located in intron 6).

1.8. *PLEKHB1* and *RAB6A* genes

These two genes located in chromosome 11q13.5 were genotyped in both the Spanish and the German samples. Particularly, eight SNPs (three from *PLEKHB1*, four from *RAB6A* and one located in the intergenic region between both genes) were genotyped in the Spanish sample, while fourteen SNPs (seven from *PLEKHB1*, six from *RAB6A* and another one in the intergenic region) were genotyped in the German sample.

Table R10 and R11 show the minor allele frequencies and the results from tests of Hardy-Weinberg Equilibrium for both the Spanish and the German samples. One SNP, rs3182788, clearly violated HWE in the Spanish Sample, as well as in the German controls, suggesting the existence of a failure during the genotyping process. Several SNPs were also out of HWE only in the German affected subset (rs4944850, rs940828, rs11235880 and rs7127066, see table R11). Furthermore, the non-synonymous change rs3203705 was monomorphic in both the Spanish and the German samples for allele A. The SNP rs11538627, genotyped only in the German sample, was also found to be monomorphic for allele A.

Table R10. Frequency and HWE data for *PLEKHB1* and *RAB6A* SNPs in the Spanish sample.

SNP	Marker order	MAF (controls)[a]	HWE *P* value (controls)	MAF (patients)[b]	HWE *P* value (patients)
rs663303	M1	0.092 (T)	1.0	0.137 (T)	1.0
rs940828	M2	0.206 (G)	0.3574	0.216 (G)	0.4121
rs3741147	M3	0.196 (G)	0.3081	0.154 (G)	1.0
rs12274970	M4	0.32 (C)	0.1059	0.304 (C)	0.303
rs3182788	M5	0.474 (T)	**3.13E-21 (0.000000)**	0.465 (T)	**3.94E-11 (0.000000)**
rs3203705	M6	Monomorphic (A)	N/A	Monomorphic (A)	N/A
rs11235876	M7	0.498 (A)	0.3272	0.487 (A)	0.8093
rs7127066	M8	0.224 (C)	1.0	0.241 (C)	0.9297

Significant *P* values (P < 0.05) are indicated in bold and corrected values are indicated in brackets.
a. The minor allele in control subjects is indicated in brackets.
b. The minor allele in patients is indicated in brackets.
Abbreviation: MAF, minor allele frequency; HWE, Hardy-Weinberg Equilibrium; N/A, not applicable.

Table R11. Frequency and HWE data for *PLEKHB1* and *RAB6A* SNPs in the German sample.

SNP	Marker order	MAF (controls)[a]	HWE *P* value (controls)	MAF (patients)[b]	HWE *P* value (patients)
rs663303	M1	0.127 (T)	0.9732	0.119 (T)	0.1478
rs4944850	M2	0.154 (C)	0.62	0.14 (C)	**0.0263** (0.236)
rs11538627	M3	Monomorphic (A)	N/A	Monomorphic (A)	N/A
rs591804	M4	0.325 (G)	0.9475	0.312 (G)	0.8578
rs6592527	M5	0.218 (C)	0.5046	0.248 (C)	0.1129
rs940828	M6	0.212 (G)	0.3934	0.234 (G)	**0.01** (0.12)
rs3741147	M7	0.111 (G)	1.0	0.114 (G)	0.7339
rs12274970	M8	0.235 (C)	0.7606	0.221 (C)	0.4221
rs3182788	M9	0.341 (T)	**8.3E-06 (0.000092)**	0.24 (T)	0.0511
rs10736793	M10	0.171 (A)	0.7925	0.163 (A)	0.3683
rs3203705	M11	Monomorphic (A)	N/A	Monomorphic (A)	N/A
rs11235876	M12	0.42 (A)	0.1951	0.434 (A)	0.7516
rs11235880	M13	0.179 (A)	0.6455	0.194 (A)	**0.0147** (0.147)
rs7127066	M14	0.239 (C)	0.6869	0.255 (C)	**0.0104** (0.12)

Significant *P* values (P < 0.05) are indicated in bold and corrected values are indicated in brackets.
a. The minor allele in control subjects is indicated in brackets.
b. The minor allele in patients is indicated in brackets.
Abbreviation: MAF, minor allele frequency; HWE, Hardy-Weinberg Equilibrium; N/A, not applicable.

The monomorphic SNPs as well as rs3182788 were subsequently excluded from the analysis. Finally, this association analysis included 6 SNPs in the Spanish sample and 11 SNPs in the German sample.

2. Linkage disequilibrium analysis

The LD structure for all samples and genes (with the exception of *TPH2* and *NOS1* genes, because only one polymorphism was studied) can be consulted below.

2.1. *SLC6A4* gene

Table R12 shows the linkage disequilibrium (LD) values observed in the control sample. The r^2 values present a wide range which oscillates between 0.0 and 0.98. A representation of the LD pattern which includes these r^2 values is also shown in figure R1. According to Gabriel *et al.* criteria (Gabriel *et al.*, 2002), two LD blocks were observed. Polymorphism 5-HTTLPR showed the lowest LD values (both r^2 and D') when compared with the rest of the SNPs.
The patients' LD structure is not shown since it does not differ significantly from the controls' LD pattern.

Table R12. Linkage disequilibrium values for *SLC6A4* polymorphisms in the Spanish controls.

	rs3813034	**rs1042173**	**rs140700**	**rs2020942**	**STin2**	**rs2020939**	**rs2020936**	**rs2066713**	**rs4251417**	**5-HTTLPR**	**rs12945042**
rs3813034	-	0.954	0.083	0.308	0.304	0.786	0.167	0.291	0.087	0.082	0.174
rs1042173	0.982	-	0.085	0.323	0.318	0.823	0.169	0.305	0.086	0.08	0.188
rs140700	1.0	1.0	-	0.043	0.043	0.078	0.341	0.043	0.0070	0.0	0.141
rs2020942	0.843	0.858	1.0	-	0.98	0.385	0.122	0.961	0.037	0.118	0.196
STin2	0.839	0.854	1.0	0.993	-	0.384	0.111	0.966	0.037	0.126	0.205
rs2020939	0.938	0.965	1.0	1.0	1.0	-	0.206	0.367	0.096	0.133	0.236
rs2020936	0.832	0.833	1.0	0.962	0.922	0.973	-	0.132	0.021	0.0	0.0050
rs2066713	0.819	0.834	1.0	0.98	0.986	0.973	1.0	-	0.015	0.13	0.201
rs4251417	1.0	1.0	1.0	1.0	1.0	1.0	1.0	0.621	-	0.086	0.018
5-HTTLPR	0.316	0.311	0.026	0.472	0.493	0.427	0.027	0.497	1.0	-	0.128
rs12945042	0.7	0.721	0.776	0.488	0.496	0.845	0.082	0.496	0.754	0.556	-

The values over the diagonal correspond to the r^2 values, while the D' values can be found below the diagonal.

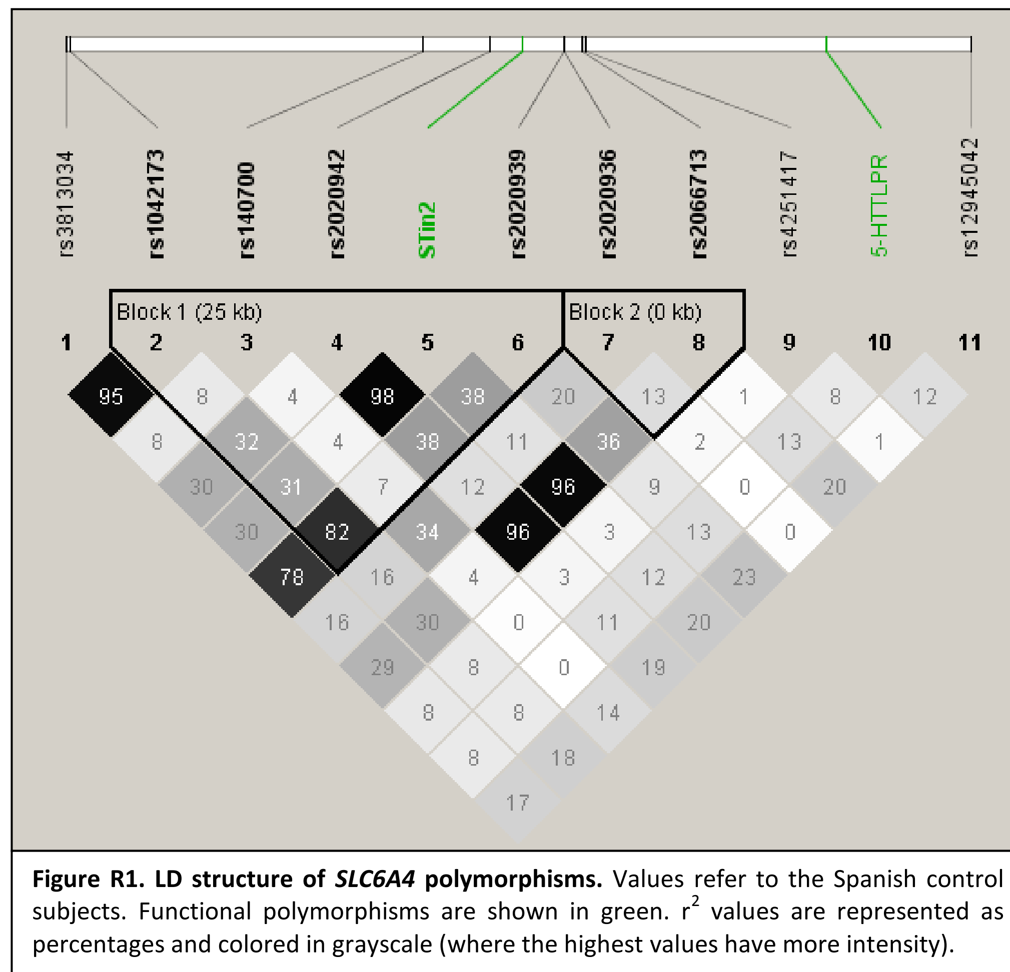


Figure R1. LD structure of *SLC6A4* polymorphisms. Values refer to the Spanish control subjects. Functional polymorphisms are shown in green. r^2 values are represented as percentages and colored in grayscale (where the highest values have more intensity).

2.2. *HTR2A* gene

Table R13 shows the linkage disequilibrium (LD) values observed in the control sample. As it can be seen, both SNPs are in almost complete LD and conform a block according to the criteria from Gabriel *et al.,* 2002 (see figure R2). The sample of schizophrenic patients does not differ from the control one (data not shown).

Table R13. Linkage disequilibrium values for *HTR2A* polymorphisms in the Spanish controls.

	rs6313	rs6311
rs6313	-	0.913
rs6311	0.975	-

The values over the diagonal correspond to the r^2 values, while the D' values can be found below the diagonal.

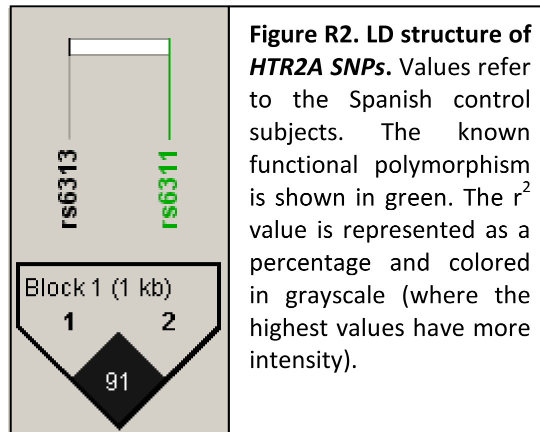


Figure R2. LD structure of *HTR2A SNPs*. Values refer to the Spanish control subjects. The known functional polymorphism is shown in green. The r^2 value is represented as a percentage and colored in grayscale (where the highest values have more intensity).

2.3. *STMN1* gene

The LD values (D’ and r2) for both SNPs from the *STMN1* gene are shown in table R14 and figure R3. Values correspond to the control sample, but the LD pattern observed in patients was extremely similar.

As expected, LD values are low, even for D’ parameter, since these two SNPs were selected as tagSNPs.

Table R14. Linkage disequilibrium values for *STMN1* polymorphisms in the Spanish controls.

	rs12037513	rs182455
rs12037513	-	0.081
rs182455	0.39	-

The value over the diagonal correspond to the r^2 value, while the D' value can be found below the diagonal.

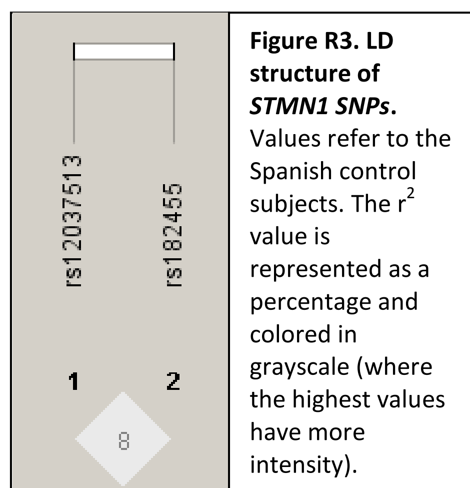


Figure R3. LD structure of *STMN1 SNPs*. Values refer to the Spanish control subjects. The r^2 value is represented as a percentage and colored in grayscale (where the highest values have more intensity).

2.4. *ASPM* gene

The LD structure observed in the Spanish control subjects is shown at table R15 and figure R4. D' values were very high and all the SNPs formed a LD block (figure R10). By contrast, r^2 values were more variable and ranged between 0.008 (between rs12138336 and rs4915337) and 1.0 (between rs6700180 and rs10922163). The highest r^2 values were concentrated around two regions: on the one hand, between rs6700180 (intron 19) and rs10922163 (intron 17); on the other hand, between rs4915337 (exon 14) and rs9726778 (promoter). The LD pattern observed in patients was also very similar (data not shown).

Table R15. Linkage disequilibrium values for the *ASPM* polymorphisms in the Spanish controls.

	rs6700180	rs3762271	rs12138336	rs41310927	rs10922163	rs4915337	rs6677082	rs9726778
rs6700180	-	0.604	0.087	0.577	1.0	0.092	0.104	0.105
rs3762271	0.991	-	0.054	0.896	0.608	0.083	0.096	0.081
rs12138336	1.0	1.0	-	0.051	0.094	0.0080	0.01	0.0090
rs41310927	1.0	0.975	1.0	-	0.581	0.079	0.09	0.076
rs10922163	1.0	0.991	1.0	1.0	-	0.087	0.101	0.099
rs4915337	1.0	1.0	1.0	1.0	1.0	-	0.86	0.827
rs6677082	1.0	1.0	1.0	1.0	1.0	1.0	-	0.922
rs9726778	1.0	0.926	1.0	0.924	1.0	0.967	0.968	-

The values over the diagonal correspond to the r^2 values, while the D' values can be found below the diagonal.

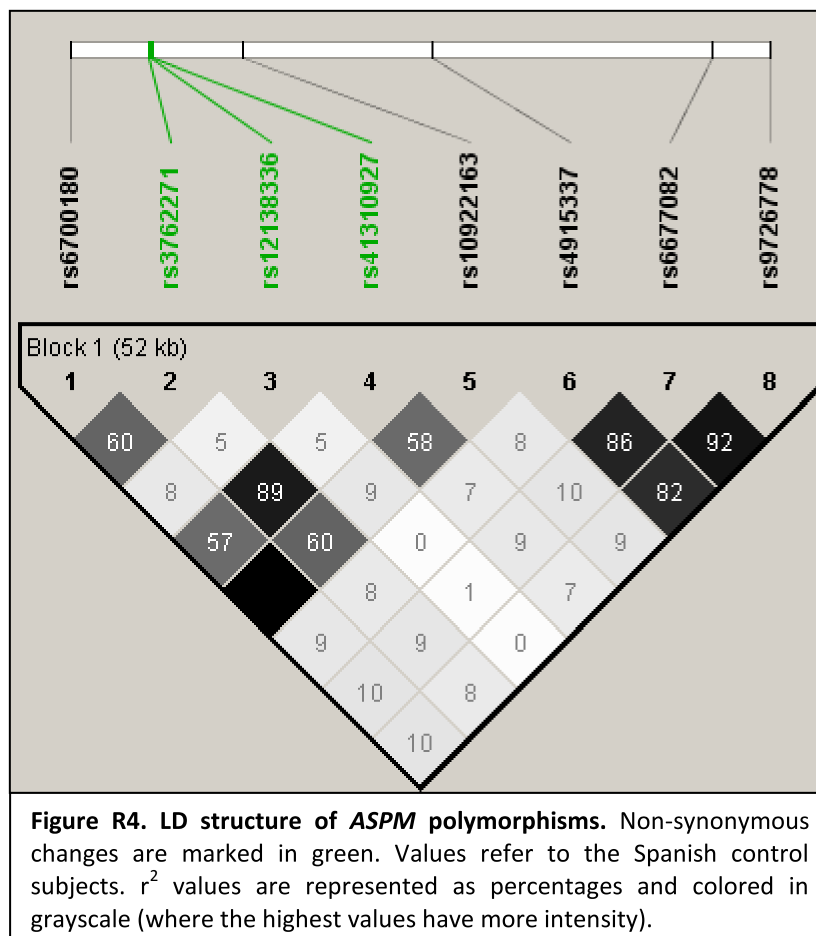


Figure R4. LD structure of *ASPM* polymorphisms. Non-synonymous changes are marked in green. Values refer to the Spanish control subjects. r^2 values are represented as percentages and colored in grayscale (where the highest values have more intensity).

With respect to the LD structure in the North-American control subjects, it was very similar to the structure observed in the Spanish subjects (table R16). All the SNPs conformed a block according to Gabriel *et al.* (2002) criteria (figure R5). The highest r^2 value was found between the polymorphisms rs3762271 and rs41310927 (both very close to each other, in exon 18). By contrast, the lowest r^2 value (0.0090) was found between rs12138336 (exon 18) and rs6677082 (exon 3).

Table R16. Linkage disequilibrium values for *ASPM* polymorphisms in the American controls.

	rs3762271	rs12138336	rs41310927	rs10922163	rs12116571	rs6677082
rs3762271	-	0.057	1.000	0.639	0.106	0.074
rs12138336	1.000	-	0.058	0.092	0.012	0.0090
rs41310927	1.000	1.000	-	0.644	0.109	0.075
rs10922163	1.000	1.000	1.000	-	0.164	0.106
rs12116571	1.000	0.997	1.000	1.000	-	0.017
rs6677082	0.951	0.998	0.954	1.000	1.000	-

The values over the diagonal correspond to the r^2 values, while the D' values can be found below the diagonal.

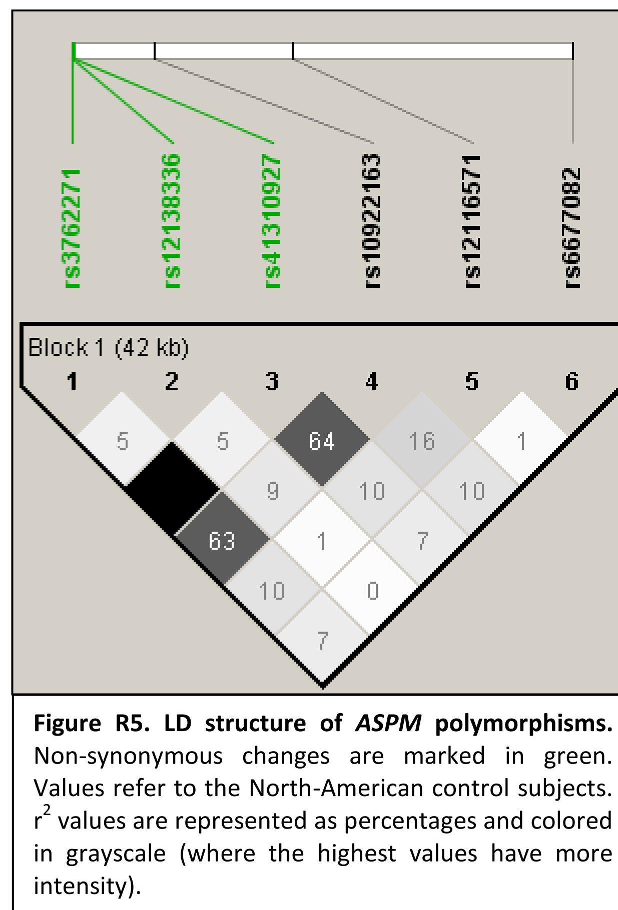


Figure R5. LD structure of *ASPM* polymorphisms. Non-synonymous changes are marked in green. Values refer to the North-American control subjects. r^2 values are represented as percentages and colored in grayscale (where the highest values have more intensity).

2.5. *PDE4D* gene

Table R17 and figure R6 show the information about the LD pattern in the healthy subjects from Spanish sample. Although D’ values are 1.0 for many pairwise values, r^2 values (which are more restrictive than D’ values) are generally lower and oscillate between 0.0020 and 0.746. Moreover, according to Gabriel *et al.* (2002) criteria (which are based in D’ values), two blocks (one of 8 kb and another of 16 kb) were detected. The LD structure in patients is not shown because it is very similar to the structure in the control sample.

Table R17. Linkage disequilibrium values for *PDE4D* polymorphisms in the Spanish controls.

	rs17291089	rs829259	rs17719378	rs10055954	rs10461656	rs7713345	rs12656462	rs4700316	rs7714708
rs17291089	-	0.106	0.037	0.012	0.031	0.013	0.0020	0.015	0.034
rs829259	1.0	-	0.357	0.291	0.746	0.308	0.13	0.028	0.344
rs17719378	1.0	1.0	-	0.104	0.267	0.111	0.047	0.087	0.256
rs10055954	1.0	1.0	1.0	-	0.39	0.036	0.448	0.301	0.095
rs10461656	1.0	1.0	1.0	1.0	-	0.414	0.175	0.056	0.505
rs7713345	1.0	1.0	1.0	1.0	1.0	-	0.016	0.028	0.342
rs12656462	0.688	1.0	1.0	1.0	1.0	1.0	-	0.012	0.0
rs4700316	1.0	0.278	0.816	0.613	0.34	0.789	0.18	-	0.435
rs7714708	1.0	0.641	0.925	0.523	0.752	0.963	0.102	1.0	-

The values over the diagonal correspond to the r^2 values, while the D' values can be found below the diagonal.

Figure R6. LD structure of *PDE4D* polymorphisms. Values refer to the Spanish control subjects. r^2 values are represented as percentages and colored in grayscale (where the highest values have more intensity).

The information about the LD pattern in the German controls is shown in table R18 and figure R7. As it can be seen, LD values are similar to those obtained with the Spanish controls.

Table R18. Linkage disequilibrium values for *PDE4D* polymorphisms in the German controls.

	rs17291089	rs829259	rs17719378	rs10055954	rs10461656	rs7713345	rs12656462	rs10056492	rs4700316	rs7714708
rs17291089	-	0.104	0.038	0.014	0.035	0.014	0.0070	0.0080	0.017	0.04
rs829259	1.0	-	0.368	0.304	0.749	0.292	0.145	0.029	0.011	0.25
rs17719378	1.0	1.0	-	0.112	0.275	0.107	0.053	0.06	0.113	0.281
rs10055954	1.0	1.0	1.0	-	0.407	0.041	0.478	0.169	0.188	0.05
rs10461656	1.0	1.0	1.0	1.0	-	0.391	0.193	0.044	0.033	0.397
rs7713345	1.0	1.0	1.0	1.0	1.0	-	0.02	0.018	0.038	0.334
rs12656462	1.0	1.0	1.0	1.0	1.0	1.0	-	0.013	0.0010	0.01
rs10056492	0.871	0.362	0.84	0.471	0.387	0.757	1.0	-	0.603	0.257
rs4700316	1.0	0.172	0.919	0.476	0.26	0.872	0.038	1.0	-	0.438
rs7714708	1.0	0.547	0.957	0.37	0.666	0.978	0.418	1.0	1.0	-

The values over the diagonal correspond to the r^2 values, while the D' values can be found below the diagonal.

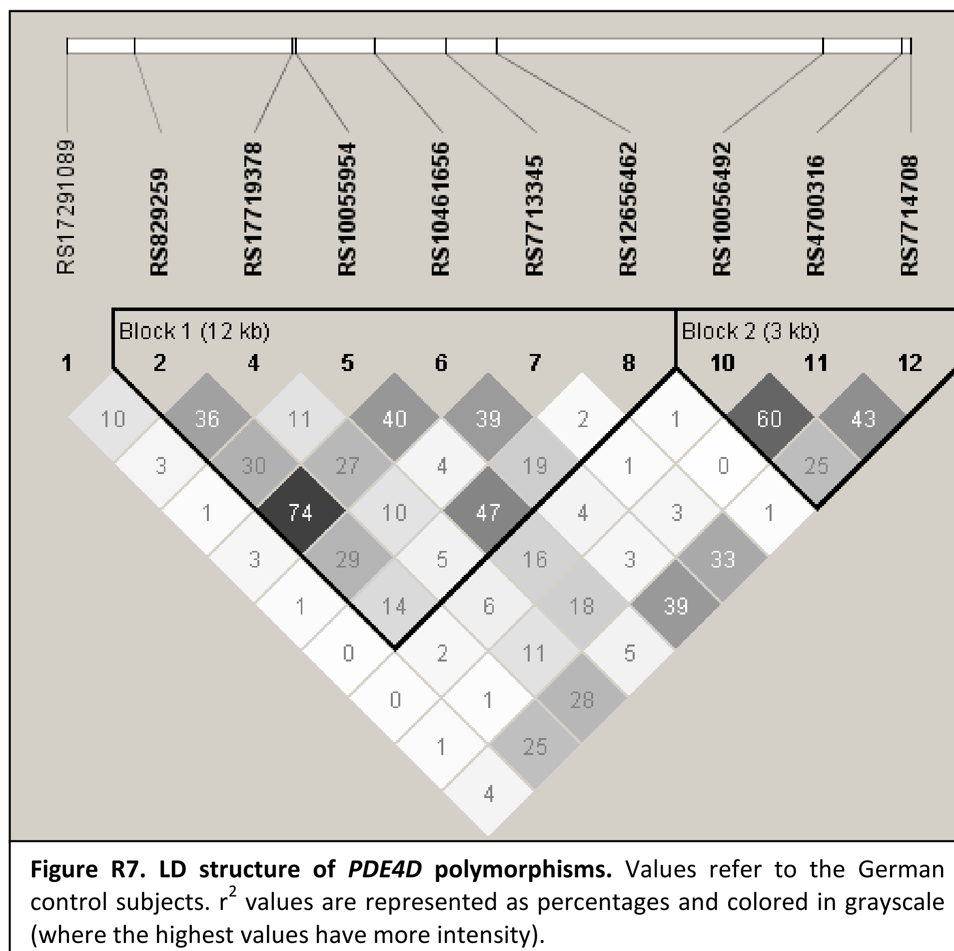


Figure R7. LD structure of *PDE4D* polymorphisms. Values refer to the German control subjects. r^2 values are represented as percentages and colored in grayscale (where the highest values have more intensity).

2.6. *PLEKHB1* and *RAB6A* genes

The LD values and the LD structure in the control Spanish group can be found at table R19 and figure R8. As it can be seen, LD between these markers is low. D' values ranged between 0.241 and 1.0. Moreover, as expected, the r^2 values were lower than D' values and ranged between 0.001 and 0.436. The highest pairwise LD value was found between markers rs663303 and rs940828 (D' = 1.0; r^2 = 0.436), which also constituted a block according to Gabriel *et al.* (2002) criteria (figure R8). The LD structure in patients (data not shown) was found to be highly similar to the pattern observed in the control sample.

Table R19. Linkage disequilibrium values for *PLEKHB1* and *RAB6A* SNPs in the Spanish controls.

	rs663303	rs940828	rs3741147	rs12274970	rs11235876	rs7127066
rs663303	-	0.436	0.025	0.013	0.07	0.023
rs940828	1.0	-	0.0060	0.0010	0.185	0.326
rs3741147	1.0	0.315	-	0.325	0.236	0.013
rs12274970	0.241	0.108	0.788	-	0.471	0.105
rs11235876	0.806	0.832	1.0	1.0	-	0.303
rs7127066	0.249	0.61	0.449	0.407	1.0	-

The values over the diagonal correspond to the r^2 values, while the D' values can be found below the diagonal.

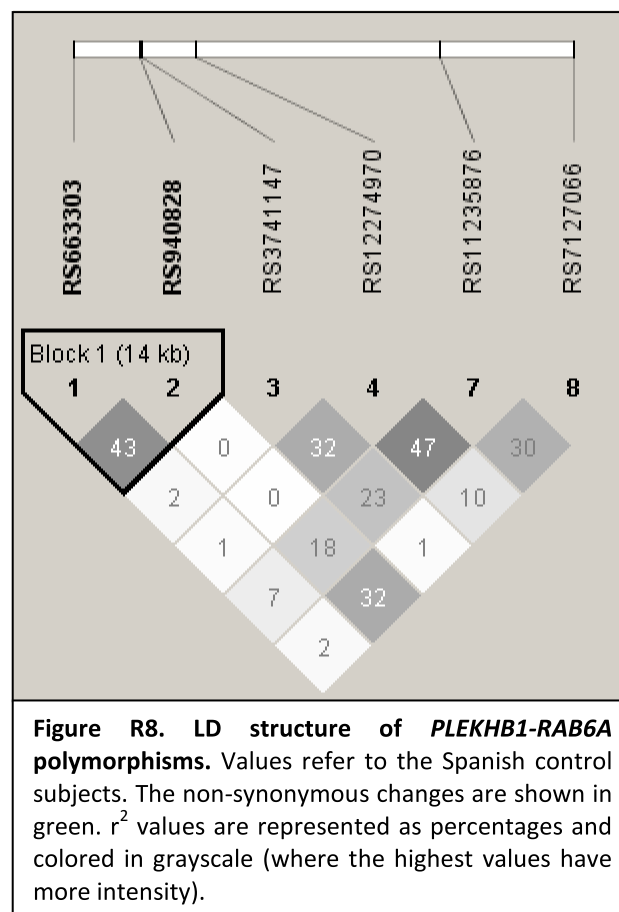


Figure R8. LD structure of *PLEKHB1-RAB6A* polymorphisms. Values refer to the Spanish control subjects. The non-synonymous changes are shown in green. r^2 values are represented as percentages and colored in grayscale (where the highest values have more intensity).

Information about the LD pattern in the German controls is shown in table R20 and figure R9. On the whole, for those LD values which could be compared to the Spanish sample, there were great similarities, with a slight trend to higher values in the German sample. The highest LD value was found between the markers rsrs6592527 and rs940828 (r^2 = 0.918). Moreover, three LD blocks were observed (see figure R9): block 1: from rs663303 (5' upstream of *PLEKHB1)* to rs591804 (intron 4 from *PLEKHB1*); block 2: from rs6592527 to rs940828 (both in intron 5 from *PLEKHB1* gene); and block 3: from rs10736793 (intron 6 of *RAB6A* gene) to rs7127066 (intron 1 of *RAB6A* gene).

Table R20. Linkage disequilibrium values for *PLEKHB1* and *RAB6A* polymorphisms in the German controls.

	rs663303	**rs4944850**	**rs591804**	**rs6592527**	**rs940828**	**rs3741147**	**rs12274970**	**rs10736793**	**rs11235876**	**rs11235880**	**rs7127066**
rs663303	-	0.025	0.259	0.459	0.519	0.018	0.124	0.014	0.178	0.183	0.135
rs4944850	1.0	-	0.385	0.051	0.046	0.462	0.192	0.247	0.045	0.016	0.029
rs591804	0.956	1.0	-	0.035	0.054	0.147	0.506	0.177	0.29	0.164	0.084
rs6592527	0.967	1.0	0.248	-	0.918	0.01	0.0090	0.0010	0.371	0.2	0.41
rs940828	0.979	1.0	0.323	1.0	-	0.0040	0.03	0.0	0.358	0.223	0.465
rs3741147	1.0	0.801	0.734	0.52	0.354	-	0.231	0.384	0.157	0.0060	0.0090
rs12274970	0.512	0.561	0.899	0.101	0.184	0.754	-	0.176	0.424	0.27	0.175
rs10736793	0.145	0.524	0.643	0.115	0.0040	0.778	0.508	-	0.282	0.045	0.063
rs11235876	0.943	0.421	0.664	0.985	0.98	0.956	1.0	1.0	-	0.289	0.433
rs11235880	0.542	0.629	0.601	0.505	0.509	0.446	0.61	1.0	0.982	-	0.704
rs7127066	0.54	0.721	0.364	0.67	0.736	0.473	0.423	1.0	1.0	0.989	-

The values over the diagonal correspond to the r^2 values, while the D' values can be found below the diagonal.

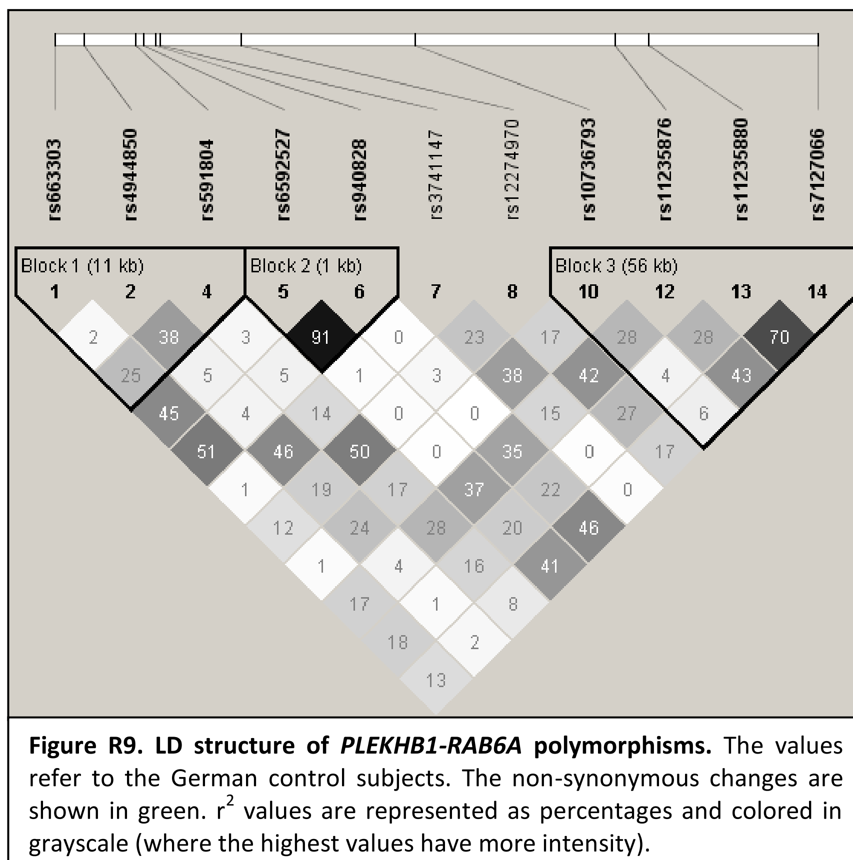


Figure R9. LD structure of *PLEKHB1-RAB6A* polymorphisms. The values refer to the German control subjects. The non-synonymous changes are shown in green. r^2 values are represented as percentages and colored in grayscale (where the highest values have more intensity).

3. Case-control and family-based association analyses

The allelic, genotypic and haplotypic frequencies of both patients and controls were compared in order to detect any association with psychosis or schizophrenia.

With regard to the case-control analyses, several comparisons were made on each sample separately:

<u>Spanish sample</u>

1. **Controls (N = 342) versus all patients (N=298).**
2. **Controls (N = 342) versus hallucinatory patients (N = 220).**
3. **Controls (N = 342) versus schizophrenic patients (N = 218).**
4. Controls (N = 342) versus patients with no AH (N = 77).
5. Hallucinatory patients (N = 220) versus patients with no AH (N = 77).
6. Controls (N = 342) versus schizophrenic patients with AH (N = 183).
7. Controls (N = 342) versus patients with chronic AH (n = 81).
8. Patients with chronic AH (n = 81) versus patients with no AH (N = 77).

<u>German sample</u>

1. **Controls (N = 540) versus all patients (N=336).**
2. **Controls (N = 540) versus schizophrenic patients (N = 247).**
3. Controls (N = 540) versus bipolar patients (N = 89).

<u>American sample</u>

1. **Controls (N = 483) versus the whole sample of patients (all of them schizophrenics or with schizoaffective disorder) (N = 498).**

However, for the case-control haplotype association analysis, only the comparisons marked in bold were made, to ensure that sample sizes were large enough to make a reliable estimation of haplotype frequencies.

Moreover, for those genes with genotypic information for more than one sample, an additional case-control analysis of the genotypic frequencies with a pooled sample (result of the combination of individuals from different country of origin) was also performed.

Finally, to simplify the information given in this section, only the complete results for the first two comparisons will be shown. Only in case there is a significant result for any of the other comparisons, then it will be indicated in the text or in a summary table.

3.1. Estimation of the statistical power

Prior to the association analysis, the statistical power to detect association was calculated with Quanto software. These estimations are summarized in table R21. Particularly, this power ranged between 0.08 and 0.99 depending on several factors, such as the sample size, the risk associated to each allele and the frequency of this allele. Thus, for those polymorphisms with a

low MAF, the statistical power to detect association with the disease is expected to be lower, especially if the expected risk is low.

Table R21. Estimation of the statistical power associated to the different case-control and family-based studies.

Type of study	Sample	N	Frequency of the risk allele[a]	Statistical Power	
				OR = 1.3	OR = 2
Case-control	Spanish	298 patients 348 controls	0.01	0.076	0.2834
			0.03	0.1334	0.6839
			0.05	0.1891	0.8743
			0.1	0.3366	0.9919
			0.2	0.4938	0.9997
			0.3	0.5943	0.9998
			0.4	0.6405	0.9999
			0.5	0.6480	0.9999
Case-control	German	330 patients 534 controls	0.01	0.0872	0.3831
			0.03	0.1614	0.8053
			0.05	0.2328	0.9460
			0.1	0.3889	0.9977
			0.2	0.5959	0.9999
			0.3	0.7008	0.9999
			0.4	0.7455	0.9999
			0.5	0.7509	0.9999
Case-control	American	498 patients 483 controls	0.01	0.09	0.4355
			0.03	0.1832	0.8626
			0.05	0.2681	0.9715
			0.1	0.4489	0.9994
			0.2	0.6729	0.9999
			0.3	0.7768	0.9999
			0.4	0.8189	0.9999
			0.5	0.8245	0.9999
Family-based	American	400 families	0.01	0.086	0.3718
			0.03	0.1584	0.7914
			0.05	0.2280	0.9390
			0.1	0.3808	0.9971
			0.2	0.5859	0.9999
			0.3	0.6912	0.9999
			0.4	0.7369	0.9999
			0.5	0.7429	0.9999

a. The risk allele was considered to be the minor allele.
Abbreviation: OR, Odds Ratio.

3.2. *SLC6A4* gene

Prior to the statistical analyses, genotypes of 5-HTTLPR promoter polymorphism were grouped according to the level of activity detected in previous reports for each of the four alleles (Kraft *et al.,* 2005; Hu *et al.,* 2006). Therefore, three groups of genotypes were considered: "high expression genotypes" (L_A/L_A), "medium expression genotypes" (L_A/L_G, L_A/S_A and L_A/S_G) and "low expression genotypes" (L_G/L_G, L_G/S_A, L_G/S_G, S_A/S_G, S_A/S_A and S_G/S_G). With this strategy, genotypic groups with very low frequency are avoided and the statistical power is increased. However, for the haplotype and allele analysis, the four alleles of 5-HTTLPR were considered separately.

All the results from the case-control analysis of this gene can be found below.

3.2.1. Allelic frequencies.

The results from the comparisons of the allelic frequencies between a) controls and all patients and b) controls and hallucinatory patients are shown in tables R22 and R23, respectively. As it can be seen, SNPs rs140700 and rs2020936 were found to be associated with psychosis when all patients were considered (table R22). Particularly, allele C from rs2020936 and allele A from rs140700 were significantly less frequent in the psychotic group. However, only the association with rs2020936 remained significant when the subset of hallucinatory patients was compared with the controls (table R23). In any case, the associations were in general weak and after multiple-testing correction the *P* values did not remain significant.

Table R22. Comparison of the allelic frequencies of *SLC6A4* polymorphisms in psychotic patients and healthy controls from the Spanish sample.

Polymorphism	Allele	Patients frequency	Controls frequency	OR (95% CI)	χ^2	P[a]
rs3813034	G	0.4715	0.4693	1	0.006	0.938 (1)
	T	0.5285	0.5307	0.991 (0.796-1.235)		
rs1042173	G	0.4815	0.4721	1	0.113	0.737 (1)
	T	0.5185	0.5279	0.963 (0.773-1.2)		
rs140700	A	0.04833	0.08254	1	5.45	**0.0196** (0.2152)
	G	0.9517	0.9175	1.772(1.09-2.879)		
rs2020942	A	0.3658	0.3284	1	1.959	0.162 (1)
	G	0.6342	0.6716	0.848 (0.673-1.068)		
STin2	12 rep	0.6429	0.6776	1	1.668	0.197 (1)
	10 rep	0.3571	0.3224	1.168 (0.923-1.478)		
rs2020939	C	0.5406	0.558	1	0.359	0.549 (1)
	T	0.4594	0.442	1.073 (0.852-1.35)		
rs2020936	C	0.1644	0.2141	1	5.075	**0.0243** (0.2427)
	T	0.8356	0.7859	1.384 (1.042-1.838)		
rs2066713	C	0.646	0.6711	1	0.892	0.345 (1)
	T	0.354	0.3289	1.118 (0.887-1.409)		
rs4251417	A	0.06506	0.07166	1	0.198	0.657 (1)
	G	0.9349	0.9283	1.109 (0.702-1.753)		

Table R22 (continuation). Comparison of the allelic frequencies of *SLC6A4* polymorphisms in psychotic patients and healthy controls from the Spanish sample.

Polymorphism	Allele	Patients frequency	Controls frequency	OR (95% CI)	χ^2	P[a]
5-HTTLPR (including rs25531)	L_A	0.4505	0.4797	1	1.104	0.576 (1)
	L_G	0.04785	0.05072	1.005 (0.599 - 1.686)		
	S_A	0.4901	0.4638	1.125 (0.899 - 1.409)		
	S_G	0.01155	0.005797	2.122 (0.615 - 7.324)		
rs12945042	G	0.7286	0.719	1	0.133	0.715 (1)
	A	0.2714	0.281	0.9532 (0.737-1.233)		

Significant *P* values (P < 0.05) are indicated in bold.
a. The corrected *P* value is indicated in brackets.
Abbreviations: OR, Odds Ratio; CI, confidence intervals; rep, repeats.

Table R23. Comparison of the allelic frequencies of *SLC6A4* polymorphisms in hallucinatory psychotic patients and healthy controls from the Spanish sample.

Polymorphism	Allele	Hallucinatory patients frequency	Controls frequency	OR (95% CI)	χ^2	P[a]
rs3813034	G	0.4614	0.4693	1	0.0677	0.795 (1)
	T	0.5386	0.5307	1.032 (0.812 - 1.313)		
rs1042173	G	0.4727	0.4721	1	0.00037	0.985 (1)
	T	0.5273	0.5279	0.998 (0.785 - 1.268)		
rs140700	A	0.05122	0.08254	1	3.733	0.0534 (0.5335)
	G	0.9488	0.9175	1.667 (0.988 - 2.811)		
rs2020942	A	0.3795	0.3284	1	3.076	0.0795 (0.5171)
	G	0.6205	0.6716	0.799 (0.623 - 1.027)		
STin2	12 rep	0.6297	0.6776	1	2.651	0.1035 (0.828)
	10 rep	0.3703	0.3224	1.236 (0.958 - 1.595)		
rs2020939	C	0.5463	0.558	1	0.137	0.711 (1)
	T	0.4537	0.442	1.048 (0.817 - 1.345)		
rs2020936	C	0.1659	0.2141	1	3.95	**0.0469** (0.5156)
	T	0.8341	0.7859	1.369 (1.004 - 1.868)		
rs2066713	C	0.6364	0.6711	1	1.431	0.232 (1)
	T	0.3636	0.3289	1.166 (0.907 - 1.499)		
rs4251417	A	0.05122	0.07166	1	1.74	0.187 (1)
	G	0.9488	0.9283	1.43 (0.839 - 2.438)		
5-HTTLPR (including rs25531)	L_A	0.4467	0.4797	1	2.156	0.340 (1)
	L_G	0.04	0.05072	0.847 (0.467 - 1.535)		
	S_A	0.5022	0.4638	1.163 (0.911 - 1.485)		
	S_G	0.01111	0.005797	2.059 (0.546 - 7.756)		
rs12945042	G	0.7293	0.719	1	0.129	0.719 (1)
	A	0.2707	0.281	0.950 (0.719 - 1.255)		

Significant *P* values (P < 0.05) are indicated in bold.
a. The corrected *P* value is indicated in brackets.
Abbreviations: OR, Odds Ratio; CI, confidence intervals; rep, repeats.

With regard to the remaining case-control comparisons, there were some significant associations (table R24). However, neither of the significant values resisted the sequential Bonferroni correction. Moreover, the validity of the results presented in table R24 is limited because of the small number of patients without AH (N = 77) and patients with chronic AH (N = 81).

Table R24. Summary of the significant associations found for the remaining case-control comparisons.

Comparison	Polymorphism	*P* Value[a]
Patients with AH vs patients without AH	rs4251417	**0.0245** (0.269)
Patients with cronic AH vs controls	rs2020942	**0.0060** (0.066)
Patients with cronic AH vs controls	STin2	**0.0072** (0.072)
Patients with cronic AH vs controls	rs2020936	**0.0332** (0.299)
Patients with cronic AH vs controls	rs2066713	**0.0437** (0.349)
Patients with cronic AH vs patients without AH	rs2020942	**0.0285** (0.314)
Patients with cronic AH vs patients without AH	STin2	**0.0314** (0.314)

Significant *P* values (P < 0.05) are indicated in bold.
a. The corrected *P* value is indicated in brackets.
Abbreviations: AH, auditory hallucinations; vs, versus.

3.2.2. Genotypic frequencies.

Tables R25 and R26 show the results from the case-control association analyses. In coherence with the comparisons of the allelic frequencies (section 3.2.1), polymorphisms rs140700 and rs2020936 appeared to be associated with psychosis when all patients were considered (table R25: P = 0.017 and P = 0.0045, respectively) but also when the subsample of patients with AH was compared to the genotypic distribution in healthy subjects (table R26: P = 0.047 for rs140700 and P = 0.014 for rs2020936). Regarding rs140700, genotypes with the A allele were less frequent in the patients group, while, with regard to rs2020936, T/C heterozygotes were less frequent than homozygotes T/T in the affected group. Interestingly, after correction for multiple testing, the differences in the genotypic distribution of rs2020936 between all patients and controls still remained significant (p = 0.0495, see table R25).

Table R25. Comparison of the genotypic distributions of *SLC6A4* polymorphisms in psychotic patients and healthy controls from the Spanish sample.

Polymorphism	Genotype	Controls frequency	Patients frequency	OR (95% CI)	*P* value[b]	AIC	Model
rs3813034	T/T-G/T	270 (79%)	230 (77.2%)	1.00	0.59 (1)	887.9	Recessive
	G/G	72 (21.1%)	68 (22.8%)	1.11 (0.76-1.61)			
rs1042173	T/T-T/G	268 (78.6%)	228 (76.5%)	1.00	0.53 (1)	886.5	Recessive
	G/G	73 (21.4%)	70 (23.5%)	1.13 (0.78-1.64)			
rs140700	G/G	265 (84.1%)	243 (90.3%)	0.56(0.34-0.91)[a]	**0.017** (0.17)	804.3	Additive
	A/G	48 (15.2%)	26 (9.7%)				
	A/A	2 (0.6%)	0 (0%)				
rs2020942	G/G	156 (45.8%)	119 (39.9%)	1.00	0.14 (1)	884.7	Dominant
	A/G-A/A	185 (54.2%)	179 (60.1%)	1.27 (0.93-1.74)			

Table R25 (continuation). Comparison of the genotypic distributions of *SLC6A4* polymorphisms in psychotic patients and healthy controls from the Spanish sample.

Polymorphism	Genotype	Controls frequency	Patients frequency	OR (95% CI)	*P* value [b]	AIC	Model
STin2	12/12	156 (46.6%)	118 (41.1%)	1.00	0.17 (1)	860.7	Dominant
	10/12-10/10	179 (53.4%)	169 (58.9%)	1.25 (0.91-1.72)			
rs2020939	C/C-C/T	259 (81.2%)	214 (79%)	1.00	0.5 (1)	817.5	Recessive
	T/T	60 (18.8%)	57 (21%)	1.15(0.77-1.72)			
rs2020936	T/T	204 (59.8%)	211 (70.8%)	1.00	**0.0045 (0.0495)**	878.1	Codominant
	T/C	128 (37.5%)	76 (25.5%)	0.57 (0.41-0.81)			
	C/C	9 (2.6%)	11 (3.7%)	1.18 (0.48-2.91)			
rs2066713	C/C	156 (45.6%)	121 (40.6%)	1.00	0.2 (1)	886.6	Dominant
	C/T-T/T	186 (54.4%)	177 (59.4%)	1.23 (0.90-1.68)			
rs4251417	G/G	269 (85.7%)	234 (87%)	1.00	0.64 (1)	808.5	N/A
	A/G	45 (14.3%)	35 (13%)	0.89 (0.56-1.44)			
5-HTTLPR (including rs25531)	low/low	257 (75.4%)	239 (80.2%)	1.00	0.14 (1)	884.8	Recessive
	high/low-high/high	84 (24.6%)	59 (19.8%)	0.76 (0.52-1.10)			
rs12945042	G/G-G/A	285 (90.5%)	253 (94%)	1.00	0.11 (0.99)	807.4	Recessive
	A/A	30 (9.5%)	16 (6%)	0.60 (0.32-1.13)			

Significant *P* values (P < 0.05) are indicated in bold.
a. The indicated value corresponds to the difference which can be attributed to each allele in the additive model.
b. The corrected *P* value is indicated in brackets
Abbreviations: OR, Odds Ratio; CI, confidence intervals; N/A, not applicable.

Table R26. Comparison of the genotypic distributions of *SLC6A4* polymorphisms in hallucinatory patients and healthy controls from the Spanish sample.

Polymorphism	Genotype	Controls frequency	Hallucinatory patients frequency	OR (95% CI)	*P* Value[b]	AIC	Model
rs3813034	T/T	93 (27.2 %)	67 (30.4%)	1.00	0.4 (1)	755.7	Dominant
	G/T-G/G	249 (72.8%)	153 (69.5%)	0.85 (0.59-1.24)			
rs1042173	T/T-T/G	268 (78.6%)	168 (76.4%)	1.00	0.54 (1)	755	Recessive
	G/G	73 (21.4%)	52 (23.6%)	1.14 (0.76-1.70)			
rs140700	G/G	265 (84.1%)	184 (89.8%)	0.59 (0.35-0.04)[a]	**0.047** (0.47)	697.5	Additive
	A/G-A/A	48 (15.2%)	21 (10.2%)				
	A/A	2 (0.6%)	0 (0%)				
rs2020942	G/G	156 (45.8%)	86 (39.1%)	1.24 (0.27-1.59)[a]	0.084 (0.756)	752.4	Additive
	A/G	146 (42.8%)	101 (45.9%)				
	A/A	39 (11.4%)	33 (15%)				
STin2	12rep/12rep	156 (46.6%)	85 (40.1)	1.23 (0.96-1.58)[a]	0.11 (0.88)	731.8	Additive
	10rep/12rep	142 (42.4%)	97 (45.8%)				
	10rep/10rep	37 (11%)	30 (14.2%)				
rs2020939	C/C-C/T	259 (81.2%)	160 (78%)	1.00	0.38 (1)	704.7	Recessive
	T/T	60 (18.8%)	45 (21.9%)	1.21(0.79-1.87)			
rs2020936	T/T	204 (59.8%)	154 (70%)	1.00	**0.014** (0.154)	749.3	Dominant
	T/C-C/C	137 (40.2%)	66 (30%)	0.64 (0.44-0.92)			
rs2066713	C/C	156 (45.6%)	88 (40%)	1.00	0.19 (1)	754.7	Dominant
	C/T-T/T	186 (54.4%)	132 (60%)	1.26 (0.89-1.77)			

Table R26 (continuation). Comparison of the genotypic distributions of *SLC6A4* polymorphisms in hallucinatory patients and healthy controls from the Spanish sample.

Polymorphism	Genotype	Controls frequency	Hallucinatory patients frequency	OR (95% CI)	*P* Value[b]	AIC	Model
rs4251417	G/G	269 (85.7%)	184 (89.8%)	1.00	0.17 (1)	698.5	N/A
	A/G	45 (14.3%)	21 (10.2%)	0.68 (0.39-1.18)			
5-HTTLPR (including rs25531)*	low/low	257 (75.4%)	176 (80%)	1.00	0.2 (1)	753.8	Recessive
	high/low-high/high	84 (24.6%)	44(20%)	0.76 (0.51-1.15)			
rs12945042	G/G-G/A	285 (90.5%)	190 (92.7%)	1.00	0.38 (1)	700.6	Recessive
	A/A	30 (9.5%)	15 (7.3%)	0.75 (0.39-1.43)			

Significant *P* values (P < 0.05) are indicated in bold.
a. The indicated value corresponds to the difference which can be attributed to each allele in the additive model.
b. The corrected *P* value is indicated in brackets.
Abbreviations: OR, Odds Ratio; CI, confidence intervals; N/A, not applicable.

Regarding the remaining case-control comparisons, there were some significant associations which are shown in table R27. However, neither of the significant values resisted the sequential Bonferroni correction. Furthermore, like the comparisons of the allelic frequencies, due to the small number of patients without AH (N = 77) and patients with chronic AH (N = 81), the reliability of some of the results shown in table R27 should be considered to be very low.

Table R27. Summary of the significant associations found for the remaining case-control comparisons.

Comparison	Polymorphism	*P* Value[a]	Model
Patients without AH vs controls	rs2020936	**0.022** (0.22)	Codominant
Patients without AH vs controls	rs12945042	**0.011** (0.121)	Codominant
Patients without AH vs patients with AH	rs4251417	**0.019** (0.209)	N/A
Schizophrenic patients vs controls	rs2020936	**0.022** (0.242)	Codominant
Schizophrenics with AH vs controls	rs2020936	**0.021** (0.231)	Additive
Schizophrenics with AH vs controls	rs140700	**0.049** (0.49)	Dominant
Patients with cronic AH vs controls	rs2020942	**0.0071** (0.07881)	Additive
Patients with cronic AH vs controls	STin2	**0.0084** (0.084)	Additive
Patients with cronic AH vs controls	rs2020936	**0.015** (0.135)	Dominant
Patients with cronic AH vs controls	rs2066713	**0.046** (0.368)	Additive
Patients with cronic AH vs patients without AH	rs3813034	**0.039** (0.312)	Dominant
Patients with cronic AH vs patients without AH	rs2020942	0.06 (0.176)	Recessive
Patients with cronic AH vs patients without AH	Stin2	**0.029** (0.261)	Recessive
Patients with cronic AH vs patients without AH	rs2020939	**0.018** (0.18)	Codominant
Patients with cronic AH vs patients without AH	rs4251417	**0.048** (0.336)	N/A

Significant *P* values (P < 0.05) are indicated in bold.
a. The corrected *P* value is indicated in brackets.
Abbreviations: AH, auditory hallucinations; vs, versus; N/A, not applicable.

3.2.3. Haplotype analysis.

The most common 11-marker haplotypes detected in our sample can be consulted at table R28.

Table R28. Overview of the most common *SLC6A4* haplotypes observed in the Spanish sample.

Haplotype[a]	Patients frequency	Controls frequency
G-G-G-G-12rep-T-T-C-G-S_A-G	0.323	0.3047
T-T-G-A-10rep-C-T-T-G-L_A-A	0.1372	0.1447
T-T-G-A-10rep-C-T-T-G-S_A-G	0.0659	0.07106
G-G-G-G-12rep-T-T-C-A-L_A-G	0.05848	0.06504
T-T-G-G-12rep-C-C-C-G-L_A-G	0.04269	0.05642
T-T-G-A-10rep-C-T-T-G-L_A-G	0.0687	0.0543
T-T-G-G-12rep-C-C-C-G-S_A-G	0.06713	0.04565
G-G-G-G-12rep-T-T-C-G-L_A-G	0.02738	0.04309
T-T-A-G-12rep-C-C-C-G-L_A-A	0.0134	0.04147
T-T-A-G-12rep-C-C-C-G-S_A-A	0.01771	0.03379
All rare (freq < 0.03)	0.17841	0.13978

Note: global *P* value (uncorrected) was 0.084 (ns). The patients' data refer to the whole sample of psychotic patients.
a. The order of the polymorphisms corresponds to the order in the previous tables (R22 to R27).
Abbreviations: freq, frequency.

However, to reduce the number of polymorphisms and therefore to increase the power of our results, three SNPs were discarded from the haplotype association analysis because they were in very high LD (r^2 > 0.94) with other polymorphisms (see table R12). The eliminated polymorphisms were rs1042173 (located in the 3'UTR region), STin2 (intron 2) and rs2066713 (intron 1A). Thus, the haplotype analysis was finally performed with 8 polymorphisms and sliding windows of two, three and four-marker haplotypes, as it was explained in the Materials and Methods section. The haplotypes which were significantly associated with the disease can be found at tables R29 (all patients compared to controls) and R30 (hallucinatory patients compared to controls).

Table R29. *SLC6A4* haplotypes significantly associated with psychosis in the Spanish sample.

HAPLOTYPE[a]															
rs3813034	rs1042173	**rs1140700**	**rs2020942**	STin2	**rs2020939**	**rs2020936**	rs2066713	**rs4251417**	**5-HTTLPR**	**rs12945042**	**Patients frequency**	**Controls frequency**	**df**	**Global *P* value**	**Haplotype *P* value[b]**
T		A									0.0466	0.0825	2	**0.0361**	**0.0139** (0.06194)
		A	G								0.0466	0.0825	2	**0.0263**	**0.0139** (0.06194)
T		A	G								0.0437	0.0825	4	**0.0458**	**0.0139** (0.09191)
T		A	G		C						0.0407	0.0861	3	**0.0211**	**0.01058** (> 0.06)
		A	G		C	C					0.0463	0.0861	3	**0.0480**	**0.00869** (0.06693)

Significant *P* values (P < 0.05) are indicated in bold.
a. The polymorphisms which were discarded from the analysis are shown in grey.
b. The corrected *P* value after 1000 permutations is indicated in brackets.
Abbreviation: df, degrees of freedom.

Table R30. *SLC6A4* haplotypes significantly associated with hallucinatory psychosis in the Spanish sample.

HAPLOTYPE[a]											Hallucinatory patients frequency	Controls frequency	df	Global *P* value	Haplotype *P* value[b]
rs3813034	rs1042173	rs140700	rs2020942	STin2	rs2020939	rs2020936	rs2066713	rs4251417	5-HTTLPR	rs12945042					
		A	G								0.0490	0.0825	2	**0.0387**	**0.0379** (0.1029)

Significant *P* values (P < 0.05) are indicated in bold.
a. The polymorphisms which were discarded from the analysis are shown in grey.
b. The corrected *P* value after 1000 permutations is indicated in brackets.
Abbreviation: df, degrees of freedom.

According to the information provided by tables R29 and R30, several protective haplotypes were found (five haplotypes when all patients were considered and one haplotype for the comparison between hallucinatory patients and controls). However, after a 1000-permutation run, all results became not significant, although a trend can be still observed in many cases.
With regard to the comparison between the haplotype frequencies of controls and the subset of schizophrenic patients (according to DSM-IV criteria), a protective haplotype (rs3813034 T/rs140700 A/rs2020942 G/rs2020939 C) was found (global p = 0.02873; haplotype p = 0.2424, not significant after 1000 permutations). This haplotype had been also detected when all patients were considered (see table R29).

It should be noted that all those protective haplotypes, which were more frequent in controls, were variable combinations of the same SNPs and alleles. Interestingly, allele A from SNP rs140700 (previously found to be associated with psychosis in the analysis of allelic and genotypic frequencies) was present in all haplotypes.

3.3. *HTR2A* gene

3.3.1. Allelic frequencies.

Tables R31 and R32 contain the results from the case-control analysis of the allelic frequencies between all patients and controls (table R31) and the patients with AH and controls (table R32). In this case, the results were negative for both comparisons, as well as for the remaining comparisons (data not shown).

Table R31. Comparison of the allelic frequencies of *HTR2A* SNPs in psychotic patients and healthy controls from the Spanish sample.

Polymorphism	Allele	Patients frequency	Controls frequency	OR (95% CI)	χ^2	P[a]
rs6313	C	0.5654	0.5614	1	0.0211	0.8846 (1)
	T	0.4346	0.4386	0.9837 (0.7883 - 1.228)		
rs6311	A	0.4631	0.4488	1	0.261	0.6094 (1)
	G	0.5369	0.5512	0.9441 (0.7573 - 1.177)		

a. The corrected *P* value is indicated in brackets.
Abbreviations: OR, Odds Ratio; CI, confidence intervals.

Table R32. Comparison of the allelic frequencies of *HTR2A* SNPs in hallucinatory psychotic patients and healthy controls from the Spanish sample.

Polymorphism	Allele	Hallucinatory patients frequency	Controls frequency	OR (95% CI)	χ^2	P[a]
rs6313	C	0.5568	0.5614	1	0.0228	0.8799 (0.88)
	T	0.4432	0.4386	1.019 (0.8004 - 1.297)		
rs6311	A	0.4773	0.4488	1	0.872	0.3504 (0.7)
	G	0.5227	0.5512	0.8919 (0.7014 - 1.134)		

a. The corrected *P* value is indicated in brackets.
Abbreviations: OR, Odds Ratio; CI, confidence intervals.

3.3.2. Genotypic frequencies.

The results were negative for the 8 case-control comparisons. The complete results for the two main comparisons (all patients vs. controls and hallucinatory patients vs. controls) can be found at tables R33 and R34, respectively.

Table R33. Comparison of the genotypic distributions of the two *HTR2A* polymorphisms in psychotic patients and healthy controls from the Spanish sample.

Polymorphism	Genotype	Controls frequency	Patients frequency	OR (95% CI)	*P* Value[b]	AIC	Model
rs6313	C/C	105 (30.7%)	95 (31.9%)	1.00	0.75 (1)	888.1	Dominant
	T/C - T/T	237 (69.3%)	203 (68.1%)	0.95 (0.68-1.32)			
rs6311	G/G	96 (28.1%)	79 (26.5%)	1.07 (0.85-1.34)[a]	0.59 (1)	887.9	Additive
	A/G	185 (54.1%)	162 (54.4%)				
	A/A	61 (17.8%)	57 (19.1%)				

a. The indicated value corresponds to the difference which can be attributed to each allele in the additive model.
b. The corrected *P* value is indicated in brackets.
Abbreviations: OR, Odds Ratio; CI, confidence intervals.

Table R34. Comparison of the genotypic distributions of the two *HTR2A* polymorphisms in psychotic patients with AH and healthy controls from the Spanish sample.

Polymorphism	Genotype	Controls frequency	Hallucinatory patients frequency	OR (95% CI)	*P* Value[b]	AIC	Model
rs6313	C/C - T/C	279 (81.6%)	175 (79.5%)	1.00	0.55 (0.66)	756	Recessive
	T/T	63 (18.4%)	45 (20.4%)	1.14 (0.74 - 1.74)			
rs6311	G/G	96 (28.1%)	55 (25%)	1.13 (0.88-1.46)[a]	0.33 (0.66)	755.4	Additive
	A/G	185 (54.1%)	120 (54.5%)				
	A/A	61 (17.8%)	45 (20.4%)				

a. The indicated value corresponds to the difference which can be attributed to each allele in the additive model.
b. The corrected *P* value is indicated in brackets
Abbreviations: OR, Odds Ratio; CI, confidence intervals.

3.3.3. Haplotype analysis.

Table R35 and R36 show the haplotype frequencies estimated by the Unphased program in control subjects, all patients and the subset of patients with AH. In all samples, there are three

common haplotypes as well as a rare haplotype (frequency < 0.03). However, no differences in the haplotype frequencies between patients and controls (table R35) or between hallucinatory patients and controls (table R36) were found (global *P* values < 0.05). With regard to the comparison between schizophrenic patients and controls, the results are also negative (data not shown). However, in the three case-control comparisons, there is a risk haplotype (C-A), which shows a slight trend toward association, with a higher frequency in the patients group.

Table R35. Results from the *HTR2A* haplotype association analysis between all patients and healthy subjects.

Haplotype[a]	Patients frequency	Controls frequency	OR (95% CI)	χ^2	P
C-A	0.03026	0.01618	1	2.867	0.09043
T-A	0.4328	0.4326	0.5351 (0.2481 – 1.154)	2.564E-005	0.996
C-G	0.5352	0.5452	0.5251 (0.2443 – 1.128)	0.1304	0.718
T-G	0.001733 (rare)	0.005951 (rare)	0.1558 (0.01545 - 1.57)	N/A	N/A

Note: the global *P* value (uncorrected) was 0.244 (not significant).
a. The order of the SNPs in the haplotype is rs6313-rs6311.
Abbreviations: OR, Odds Ratio; CI, confidence intervals; N/A, not applicable.

Table R36. Results from the *HTR2A* haplotype association analysis between hallucinatory patients and healthy subjects.

Haplotype[a]	Hallucinatory patients frequency	Controls frequency	OR (95% CI)	χ^2	P
C-A	0.03645	0.01618	1	4.696	0.03024
T-A	0.4408	0.4326	0.4524 (0.2055 – 0.9957)	0.07268	0.7875
C-G	0.5204	0.5452	0.4238 (0.1932 – 0.9293)	0.6656	0.4146
T-G	0.002361 (rare)	0.005951 (rare)	0.11762 (0.0174 - 1.785)	N/A	N/A

Significant *P* values (P < 0.05) are indicated in bold.
The global *P* value (uncorrected) was 0.093 (not significant).
a. The order of the SNPs in the haplotype is rs6313-rs6311.
Abbreviations: OR, Odds Ratio; CI, confidence intervals; N/A, not applicable.

3.4. *TPH2* gene

3.4.1. Allelic frequencies.

As it can be seen at tables R37 and R38, no significant differences in the allelic frequencies were found between all psychotic patients and controls (table R37) or between hallucinatory patients and controls (table R38). With regard to the other comparisons, in coherence with the other results, no significant differences were found (data not shown).

Table R37. Comparison of the allelic frequencies of the *TPH2* SNP in psychotic patients and healthy controls from the Spanish sample.

Polymorphism	Allele	Patients frequency	Controls frequency	OR (95% CI)	χ^2	P
rs4570625	G	0.8473	0.8174	1	2.089	0.1484
	T	0.1527	0.1826	0.8069 (0.6029 - 1.08)		

Abbreviations: OR, Odds Ratio; CI, confidence intervals.

Table R38. Comparison of the allelic frequencies of the*TPH2* SNP in hallucinatory psychotic patients and healthy controls from the Spanish sample.

Polymorphism	Allele	Hallucinatory patients frequency	Controls frequency	OR (95% CI)	χ^2	P
rs4570625	G	0.8432	0.8174	1	1.275	0.2589
	T	0.1568	0.1826	0.8328 (0.6059 - 1.144)		

Abbreviations: OR, Odds Ratio; CI, confidence intervals.

3.4.2. Genotypic frequencies.

When the genotypic frequencies of rs4570625 in controls were compared to those observed in the whole group of psychotic patients (table R39) and the subsample of hallucinatory patients (table R40), no significant differences were detected.

Table R39. Comparison of the genotypic distributions of the *TPH2* SNP in psychotic patients and healthy controls from the Spanish sample.

Polymorphism	Genotype	Controls frequency	Patients frequency	OR (95% CI)	*P* Value	AIC	Model
rs4570625	G/G	243 (66.2%)	214 (71.8%)	1.00	0.12	916.3	Dominant
	G/T - T/T	124 (33.8%)	84 (28.2%)	0.77 (0.55-1.07)			

Abbreviations: OR, Odds Ratio; CI, confidence intervals.

Table R40. Comparison of the genotypic distributions of the *TPH2* SNP in psychotic patients with AH and healthy controls from the Spanish sample.

Polymorphism	Genotype	Controls frequency	Hallucinatory patients frequency	OR (95% CI)	*P* Value	AIC	Model
rs4570625	G/G	243 (66.2%)	158 (71.8%)	1.00	0.16	778.5	Dominant
	G/T - T/T	124 (33.8%)	62 (28.2%)	0.77 (0.53-1.11)			

Abbreviations: OR, Odds Ratio; CI, confidence intervals.

With regard to the other case-control comparisons, the results were once again negative (data not shown).

3.5. *NOS1* gene

3.5.1. Allelic frequencies.

Tables R41 and R42 include the results about the distribution of the allelic frequencies in controls, all the sample of patients and the subsample of hallucinatory cases. There were no differences in those frequencies between controls and all psychotic patients (table R41) or between controls and the group of patients with AH (table R42). In coherence with these results, the other case-control analyses did not give significant results (data not shown).

Table R41. Comparison of the allelic frequencies of the *NOS1* VNTR in psychotic patients and healthy controls from the Spanish sample.

Polymorphism	Allele	Patients frequency	Controls frequency	OR (95% CI)	χ^2	P
***NOS1* Ex1f-VNTR**	L	0.5276	0.5356	1	0.06727	0.7953
	S	0.4724	0.4644	1.033 (0.8096 - 1.317)		

Abbreviations: OR, Odds Ratio; CI, confidence intervals; L, long alleles; S, short alleles.

Table R42. Comparison of the allelic frequencies of the *NOS1* VNTR in hallucinatory psychotic patients and healthy controls from the Spanish sample.

Polymorphism	Allele	Hallucinatory patients frequency	Controls frequency	OR (95% CI)	χ^2	P
***NOS1* Ex1f-VNTR**	L	0.5317	0.5356	1	0.01308	0.909
	S	0.4683	0.4644	1.016 (0.7798 - 1.322)		

Abbreviations: OR, Odds Ratio; CI, confidence intervals; L, long alleles; S, short alleles.

3.5.2. Genotypic frequencies.

In this case, no differences were found in the genotypic frequencies of healthy subjects and psychotic patients (table R43) or between healthy subjects and patients with AH (table R44).

Table R43. Comparison of the genotypic distributions of the *NOS1* VNTR in psychotic patients and healthy controls from the Spanish sample.

Polymorphism	Genotype	Controls frequency	Patients frequency	OR (95% CI)	*P* Value	AIC	Model
***NOS1* Ex1f-VNTR**	L/L	80 (30%)	72 (28.4%)	1.00	0.68	725.8	Dominant
	S/L - S/S	187 (70%)	182 (71.7%)	1.08 (0.74-1.58)			

Abbreviations: OR, Odds Ratio; CI, confidence intervals; L, long alleles; S, short alleles.

Table R44. Comparison of the genotypic distributions of the *NOS1* VNTR in psychotic patients with AH and healthy controls from the Spanish sample.

Polymorphism	Genotype	Controls frequency	Hallucinatory patients frequency	OR (95% CI)	*P* Value	AIC	Model
***NOS1* Ex1f-VNTR**	L/L	80 (30%)	53 (28%)	1.00	0.66	622.5	Dominant
	S/L - S/S	187 (70%)	136 (72%)	1.10 (0.73-1.66)			

Abbreviations: OR, Odds Ratio; CI, confidence intervals; L, long alleles; S, short alleles.

Regarding the remaining case-control comparisons, the results were also negative and very similar to those shown in tables R43 and R44 (data not shown).

3.6. *STMN1* gene

3.6.1. Allelic frequencies.

The results from the association analysis of allelic frequencies are shown at tables R45 (all patients against controls) and R46 (patients with AH against controls). There were no significant differences between all patients and controls, or between hallucinatory patients and controls. The same negative findings were obtained for the other case-control comparisons (data not shown).

Table R45. Comparison of the allelic frequencies of *STMN1* SNPs in psychotic patients and healthy controls from the Spanish sample.

Polymorphism	Allele	Patients frequency	Controls frequency	OR (95% CI)	χ^2	P[a]
rs12037513	T	0.7379	0.6911	1	3.104	0.07809 (0.156)
	C	0.2621	0.3089	0.7945 (0.615 - 1.026)		
rs182455	C	0.5755	0.5503	1	0.8515	0.3561 (0.356)
	T	0.4245	0.4497	0.9025 (0.7258 - 1.122)		

a. The corrected *P* value is indicated in brackets.
Abbreviations: OR, Odds Ratio; CI, confidence intervals.

Table R46. Comparison of the allelic frequencies of *STMN1* SNPs in hallucinatory psychotic patients and healthy controls from the Spanish sample.

Polymorphism	Allele	Hallucinatory patients frequency	Controls frequency	OR (95% CI)	χ^2	P[a]
rs12037513	T	0.722	0.6911	1	1.133	0.2872 (0.3016)
	C	0.278	0.3089	0.8616 (0.6548 - 1.134)		
rs182455	C	0.5932	0.5503	1	2.064	0.1508 (0.3016)
	T	0.4068	0.4497	0.8391 (0.6606 - 1.066)		

a. The corrected *P* value is indicated in brackets.
Abbreviations: OR, Odds Ratio; CI, confidence intervals.

3.6.2. Genotypic frequencies.

Results were mainly negative for the 8 case-control comparisons. The complete results for the two main comparisons (all patients vs. controls and hallucinatory patients vs. controls) are negative and can be found at tables R47 and R48, respectively. However, despite the negative results, a trend can be observed when controls are compared with the complete sample of patients (see table R47): T/T homozygotes for rs12037513, as well as individuals with at least one copy of allele C from rs182455, tend to be more frequent in the patients group. However, this trend became significant for rs182455 when the control sample was compared with a subset of those patients diagnosed as schizophrenics according to DSM-IV criteria (see table R49).

Table R47. Comparison of the genotypic distributions of *STMN1* polymorphisms in psychotic patients and healthy controls from the Spanish sample.

SNP	Genotype	Controls frequency	Patients frequency	OR (95% CI)	*P* Value[b]	AIC	Model
rs12037513	T/T	149 (47.5%)	144 (53.5%)				
	T/C	136 (43.3%)	109 (40.5%)	0.79 (0.61-1.02)[a]	0.074 (0.124)	814.3	Additive
	C/C	29 (9.2%)	16 (6%)				
rs182455	C/C - T/C	285 (77.5%)	248 (83.2%)	1.00	0.062 (0.124)	925.4	Recessive
	T/T	83 (22.6%)	50 (16.8%)	1.07 (0.85-1.34)			

a. The indicated value corresponds to the difference which can be attributed to each allele in the additive model.
b. The corrected *P* value is indicated in brackets.
Abbreviations: OR, Odds Ratio; CI, confidence interval.

Table R48. Comparison of the genotypic distributions of *STMN1* polymorphisms in hallucinatory patients and healthy controls from the Spanish sample.

SNP	Genotype	Controls frequency	Hallucinatory patients frequency	OR (95% CI)	*P* Value[b]	AIC	Model
rs12037513	T/T	149 (47.5%)	107 (52.2%)				
	T/C	136 (43.3%)	82 (40%)	0.86 (0.65-1.13)[a]	0.28 (0.28)	699.3	Additive
	C/C	29 (9.2%)	16 (7.8%)				
rs182455	C/C - T/C	285 (77.5%)	184 (83.6%)	1.00	0.067 (0.134)	778.1	Recessive
	T/T	83 (22.6%)	36 (16.4%)	0.67 (0.44-1.04)			

a. The indicated value corresponds to the difference which can be attributed to each allele in the additive model.
b. The corrected *P* value is indicated in brackets.
Abbreviations: OR, Odds Ratio; CI, confidence interval.

Table R49. Comparison of the genotypic distributions of *STMN1* polymorphisms in schizophrenic patients and healthy controls from the Spanish sample.

SNP	Genotype	Controls frequency	Schizophrenic patients frequency	OR (95% CI)	*P* Value[b]	AIC	Model
rs12037513	T/T	149 (47.5%)	107 (52.2%)				
	T/C	136 (43.3%)	82 (40%)	0.86 (0.65-1.13)[a]	0.28 (0.28)	699.3	Additive
	C/C	29 (9.2%)	16 (7.8%)				
rs182455	C/C - T/C	285 (77.5%)	185 (84.9%)	1.00	**0.027** (0.54)	772.7	Recessive
	T/T	83 (22.6%)	33 (15.1%)	0.61 (0.39-0.95)			

Significant *P* values (P < 0.05) are indicated in bold.
a. The indicated value corresponds to the difference which can be attributed to each allele in the additive model.
b. The corrected *P* value is indicated in brackets.
Abbreviations: OR, Odds Ratio; CI, confidence interval.

3.6.3. Haplotype analysis.

Tables R50 and R51 show the comparisons of the haplotype frequencies (estimated by the Unphased program) between a) control subjects and all patients and b) control subjects and the subset of patients with AH. Four common haplotypes (frequency > 0.03) were detected. However, there were no significant differences in the haplotype distribution between patients and controls (table R50) or between hallucinatory patients and controls (table R51). Regarding

the case-control association study between schizophrenic patients and controls, the results are similar and also negative (data not shown).

Table R50. Results from the *STMN1* haplotype association analysis between all patients and healthy subjects.

Haplotype[a]	Patients frequency	Controls frequency	OR (95% CI)	χ^2	P
T-C	0.4823	0.4437	1	1.906	0.1675
T-T	0.2584	0.2474	0.9611 (0.7025 - 1.315)	0.1974	0.6568
C-C	0.09049	0.104	0.8001 (0.4897 - 1.307)	0.809	0.3684
C-T	0.1688	0.2049	0.7583 (0.5472 - 1.051)	2.769	0.09609

Note: the global *P* value (uncorrected) was 0.3055 (not significant).
a. The order of the SNPs in the haplotype is rs12037513-rs182455.
Abbreviations: OR, Odds Ratio; CI, confidence intervals.

Table R51. Results from the *STMN1* haplotype association analysis between hallucinatory patients and healthy subjects.

Haplotype[a]	Hallucinatory patients frequency	Controls frequency	OR (95% CI)	χ^2	P
C-A	0.4743	0.4437	1	1.373	0.2412
T-A	0.2512	0.2474	0.9503 (0.6743 – 1.339)	0.002098	0.9635
C-G	0.1115	0.104	1.003 (0.6078 – 1.655)	0.0192	0.8898
T-G	0.163	0.2049	0.7443 (0.5197 - 1.066)	2.516	0.1127

Note: the global *P* value (uncorrected) was 0.444 (not significant).
a. The order of the SNPs in the haplotype is rs12037513-rs182455.
Abbreviations: OR, Odds Ratio; CI, confidence intervals.

3.7. *ASPM* gene

3.7.1. Case-control analysis of the allelic frequencies.

3.7.1.1. Spanish Sample

The results from the case-control comparisons of the allelic frequencies can be found at tables R52 (all patients against controls) and R53 (hallucinatory patients against controls). Unfortunately, no differences were found for any SNP or case-control comparison.

Table R52. Comparison of the allelic frequencies of *ASPM* polymorphisms in psychotic patients and healthy controls from the Spanish sample.

Polymorphism	Allele	Patients frequency	Controls frequency	OR (95% CI)	χ^2	P[a]
rs6700180	T	0.4927	0.4624	1	1086	0.2973 (1)
	C	0.5073	0.5376	0.8855 (0.7046 - 1.113)		
rs3762271	A	0.3977	0.4267	1	1106	0.293 (1)
	C	0.6023	0.5733	1.127 (0.9016 - 1.41)		
rs12138336	C	0.08725	0.06745	1	1761	0.1845 (1)
	G	0.9128	0.9326	0.7567 (0.5007 - 1.143)		

Table R52 (continuation). Comparison of the allelic frequencies of *ASPM* polymorphisms in psychotic patients and healthy controls from the Spanish sample.

Polymorphism	Allele	Patients frequency	Controls frequency	OR (95% CI)	χ^2	P[a]
rs41310927	A	0.6023	0.5881	1	0.2786	0.5976 (1)
	G	0.3977	0.4119	0.9425 (0.7564 - 1.174)		
rs10922163	G	0.4926	0.4524	1	1.881	0.1703 (1)
	A	0.5074	0.5476	0.851 (0.6758 - 1.072)		
rs4915337	A	0.8909	0.9032	1	0.5219	0.47 (1)
	T	0.1091	0.09677	1.142 (0.7958 - 1.64)		
rs6677082	T	0.8959	0.8921	1	0.04526	0.8315 (1)
	C	0.1041	0.1079	0.9602 (0.6606 - 1.396)		
rs9726778	C	0.1208	0.1085	1	0.4756	0.4904 (1)
	G	0.8792	0.8915	0.8858 (0.6274 - 1.251)		

a. The corrected *P* value is indicated in brackets.
Abbreviations: OR, Odds Ratio; CI, confidence intervals.

Table R53. Comparison of the allelic frequencies of *ASPM* polymorphisms in hallucinatory psychotic patients and healthy controls from the Spanish sample.

Polymorphism	Allele	Hallucinatory patients frequency	Controls frequency	OR (95% CI)	χ^2	P[a]
rs6700180	T	0.5	0.4624	1	1428	0.232 (1)
	C	0.5	0.5376	0.8601 (0.6716 - 1.101)		
rs3762271	A	0.4023	0.4267	1	0.6556	0.4181 (1)
	C	0.5977	0.5733	1.106 (0.8668 - 1.411)		
rs12138336	C	0.07955	0.06745	1	0.5843	0.4446 (1)
	G	0.9205	0.9326	0.8369 (0.53 - 1.322)		
rs41310927	A	0.5932	0.5881	1	0.02971	0.8632 (1)
	G	0.4068	0.4119	0.9791 (0.7701 - 1.245)		
rs10922163	G	0.4927	0.4524	1	1.62	0.2031 (1)
	A	0.5073	0.5476	0.8506 (0.663 - 1.091)		
rs4915337	A	0.9	0.9032	1	0.03147	0.8592 (1)
	T	0.1	0.09677	1.037 (0.6939 - 1.55)		
rs6677082	T	0.9	0.8921	1	0.1667	0.683 (1)
	C	0.1	0.1079	0.9183 (0.6099 - 1.383)		
rs9726778	C	0.1114	0.1085	1	0.0224	0.881 (1)
	G	0.8886	0.8915	0.9712 (0.6624 - 1.424)		

a. The corrected *P* value is indicated in brackets.
Abbreviations: OR, Odds Ratio; CI, confidence intervals.

3.7.1.2. American Sample

As it can be seen at table R54, there were no significant differences in the allelic frequencies of any SNP between schizophrenic patients and healthy subjects. These results are in accordance with the results from the Spanish subjects.

Table R54. Comparison of the allelic frequencies of *ASPM* polymorphisms in schizophrenic patients and healthy controls from the American sample.

Polymorphism	Allele	Patients frequency	Controls frequency	OR (95% CI)	χ^2	P[a]
rs3762271	C	0.6094	0.5881	1	0.803	0.3702 (0.744)
	A	0.3906	0.4119	0.9152 (0.754 - 1.111)		
rs12138336	G	0.9146	0.9291	1	1.225	0.2684 (0.744)
	C	0.08537	0.07094	1.222 (0.8563 - 1.745)		
rs41310927	A	0.606	0.5767	1	1.519	0.2177 (0.744)
	G	0.394	0.4233	0.8855 (0.7298 - 1.074)		
rs10922163	A	0.4882	0.5299	1	2.985	0.0840 (0.504)
	G	0.5118	0.4701	1.181 (0.9778 - 1.428)		
rs12116571	G	0.8925	0.8658	1	2.85	0.09137 (0.504)
	A	0.1075	0.1342	0.7772 (0.5793 - 1.043)		
rs6677082	T	0.9159	0.8973	1	1.749	0.186 (0.744)
	C	0.08413	0.1027	0.8025 (0.5787 - 1.113)		

a. The corrected *P* value is indicated in brackets.
Abbreviations: OR, Odds Ratio; CI, confidence intervals.

3.7.2. Case-control analysis of the genotypic frequencies.

3.7.2.1. Spanish Sample

Genotypic frequencies of the different subsets were compared and the main results can be found at tables R55 (all patients against control subjects) and R56 (hallucinatory patients against control subjects). There were significant differences in the genotypic frequencies of two SNPs: rs6700180 (intron 19) and rs10922163 (intron 17). Positive associations were found when the whole sample of patients was considered (table R55) but also when the subset of patients with hallucinations was compared to the control group (table R56). Interestingly, almost all the significant *P* values resisted the sequential Bonferroni correction (the only exception was rs6700180 when all patients were compared to controls, but a clear trend is still observed).

Table R55. Comparison of the genotypic distributions of *ASPM* polymorphisms in psychotic patients and healthy controls from the Spanish sample.

Marker	Genotype	Controls frequency	Patients frequency	OR (95% CI)	*P* Value[b]	AIC	Model
rs6700180	C/C	102 (32%)	62 (22.9%)	1.00	**0.0091** (0.0637)	810.6	Codominant
	T/C	139 (43.6%)	151 (55.7%)	1.79 (1.21-2.64)			
	T/T	78 (24.4%)	58 (21.4%)	1.22 (0.77-1.95)			
rs3762271	C/C - A/C	275 (80.7%)	253 (84.9%)	1.00	0.16 (0.84)	884.9	Recessive
	A/A	66 (19.4%)	45 (15.1%)	0.74 (0.49-1.12)			
rs12138336	G/G	298 (87.4%)	248 (83.2%)	1.00	0.14 (0.84)	884.7	Dominant
	C/G - C/C	43 (12.6%)	50 (16.8%)	1.40 (0.90-2.17)			
rs41310927	A/A - A/G	302 (81.8%)	253 (84.9%)	1.00	0.29 (1)	920	Recessive
	G/G	67 (18.2%)	45 (15.1%)	0.80 (0.53-1.21)			

Table R55 (continuation). Comparison of the genotypic distributions of *ASPM* polymorphisms in psychotic patients and healthy controls from the Spanish sample.

Marker	Genotype	Controls frequency	Patients frequency	OR (95% CI)	*P* Value[b]	AIC	Model
rs10922163	A/A	104 (33%)	61 (22.7%)	1.00	**0.005 (0.04)**	801.4	Codominant
	G/A	137 (43.5%)	151 (56.1%)	1.88 (1.27-2.78)			
	G/G	74 (23.5%)	57 (21.2%)	1.31 (0.82-2.10)			
rs4915337	A/A	277 (81.2%)	235 (78.9%)	1.15 (0.79-1.67)[a]	0.46 (1)	886.4	Additive
	A/T	62 (18.2%)	61 (20.5%)				
	T/T	2 (0.6%)	2 (0.7%)				
rs6677082	T/T	248 (78.7%)	214 (79.5%)	0.96 (0.65-1.41)[a]	0.82 (1)	809.9	Additive
	T/C	66 (20.9%)	54 (20.1%)				
	C/C	1 (0.3%)	1 (0.4%)				
rs9726778	G/G	269 (78.9%)	229 (76.8%)	1.14 (0.80-1.62)[a]	0.48 (1)	886.4	Additive
	C/G	70 (20.5%)	66 (22.1%)				
	C/C	2 (0.6%)	3 (1%)				

Significant *P* values (P < 0.05) are indicated in bold.
a. The indicated value corresponds to the difference which can be attributed to each allele in the additive model.
b. The corrected *P* value is indicated in brackets.
Abbreviations: OR, Odds Ratio; CI, confidence intervals.

Table R56. Comparison of the genotypic distributions of *ASPM* SNPs in hallucinatory patients and healthy controls from the Spanish sample.

Marker	Genotype	Controls frequency	Hallucinatory patients frequency	OR (95% CI)	*P* Value[b]	AIC	Model
rs6700180	C/C	102 (32%)	43 (21%)	1.00	**0.0031 (0.0217)**	695.9	Codominant
	T/C	139 (43.6%)	119 (58%)	2.03 (1.32-3.13)			
	T/T	78 (24.4%)	43 (21%)	1.31 (0.78-2.19)			
rs3762271	C/C - A/C	275 (80.7%)	187 (85%)	1.00	0.18 (1)	753.6	Recessive
	A/A	66 (19.4%)	33 (15%)	0.74 (0.47-1.16)			
rs12138336	G/G	298 (87.4%)	186 (84.5%)	1.00	0.34 (1)	754.5	Dominant
	C/G - C/C	43 (12.6%)	34 (15.4%)	1.27 (0.78-2.06)			
rs41310927	A/A - A/G	302 (81.8%)	186 (84.5%)	1.00	0.4 (1)	781.7	Recessive
	G/G	67 (18.2%)	34 (15.4%)	0.82 (0.52-1.29)			
rs10922163	A/A	104 (33%)	44 (21.5%)	1.00	**0.0021 (0.0168)**	691.1	Codominant
	G/A	137 (43.5%)	120 (58.5%)	2.07 (1.35-3.18)			
	G/G	74 (23.5%)	41 (20%)	1.31 (0.78-2.20)			
rs4915337	A/A - A/T	339 (99.4%)	219 (99.5%)	1.00	0.83 (1)	755.4	Recessive
	T/T	2 (0.6%)	1 (0.4%)	0.77 (0.07-8.59)			
rs6677082	T/T	248 (78.7%)	165 (80.5%)	0.91 (0.60-1.40)[a]	0.67 (1)	701.2	Additive
	T/C	66 (20.9%)	39 (19%)				
	C/C	1 (0.3%)	1 (0.5%)				
rs9726778	G/G - C/G	339 (99.4%)	219 (99.5%)	1.00	0.83 (1)	755.4	Recessive
	C/C	2 (0.6%)	1 (0.4%)	0.77 (0.07-8.59)			

Significant *P* values (P < 0.05) are indicated in bold.
a. The indicated value corresponds to the difference which can be attributed to each allele in the additive model.
b. The corrected *P* value is indicated in brackets
Abbreviations: OR, Odds Ratio; CI, confidence intervals.

Moreover, there were some significant associations in the remaining comparisons, which also involved SNPs rs6700180 and rs10922163. These findings are summarized in table R57.

Table R57. Significant results from the case-control association analysis which compared the genotypic distribution of *ASPM* polymorphisms between different groups from the Spanish sample.

Comparison	Polymorphism	*P* Value[a]	Model
Schizophrenic patients vs controls	rs6700180	**0.0018 (0.0112)**	Codominant
Schizophrenic patients vs controls	rs10922163	**0.0014 (0.0126)**	Codominant
Schizophrenic patients with AH vs controls	rs6700180	**0.0015 (0.012)**	Codominant
Schizophrenic patients with AH vs controls	rs10922163	**0.0016 (0.012)**	Dominant
Patients with chronic AH vs controls	rs6700180	**0.011** (0.077)	Dominant
Patients with chronic AH vs controls	rs10922163	**0.0058 (0.046)**	Dominant

Significant *P* values ($P < 0.05$) are indicated in bold.
a. The corrected *P* value is indicated in brackets.
Abbreviations: vs, versus; AH, auditory hallucinations.

However, these findings should be taken cautiously, since the two significant SNPs are not in HWE in patients and controls, therefore they could be representing a false positive.

3.7.2.2. American Sample

We also found significant differences in the case-control association analysis (table R58). SNP rs12116571 gave the most significant association (P = 0.001 for the Fisher's exact test; P = 0.008 for the logistic regression analysis), while the genotypic frequencies between cases and controls also differed slightly for two other SNPs: rs10922163 (logistic regression P = 0.042) and rs6677082 (Fisher's exact test P = 0.03). However, only the significant associations of rs12116571 survived the correction for multiple testing. With regard to SNP rs10922163, this SNP was also found to be associated with schizophrenia in the Spanish sample and in both cases the A/A individuals were significantly less frequent in the affected group.

Table R58. Case-control association analysis for *ASPM* SNPs in the American Sample.

SNP	Genotype	CONTROLS frequency	CASES frequency	Fisher's exact test *P* value[a,b]	OR (95% CI)	Logistic regression *P* value[b]
rs3762271	C/C	149 (34.57%)	151 (37.94%)	0.580 (1)	0.927 (0.672-1.279)[c]	0.646 (1)[c]
	C/A	209 (48.49%)	185 (46.48%)		0.831 (0.541 - 1.279)[d]	0.401 (0.813)[d]
	A/A	73 (16.94%)	62 (15.58%)			
rs12138336	G/G	371 (86.08%)	324 (83.08%)	0.417 (1)	1.263 (0.838 - 1.901)[c]	0.264 (1)[c]
	C/G	58 (13.46%)	65 (16.67%)		0.659 (0.0386-11.254)[d]	0.774 (0.813)[d]
	C/C	2 (0.46%)	1 (0.26%)			

Table R58 (continuation). Case-control association analysis for *ASPM* SNPs in the American Sample.

SNP	Genotype	CONTROLS frequency	CASES frequency	Fisher's exact test *P* value[a,b]	OR (95% CI)	Logistic regression *P* value[b]
rs41310927	A/A	143 (33.18%)	148 (37.47%)	0.397 (1)	0.910 (0.659 - 1.257)[c]	0.568 (1)[c]
	A/G	211 (48.96%)	185 (46.84%)		0.786 (0.512 - 1.206)[d]	0.271 (0.813)[d]
	G/G	77 (17.87%)	62 (15.70%)			
rs10922163	A/A	119 (27.74%)	89 (21.92%)	0.135 (0.54)	1.441 (1.012 - 2.049)[c]	**0.042** (0.21)[c]
	A/G	218 (50.82%)	217 (53.45%)		1.481 (0.975 - 2.248)[d]	0.065 (0.325)[d]
	G/G	92 (21.45%)	100 (24.63)			
rs12116571	G/G	321 (75.65%)	320 (81.42%)	**0.001 (0.006)**	0.604 (0.416 - 0.877)[c]	**0.008 (0.048)**[c]
	G/A	105 (24.42%)	61 (15.52%)		2.508 (0.769 - 8.179)[d]	0.127 (0.508)[d]
	A/A	4 (0.93%)	12 (3.05%)			
rs6677082	T/T	353 (80.78%)	329 (83.29%)	**0.03** (0.15)	0.829 (0.536 - 1.22)[c]	0.344 (1)[c]
	T/C	77 (17.62%)	66 (16.71%)			
	C/C	7 (1.60%)	0 (0.00%)			

Significant *P* values (P < 0.05) are indicated in bold.
a. Results from the Fisher's exact test, which compares the genotypic frequencies of cases and controls, assuming a codominant model.
b. The corrected *P* value is indicated in brackets.
c. Results from the logistic regression analysis when the homozygotes for the most frequent allele are compared with the heterozygotes .
d. Results from the logistic regression analysis when the homozygotes for the major allele are compared with the homozygotes for the minor allele.
Abbreviations: OR, Odds Ratio.

3.7.3. Case-control haplotype analysis.

3.7.3.1. Spanish Sample

Four common 8-marker haplotypes were found in the Spanish sample (table R59), although no differences were found between patients and controls.

Table R59. Overview of the most common *ASPM* haplotypes observed in the Spanish sample.

HAPLOTYPE[a]									
rs6700180	rs3762271	rs12138336	rs41310927	rs10922163	rs4915337	rs6677082	rs9726778	Patients frequency	Controls frequency
G	A	G	G	T	A	A	G	0.385	0.4035
A	C	G	A	C	A	A	G	0.4153	0.3812
G	C	G	A	T	T	G	C	0.09735	0.09156
A	C	C	A	C	A	A	G	0.07312	0.06727
All rare (freq < 0.03)								0.02923	0.05647

Note: the global *P* value was 0.77834 (uncorrected). Patients' data refer to the whole sample of psychotic patients.
a. The SNPs which deviated from HWE are marked in grey.
Abbreviations: freq, frequency; OR, Odds Ratio; CI, confidence intervals; N/A, not applicable.

A case-control analysis of the 2, 3 and 4-marker haplotype frequencies was finally performed with 6 SNPs and eliminating those SNPs out of Hardy-Weinberg Equilibrium (rs6700180 and rs10922163). However, no differences were found between patients and controls, hallucinatory patients and controls or between schizophrenic patients and controls (data not shown). For this reason, we decided to repeat the haplotype analysis but including those two SNPs out of HWE (table R60). In this case, two protective haplotypes were found (one 3-marker haplotype and one 4-marker haplotype which was an extension of the previous one). Both haplotypes still remained significant after 1000 permutations and included allele A from rs10922163, which was shown to be less frequent in patients in the comparison of the genotypic frequencies. Moreover, the same risk haplotypes were found when the subset of hallucinatory patients was considered (data not shown).

Table R60. *ASPM* haplotypes significantly associated with psychosis in the Spanish sample.

HAPLOTYPE[a]								Patients frequency	Controls frequency	df	Global *P* value	Haplotype *P* value[b]
rs6700180	rs3762271	rs12138336	rs41310927	rs10922163	rs4915337	rs6677082	rs9726778					
			A	A	A			0.005597	0.0381	3	0.0008104	**0.0002358 (0.001995)**
		G	A	A	A			0.005597	0.0381	4	0.002275	**0.0002358 (0.003996)**

Significant *P* values (P < 0.05) are indicated in bold.
a. The SNPs which deviated from HWE are marked in grey.
b. The corrected *P* value after 1000 permutations is indicated in brackets.
Abbreviation: df, degrees of freedom.

3.7.3.2. American Sample

There were also two three-marker haplotypes which differed significantly between groups (table R61): rs41310927(allele A)/ 10922163(allele G)/ rs12116571(allele G) and rs10922163(alleleG)/ SNP5(alleleG)/ SNP6(alleleT). As it can be seen, these risk haplotypes included the three polymorphisms previously reported to be associated with schizophrenia in the CBDB case-control data set.

Table R61. *ASPM* haplotypes significantly associated with schizophrenia in the American sample.

HAPLOTYPE						Haplotype frequency	df	Z score	Global *P* value	Haplotype *P* value[a]
rs3762271	rs12138336	rs41310927	rs10922163	rs12116571	rs6677082					
		A	G	G		0.370	3	2.9197	0.0347	**0.0035 (0.014)**
			G	G	T	0.370	3	2.7875	0.0294	**0.0053 (0.0212)**

a. Significant *P* values (P < 0.05) are indicated in bold. The corrected *P* value is indicated in brackets.
Abbreviation: df, degrees of freedom.

3.7.4. Family-based analyses.

Regarding to the family-based association studies, FBAT program detected that the A allele of the SNP rs6677082 was slightly overtransmitted (Z = 2.132, P = 0.038) from parents to affected offspring (table R62). We also detected a trend for overtransmission of allele C of SNP rs10922163 (Z = -2.017, P = 0.071).

Table R62. Family based Transmission disequilibrium test results for *ASPM* polymorphisms in the American families.

Polymorphism	Overtransmitted allele	No. Families[a]	Statistic[b]	Expected statistic[c]	Z score	P[d]
rs3762271	C	144	179.000	170.281	1.206	0.21 (0.8)
rs12138336	C	64	37.000	34.000	0.688	0.53 (1)
rs41310927	A	150	187.000	176.348	1.444	0.20 (0.8)
rs10922163	C	157	173.000	157.898	2.017	0.071 (0.355)
rs12116571	G	76	108.000	106.550	0.280	0.75 (1)
rs6677082	A	71	118.000	108.667	2.132	**0.038** (0.228)

Significant *P* values (P < 0.05) are indicated in bold.
a. Number of informative families.
b. Test statistic from FBAT for the observed number of transmitted alleles.
c. Expected value of the statistic under the null hypothesis (no association).
d. The corrected *P* value is indicated in brackets.

The three-marker haplotype analysis revealed an overtransmission of two common haplotypes (table R63): rs4131027(allele A)/ rs10922163(allele G)/ rs12116571(allele G) and rs10922163(allele G)/ rs12116571(allele G)/ rs6677082(allele T). It is noteworthy that one of these two haplotypes also included SNP rs6677082 mentioned above. Moreover, these two risk haplotypes were also detected in the case-control haplotype analysis (see table R61), giving more strength to the finding.

Table R63. Significant results for the family-based haplotype analysis of *ASPM* gene in the American sample.

HAPLOTYPE						Global *P* value	Haplotype *P* value	Frequency	Obs/exp
rs3762271	rs12138336	rs41310927	rs10922163	rs12116571	rs6677082				
		A	G	G		**0.034**	**0.006**	0.394	193.981/ 175.611
			G	G	T	**0.019**	**0.006**	0.393	202.030/183.872

Significant *P* values (P < 0.05) are indicated in bold.
Abbreviation: Obs/exp, Observed/expected.

3.7.5. Pooled case-control association analysis.

The Spanish and the American samples were pooled in order to perform a case-control association analysis, as explained in the Materials and Methods section. The pooled sample included all the Spanish and American control subjects. With regard to the patients, all the American patients were included, as well as those Spanish patients diagnosed as schizophrenics. The five *ASPM* SNPs which had been genotyped in both the Spanish and the American individuals were included in this study.

The results from the association analysis are shown at table R64. According to the Cochran-Mantel-Haenszel tests for IxJxK stratified tables, the American and the Spanish samples presented a similar genotypic distribution of *ASPM* polymorphisms (heterogeneity *P* values > 0.05). Thus, the use of a pooled (American-Spanish) sample can be considered to be a valid option. With regard to the comparison of the genotypic frequencies between cases and controls, the SNP rs10922163 showed significant differences in the genotypic frequencies of schizophrenic patients and controls. Although this result did not resist the sequential Bonferroni correction, it is interesting, since this SNP was also associated with the risk for schizophrenia in both the Spanish and the American sample separately.

Table R64. Results from the case-control association analysis between *ASPM* SNPs and schizophrenia when studying the pooled sample of American and Spanish schizophrenic patients.

SNP	Genotype	Schizophrenic patients frequency	Controls frequency	Heterogeneity *P* value[a,b]	χ^2 [c]	Caco *P* value[b]	OR (95% CI)
rs3762271	C/C	228 (37.01%)	265 (34.33%)	0.6353 (1)	1.911	0.1668 (0.595)	0.898 (0.770 - 1.046)
	A/C	296 (48.05%)	368 (47.67%)				
	A/A	92 (14.94%)	139 (18.01%)				
rs12138336	G/G	504 (82.89%)	669 (86.66%)	0.9414 (1)	2.085	0.1488 (0.595)	1.23 (0.928 - 1.628)
	C/G	103(16.94%)	98 (12.69%)				
	C/C	1 (0.16%)	5 (0.65%)				
rs41310927	A/A	225 (36.70%)	275 (34.38%)	0.7769 (1)	2.016	0.1557 (0.595)	0.896 (0.769 - 1.043)
	A/G	296 (48.29%)	381 (47.63%)				
	G/G	92 (15.01%)	144 (18%)				
rs10922163	A/A	130 (21.38%)	223 (29.97%)	0.7074 (1)	4.714	**0.02993** (0.149)	1.184 (1.017 - 1.378)
	G/A	335 (55.1%)	355 (47.72%)				
	G/G	143 (23.52%)	166 (22.31%)				
rs6677082	T/T	494(82.75%)	601 (80.03%)	0.6024 (1)	2068	0.1504 (0.595)	0.826 (0.637 - 1.072)
	T/C	102 (17.09%)	143 (19.04%)				
	C/C	1 (0.17%)	7 (0.93%)				

Significant *P* values (P < 0.05) are indicated in bold.
a. Statistic for the Cochran-Mantel-Haenszel test for IxJxK stratified tables, which evaluates whether the *ASPM* SNPs vary between the Spanish and American clusters.
b. The corrected *P* value is indicated in brackets.
c. Statistic for the Cochran-Mantel-Haenszel test for 2x2xK stratified tables, which evaluates the existence of associations with the disease.
Abbreviations: OR, Odds Ratio; CI, confidence interval; Caco, case-control.

3.8. *PDE4D* gene

3.8.1. Allelic frequencies.

3.8.1.1. Spanish Sample

The results from the comparisons of the allelic frequencies between a) controls and all patients and b) controls and hallucinatory patients can be found at tables R65 and R66, respectively. According to this information, there were no significant differences in the allelic frequencies between controls and patients, or between hallucinatory patients and controls. However, rs12656462 (marker 7) shows a trend toward association, with a *P* value (uncorrected) of 0.07. However, this trend turned into a significant result when the control sample was compared to the sample of schizophrenic patients (see table R67), although it not resisted sequential Bonferroni correction.

Table R65. Comparison of the allelic frequencies of *PDE4D* polymorphisms in psychotic patients and healthy controls from the Spanish sample.

Polymorphism	Allele	Patients frequency	Controls frequency	OR (95% CI)	χ^2	P[a]
rs17291089	C	0.07237	0.0581	1	0.8542	0.3554 (1)
	A	0.9276	0.9419	0.7907 (0.48 - 1.302)		
rs829259	A	0.3777	0.3475	1	1.025	0.3114 (1)
	T	0.6223	0.6525	0.8772 (0.6806 - 1.131)		
rs17719378	G	0.369	0.3845	1	0.2612	0.6093 (1)
	A	0.631	0.6155	1.068 (0.8294 - 1.376)		
rs10055954	G	0.8515	0.8512	1	0.00020	0.9886 (1)
	C	0.1485	0.1488	0.9975 (0.7067 - 1.408)		
rs10461656	G	0.6987	0.7038	1	0.03218	0.8576 (1)
	A	0.3013	0.2962	1.025 (0.7837 - 1.34)		
rs7713345	G	0.1513	0.1314	1	0.8189	0.3655 (1)
	C	0.8487	0.8686	0.8484 (0.594 - 1.212)		
rs12656462	T	0.9258	0.9531	1	3.219	0.0727 (0.655)
	A	0.07424	0.04687	1.631 (0.9516 - 2.794)		
rs4700316	G	0.1812	0.2082	1	1.164	0.2807 (1)
	C	0.8188	0.7918	1.188 (0.8686 - 1.625)		
rs7714708	T	0.3283	0.3496	1	0.511	0.4747 (1)
	C	0.6717	0.6504	1.1 (0.8468 - 1.429)		

a. The corrected *P* value is indicated in brackets.
Abbreviations: OR, Odds Ratio; CI, confidence intervals.

Table R66. Comparison of the allelic frequencies of *PDE4D* polymorphisms in hallucinatory psychotic patients and healthy controls from the Spanish sample.

Polymorphism	Allele	Hallucinatory patients frequency	Controls frequency	OR (95% CI)	χ^2	P[a]
rs17291089	C	0.0625	0.0581	1	0.07691	0.7815 (1)
	A	0.9375	0.9419	0.9252 (0.5342 - 1.603)		
rs829259	A	0.3737	0.3475	1	0.6817	0.409 (1)
	T	0.6263	0.6525	0.8926 (0.6814 - 1.169)		
rs17719378	G	0.3763	0.3845	1	0.06363	0.8008 (1)
	A	0.6237	0.6155	1.035 (0.7915 - 1.354)		
rs10055954	G	0.8522	0.8512	1	0.00158	0.9683 (1)
	C	0.1478	0.1488	0.9926 (0.6879 - 1.432)		
rs10461656	G	0.6935	0.7038	1	0.1136	0.736 (1)
	A	0.3065	0.2962	1.05 (0.7904 - 1.395)		
rs7713345	G	0.1568	0.1314	1	1.169	0.2795 (1)
	C	0.8432	0.8686	0.8137 (0.5597 - 1.183)		
rs12656462	T	0.9274	0.9531	1	2.619	0.1056 (0.95)
	A	0.07258	0.04687	1.591 (0.9027 - 2.805)		
rs4700316	G	0.1774	0.2082	1	1.345	0.2462 (1)
	C	0.8226	0.7918	1.219 (0.872 - 1.704)		
rs7714708	T	0.3306	0.3496	1	0.3562	0.5506 (1)
	C	0.6694	0.6504	1.088 (0.8242 - 1.437)		

a. The corrected *P* value is indicated in brackets.
Abbreviations: OR, Odds Ratio; CI, confidence intervals.

Table R67. Significant results from the case-control association analysis which compared the allelic frequencies of *PDE4D* polymorphisms in schizophrenic patients and healthy controls from the Spanish sample.

Polymorphism	Allele	Schizophrenic patients frequency	Controls frequency	OR (95% CI)	χ^2	P[a]
rs12656462	T	0.918	0.9531	1	4.477	**0.03285** (0.309)
	A	0.08197	0.04687	1.815 (1.043 – 3.161)		

Significant *P* values ($P < 0.05$) are indicated in bold.
a. The corrected *P* value is indicated in brackets
Abbreviations: OR, Odds Ratio; CI, confidence intervals.

Regarding the remaining case-control comparisons, there were no significant associations.

3.8.1.2. German Sample

The results from the comparisons of the allelic frequencies between controls and the whole sample patients can be found at table R68. In agreement with the results obtained with the Spanish sample, no significant differences were found. The results were also negative when the controls were compared with the subsets of schizophrenic patients or bipolar patients, for this reason these data are not shown.

Table R68. Comparison of the allelic frequencies of *PDE4D* polymorphisms in psychotic patients and healthy controls from the German sample.

Polymorphism	Allele	Patients frequency	Controls frequency	OR (95% CI)	χ^2	P[a]
rs17291089	C	0.07034	0.06497	1	0.1863	0.666 (1)
	A	0.9297	0.935	0.9184 (0.624 - 1.352)		
rs829259	A	0.4061	0.4045	1	0.004152	0.9486 (1)
	T	0.5939	0.5955	0.9935 (0.8153 - 1.211)		
rs17719378	G	0.3308	0.3511	1	0.7475	0.3873 (1)
	A	0.6692	0.6489	1.095 (0.8918 - 1.344)		
rs10055954	G	0.8308	0.8287	1	0.01353	0.9074 (1)
	C	0.1692	0.1713	0.9848 (0.7608 - 1.275)		
rs10461656	G	0.6707	0.6629	1	0.111	0.739 (1)
	A	0.3293	0.3371	0.9656 (0.786 - 1.186)		
rs7713345	G	0.1585	0.1657	1	0.1542	0.6946 (1)
	C	0.8415	0.8343	1.054 (0.8094 - 1.374)		
rs12656462	T	0.9	0.9107	1	0.5523	0.4574 (1)
	A	0.1	0.08929	1.133 (0.8146 - 1.577)		
rs10056492	T	0.1069	0.1306	1	1.837	0.1753 (1)
	C	0.8931	0.8694	1.255 (0.9032 - 1.743)		
rs4700316	G	0.1955	0.1998	1	0.04872	0.8253 (1)
	C	0.8045	0.8002	1.028 (0.8053 - 1.312)		
rs7714708	T	0.3535	0.363	1	0.1623	0.6871 (1)
	C	0.6465	0.637	1.042 (0.8514 - 1.276)		

a. The corrected *P* value is indicated in brackets.
Abbreviations: OR, Odds Ratio; CI, confidence intervals.

3.8.2. Genotypic frequencies.

3.8.2.1. Spanish Sample

The results from the comparisons of the genotypic frequencies are summarized in tables R69 (all patients against controls) and R70 (hallucinatory patients against controls). No significant associations were found, although rs12656462, as in the comparisons of the allelic frequencies, shows a trend toward association (p = 0.066) when all patients are compared to controls (where the A/T genotype is more frequent in the patient group than in the control group). The differences for this SNP become significant when only the schizophrenic patients are considered, although the significant *P* value did not survive sequential Bonferroni correction (see table R71).

Table R69. Comparison of the genotypic distributions of *PDE4D* SNPs in psychotic patients and healthy controls from the Spanish sample.

Polymorphism	Genotype	Controls frequency	Patients frequency	OR (95% CI)	*P* Value[b]	AIC	Model
rs17291089	A/A	253 (89.1%)	197 (86%)	1.00	0.3 (1)	708.2	Dominant
	C/A-C/C	31 (10.9%)	32 (14%)	1.33 (0.78-2.25)			
rs829259	T/T-A/T	264 (89.5%)	197 (85.7%)	1.00	0.18 (1)	722	Recessive
	A/A	31 (10.5%)	33 (14.3%)	1.43 (0.84-2.41)			
rs17719378	A/A - A/G	248 (85.5%)	200 (87%)	1.00	0.64 (1)	717.7	Recessive
	G/G	42 (14.5%)	30 (13%)	0.89 (0.53-1.47)			
rs10055954	G/G-C/G	286 (99%)	227 (98.7%)	1.00	0.78 (1)	716.7	Recessive
	C/C	3 (1%)	3 (1.3%)	1.26 (0.25-6.30)			
rs10461656	G/G-A/G	268 (93.4%)	210 (91.3%)	1.00	0.38 (1)	713.6	Recessive
	A/A	19 (6.6%)	20 (8.7%)	1.34 (0.70-2.58)			
rs7713345	C/C	209 (76.3%)	167 (72.9%)	1.00	0.39 (1)	696.5	Dominant
	G/C - G/G	65 (23.7%)	62 (27.1%)	1.19 (0.80-1.79)			
rs12656462	T/T	232 (90.6%)	196 (85.2%)	1.00	0.066 (0.594)	673	N/A
	A/T	24 (9.4%)	34 (14.8%)	1.68 (0.96-2.92)			
rs4700316	C/C	174 (61.9%)	155 (67.4%)	1.00	0.2 (1)	705.6	Dominant
	C/G - G/G	107 (38.1%)	75 (32.6%)	0.79 (0.55-1.13)			
rs7714708	C/C	115 (41.7%)	104 (45%)	0.90 (0.69-1.17)[a]	0.44 (1)	702.3	Additive
	T/C	129 (46.7%)	103 (44.6%)				
	T/T	32 (11.6%)	24 (10.4%)				

a. The indicated value corresponds to the difference which can be attributed to each allele in the additive model.
b. The corrected *P* value is indicated in brackets
Abbreviations: OR, Odds Ratio; CI, confidence intervals; N/A, not applicable.

Table R70. Comparison of the genotypic distributions of *PDE4D* SNPs in hallucinatory patients and healthy controls from the Spanish sample.

SNP	Genotype	Controls frequency	Hallucinatory patients frequency	OR (95% CI)	*P* Value[b]	AIC	Model
rs17291089	A/A	253 (89.1%)	162 (88%)	1.00	0.73 (1)	631.1	Dominant
	C/A-C/C	31 (10.9%)	22 (12%)	1.11 (0.62-1.98)			
rs829259	T/T-A/T	264 (89.5%)	160 (86%)	1.00	0.26 (1)	644.6	Recessive
	A/A	31 (10.5%)	26 (14%)	1.38 (0.79-2.42)			
rs17719378	A/A - A/G	248 (85.5%)	162 (87.1%)	1.00	0.63 (1)	640.7	Recessive
	G/G	42 (14.5%)	24 (12.9%)	0.87 (0.51-1.50)			
rs10055954	G/G-C/G	286 (99%)	185 (99.5%)	1.00	0.55 (1)	639.6	Recessive
	C/C	3 (1%)	1 (0.5 %)	0.52 (0.05-4.99)			
rs10461656	G/G-A/G	268 (93.4%)	170 (91.4%)	1.00	0.42 (1)	637.3	Recessive
	A/A	19 (6.6%)	16 (8.6%)	1.33 (0.66-2.65)			
rs7713345	C/C	209 (76.3%)	133 (71.9%)	1.00	0.29 (1)	621.8	Dominant
	G/C - G/G	65 (23.7%)	52 (28.1%)	1.26 (0.82-1.92)			

Table R70 (continuation). Comparison of the genotypic distributions of *PDE4D* SNPs in hallucinatory patients and healthy controls from the Spanish sample.

SNP	Genotype	Controls frequency	Hallucinatory patients frequency	OR (95% CI)	*P* Value[b]	AIC	Model
rs12656462	T/T	232 (90.6%)	159 (85.5%)	1.00	0.097 (0.873)	602.9	N/A
	A/T	24 (9.4%)	27 (14.5%)	1.64 (0.91-2.95)			
rs4700316	C/C	174 (61.9%)	125 (67.2%)	0.81 (0.58-1.15)[a]	0.23 (1)	630.5	Additive
	C/G	97 (34.5%)	56 (30.1%)				
	G/G	10 (3.6%)	5 (2.7%)				
rs7714708	C/C	115 (41.7%)	81 (43.5%)	0.92 (0.69-1.22)[a]	0.54 (1)	626.5	Aditive
	T/C	129 (46.7%)	87 (46.8%)				
	T/T	32 (11.6%)	18 (9.7%)				

a. The indicated value corresponds to the difference which can be attributed to each allele in the additive model.
b. The corrected *P* value is indicated in brackets
Abbreviations: OR, Odds Ratio; CI, confidence intervals; N/A, not applicable.

Table R71. Significant results from the case-control association analysis which compared the genotypic distribution of *PDE4D* polymorphisms in schizophrenic patients and healthy controls from the Spanish sample.

Polymorphism	Genotype	Controls frequency	Schizophrenic patients frequency	OR (95% CI)	*P* Value[a]	AIC	Model
rs12656462	T/T	232 (90.6%)	153 (83.6%)	1.00	**0.028** (0.252)	595.6	N/A
	A/T	24 (9.4%)	30 (16.4%)	1.90 (1.07-3.37)			

a. The corrected *P* value is indicated in brackets
Abbreviations: OR, Odds Ratio; CI, confidence intervals; N/A, not applicable.

3.8.2.2. German Sample

The genotypic distribution in the whole sample of patients was compared to the distribution in the control group (table R72). However, no differences or were found. The results were very similar when only the subset of schizophrenic patients was considered (data not shown).

Table R72. Comparison of the genotypic distributions of *PDE4D* SNPs in psychiatric patients and healthy controls from the German sample.

Polymorphism	Genotype	Controls frequency	Patients frequency	OR (95% CI)	*P* Value[b]	AIC	Model
rs17291089	A/A	466 (87.8%)	283 (86.5%)	1.00	0.6 (1)	1144.2	Dominant
	C/A-C/C	65 (12.2%)	44 (13.5%)	1.11 (0.74-1.68)			
rs829259	T/T	184 (34.5%)	114 (34.5%)	1.01 (0.82-1.23)[a]	0.95 (1)	1153.1	Additive
	A/T	268 (50.2%)	164 (49.7%)				
	A/A	82 (15.4%)	52 (15.8%)				
rs17719378	A/A	220 (41.2%)	148 (44.7%)	1.00	0.31 (1)	1154	Dominant
	A/G - G/G	314 (58.8%)	183 (55.3%)	0.87 (0.66-1.14)			

Table R72 (continuation). Comparison of the genotypic distributions of *PDE4D* SNPs in psychiatric patients and healthy controls from the German sample.

Polymorphism	Genotype	Controls frequency	Patients frequency	OR (95% CI)	*P* Value[b]	AIC	Model
rs10055954	G/G	365 (68.3%)	227 (68.6%)	0.98 (0.76-1.28)[a]	0.91 (1)	1155	Additive
	C/G	155 (29%)	96 (29%)				
	C/C	14 (2.6%)	8 (2.4%)				
rs10461656	G/G	232 (43.5%)	146 (44.1%)	0.96 (0.78-1.19)[a]	0.74 (1)	1154.9	Additive
	A/G	244 (45.7%)	152 (45.9%)				
	A/A	58 (10.9%)	33 (10%)				
rs7713345	C/C	373 (69.8%)	236 (72%)	1.00	0.51 (1)	1148.8	Dominant
	G/C - G/G	161 (30.1%)	92 (28.1%)	0.90 (0.67-1.22)			
rs12656462	T/T	442 (83.1%)	267 (80.9%)	1.00	0.42 (1)	1150.6	Dominant
	A/T - A/A	90 (16.9%)	63 (19.1%)	1.16 (0.81-1.65)			
rs10056492	C/C	268 (75.3%)	267 (80.4%)	1.00	0.1 (1)	954.3	Dominant
	C/T - T/T	88 (24.7%)	65 (19.6%)	0.74 (0.52-1.07)			
rs4700316	C/C - C/G	514 (96.4%)	315 (95.5%)	1.00	0.48 (1)	1151.7	Recessive
	G/G	19 (3.6%)	15 (4.5%)	1.29 (0.65-2.57)			
rs7714708	C/C	211 (39.6%)	146 (44.1%)	1.00	0.11 (1)	1151.7	Codominant
	T/C	257 (48.2%)	136 (41.1%)	0.76 (0.57-1.03)			
	T/T	65 (12.2%)	49 (14.8%)	1.09 (0.71-1.67)			

a. The indicated value corresponds to the difference which can be attributed to each allele in the additive model.
b. The corrected *P* value is indicated in brackets
Abbreviations: OR, Odds Ratio; CI, confidence intervals.

By contrast, when we compared the genotypic frequencies of controls and bipolar patients (table R73), three SNPs appeared to be associated with the disease. These SNPs were rs10056492, rs4700316 and rs7714708, located in intron 6. Interestingly, the associations of rs10056492 and rs7714708 with bipolar disorder remained significant even after the correction for multiple testing. However, the small size of the bipolar sample (around 85 individuals, depending on the efficiency of the genotyping process) should be taken into account.

Table R73. Comparison of the genotypic distributions of *PDE4D* SNPs in bipolar patients and healthy controls from the German sample.

Polymorphism	Genotype	Controls frequency	Bipolar patients frequency	OR (95% CI)	*P* Value[b]	AIC	Model
rs17291089	A/A	466 (87.8%)	70 (82.3%)	1.00	0.18 (1)	496.6	Dominant
	C/A-C/C	65 (12.2%)	15 (17.6%)	0.65 (0.35-1.20)			
rs829259	T/T	184 (34.5%)	33 (37.9%)	1.00	0.53 (1)	506.8	Dominant
	A/T - A/A	350 (65.5%)	54 (62.1%)	1.16 (0.73-1.86)			
rs17719378	A/A	220 (41.2%)	37 (42%)	1.05 (0.74-1.48)[a]	0.79 (1)	511	Additive
	A/G	253 (47.4%)	42 (47.7%)				
	G/G	61 (11.4%)	9 (10.2%)				

Table R73 (continuation). Comparison of the genotypic distributions of *PDE4D* SNPs in bipolar patients and healthy controls from the German sample.

Polymorphism	Genotype	Controls frequency	Bipolar patients frequency	OR (95% CI)	*P* Value[b]	AIC	Model
rs10055954	G/G	365 (68.3%)	64 (72.7%)	1.26 (0.79-1.99)[a]	0.32 (1)	510.1	Additive
	C/G	155 (29%)	23 (26.1%)				
	C/C	14 (2.6%)	1 (1.1%)				
rs10461656	G/G	232 (43.5%)	46 (52.3%)	1.32 (0.93-1.89)[a]	0.12 (0.840)	508.6	Additive
	A/G	244 (45.7%)	35 (39.8%)				
	A/A	58 (10.9%)	7 (8%)				
rs7713345	C/C	373 (69.8%)	63 (73.3%)	1.22 (0.77-1.94)	0.38 (1)	502.5	Additive
	G/C	145 (27.1%)	22 (25.6%)				
	G/C	16 (3%)	1 (1.2%)				
rs12656462	T/T	442 (83.1%)	68 (77.3%)	1.00	0.2 (1)	508.8	Dominant
	A/T - A/A	90 (16.9%)	20 (22.7%)	0.69 (0.40-1.20)			
rs10056492	C/C	268 (75.3%)	80 (90.9%)	3.23 (1.52-6.83)[a]	**0.0004 (0.004)**	433.7	Additive
	C/T	83 (23.3%)	8 (9.1%)				
	T/T	5 (1.4%)	0 (0%)				
rs4700316	C/C - C/G	339 (63.6%)	67 (76.1%)	1.00	**0.019** (0.152)	505.3	Dominant
	G/G	194 (36.4%)	21 (23.9%)	1.83 (1.08-3.07)			
rs7714708	C/C	211 (39.6%)	49 (55.7%)	1.00	**0.0049 (0.041)**	502.9	Dominant
	T/C - T/T	322 (60.4%)	39 (44.3%)	1.92 (1.22-3.02)			

Significant *P* values (P < 0.05) are indicated in bold.
a. The indicated value corresponds to the difference which can be attributed to each allele in the additive model.
b. The corrected *P* value is indicated in brackets.
Abbreviations: OR, Odds Ratio; CI, confidence intervals.

3.8.3. Haplotype analysis.

3.8.3.1. Spanish Sample

The most common 9-marker haplotypes detected in our sample can be consulted at table R74. The frequencies of these seven common haplotypes did not differ between patients and controls. However, this association analysis is not robust due to the high number of markers included in the haplotype and the sample size, which can be considered low for such haplotype window size.

Table R74. Overview of the most common *PDE4D* haplotypes observed in the Spanish sample.

HAPLOTYPE									Patients frequency	Controls frequency
rs17291089	rs829259	rs17719378	rs10055954	rs10461656	rs7713345	rs12656462	rs4700316	rs7714708		
A	T	G	G	G	C	T	C	C	0.3682	0.3813
A	T	A	G	G	C	T	C	C	0.1807	0.1567
A	A	A	G	A	G	T	C	T	0.146	0.1314
A	T	A	G	G	C	T	G	T	0.07155	0.1104
A	A	A	C	A	C	T	G	T	0.07301	0.07553
C	A	A	G	G	C	T	C	C	0.07301	0.05902
A	A	A	C	A	C	A	C	C	0.04846	0.03438
All rare (freq < 0.03)									0.039067	0.05127

Note: the global *P* value (uncorrected) was 0.3581 (not significant). The patients' data refer to the whole sample of psychotic patients.
Abbreviations: freq, frequency; OR, Odds Ratio; CI, confidence intervals; N/A, not applicable.

The analysis of the haplotype frequencies was finally performed with 8 polymorphisms and sliding windows of two, three and four-marker haplotypes, as it was explained in the Materials and Methods section. Unfortunately, we did not found any haplotype significantly associated to psychosis or hallucinatory psychosis. Only when the subsample of schizophrenic patients was compared to controls (see table R75), one protective haplotype (more frequent in the control group) and one risk haplotype (more frequent in the schizophrenic group) were found. Both haplotypes involved SNPs rs12656462 and rs7713345. However, despite one of the haplotypes is more frequent in controls and the other is more frequent in patients, both haplotypes share allele C from rs7713345 and only differ in SNP rs12656462, which seems to be the SNP responsible for the significance observed in the analysis. Moreover, as it is shown in sections 3.8.1 and 3.8.2, this SNP was also associated with schizophrenia in the analysis of allelic and genotypic frequencies.

Table R75. *PDE4D* haplotypes significantly associated with schizophrenia in the Spanish sample.

HAPLOTYPE									Schizophrenic patients frequency	Controls frequency	df	Global *P* value	Haplotype *P* value[a]
rs17291089	rs829259	rs17719378	rs10055954	rs10461656	rs7713345	rs12656462	rs10056492	rs4700316					
					C	T			0.761	0.8219	2	**0.0453**	**0.0287** (0.0659)
					C	A			0.08242	0.04656	2	**0.0453**	**0.03106** (0.07)

Significant *P* values (P < 0.05) are indicated in bold.
a. The corrected *P* value after 1000 permutations is indicated in brackets.
Abbreviation: df, degrees of freedom.

3.8.3.2. German Sample

The most common 10-marker haplotypes inferred in the German samples, as well as their frequencies in both the control and the patient sample, can be found at table R76. No differences were found between both groups. Moreover, no differences in the frequencies of the 2, 3 and 4-marker haplotypes were detected between patients and controls, or between schizophrenic patients and controls (data not shown).

Table R76. Overview of the most common *PDE4D* haplotypes observed in the German sample.

HAPLOTYPE											
rs17291089	**rs829259**	**rs17719378**	**rs10055954**	**rs10461656**	**rs7713345**	**rs12656462**	**rs10056492**	**rs4700316**	**rs7714708**	**Patients frequency**	**Controls frequency**
A	T	G	G	G	C	T	C	C	C	0.326	0.3523
A	A	A	G	A	G	T	C	C	T	0.1537	0.1671
A	T	A	G	G	C	T	C	C	C	0.1879	0.1437
A	A	A	C	A	C	T	T	G	T	0.0667	0.07708
C	A	A	G	G	C	T	C	C	C	0.06677	0.06571
A	A	A	C	A	C	A	C	C	C	0.05744	0.06072
A	T	A	G	G	C	T	C	G	T	0.04683	0.04929
A	T	A	G	G	C	T	T	G	T	0.0308	0.04411
A	A	A	C	A	C	A	C	G	T	0.03728	0.02071
All rare (freq < 0.03)										0.039067	0.05127

Note: the global *P* value (uncorrected) was 0.2921 (not significant). The patients' data refer to the whole sample of psychiatric patients.
Abbreviations: freq, frequency; OR, Odds Ratio; CI, confidence intervals; N/A, not applicable.

3.8.4. Pooled case-control association analysis.

A case-control association analysis was performed by combining the German and the Spanish samples and including all control subjects and those patients diagnosed as schizophrenics. The nine SNPs which had been genotyped in both the German and Spanish subjects were included in the analysis.

The genotypic frequencies of both patients and controls were compared with a Cochran-Mantel-Haenszel test for 2x2xK stratified tables. Moreover, the heterogeneity between samples depending on their country of origin was evaluated with a Cochran-Mantel-Haenszel test for IxJxK stratified tables (for more information, see the corresponding Materials and Methods section).

The results from this association analysis can be found at table R77. Three SNPs (rs829259, rs7713345 and rs12656462) showed significant between-study heterogeneity. However, no differences were found between patients or controls for any of the nine SNPs.

Table R77. Results from the case-control association analysis between *PDE4D* SNPs and schizophrenia when studying the pooled sample of German and Spanish schizophrenic patients.

SNP	Genotype	Schizophrenic patients frequency	Controls frequency	Heterogeneity *P* value[a,b]	$\chi^{2\,c}$	Caco *P* value[b]	OR (95% CI)
rs17291089	A/A	369 (87.23%)	719 (88.22%)	0.856 (1)	0.283	0.595 (1)	1.097 (0.78 - 1.544)
	C/A	51 (12.06%)	90 (11.04%)				
	C/C	3 (0.71%)	6 (0.73%)				
rs829259	T/T	148 (34.74%)	305 (36.79%)	**0.032** (0.256)	1.449	0.229 (1)	1.112 (0.936 - 1.321)
	A/T	211 (49.53%)	411 (49.58%)				
	A/A	67 (15.73%)	113 (13.63%)				
rs17719378	A/A	188 (44.13%)	329 (39.98%)	0.134 (0.668)	1.299	0.254 (1)	0.902 (0.756 - 1.077)
	A/G	186 (43.66%)	392 (47.57%)				
	G/G	52 (12.21%)	103 (12.5%)				
rs10055954	G/G	290 (68.08%)	571 (69.38%)	0.326 (1)	0.085	0.771 (1)	1.034 (0.826 - 1.295)
	G/C	126 (29.58%)	235 (28.55%)				
	C/C	10 (2.35%)	17 (2.07%)				
rs10461656	G/G	184 (43.19%)	368 (44.82%)	0.059 (0.357)	0.646	0.422 (1)	1.077 (0.899 - 1.289)
	A/G	198 (46.48%)	376 (45.80%)				
	A/A	44 (10.33%)	77 (9.38%)				
rs7713345	C/C	304 (71.70%)	582 (72.03%)	**0.043** (0.303)	0.922	0.337 (1)	1.122 (0.888 - 1.417)
	G/C	103(24.29%)	203 (25.12%)				
	G/G	17 (4.01%)	23 (2.85%)				
rs12656462	T/T	352 (82.82%)	674 (85.53%)	**0.025** (0.224)	1.417	0.234 (1)	1.206 (0.887 - 1.639)
	A/T	70 (16.47%)	109 (13.83%)				
	A/A	3 (0.71%)	5 (0.63%)				
rs4700316	C/C	269 (63.29%)	513 (63.02%)	0.929 (1)	0.0043	0.998 (1)	1 (0.811 - 1.233)
	C/G	136 (32.00%)	272 (33.42%)				
	G/G	20 (4.71%)	29 (3.56%)				
rs7714708	C/C	175 (41.08%)	326 (40.30%)	0.253 (1)	0.308	0.579 (1)	1.051 (0.881 - 1.254)
	T/C	189 (44.37%)	386 (47.71%)				
	T/T	62 (14.55%)	97 (11.99%)				

Significant *P* values ($P < 0.05$) are indicated in bold.
a. Statistic for the Cochran-Mantel-Haenszel test for IxJxK stratified tables, which evaluates whether the *PDE4D* SNPs vary between the German and Spanish clusters.
b. The corrected *P* value is indicated in brackets.
c. Statistic for the Cochran-Mantel-Haenszel test for 2x2xK stratified tables, which evaluates the existence of associations with the disease.
Abbreviations: OR, Odds Ratio; CI, confidence interval; Caco, case-control.

3.9. *PLEKHB1* and *RAB6A* genes

3.9.1. Allelic frequencies.

3.9.1.1. Spanish Sample

The following two tables show the results from the comparisons of the allelic frequencies between the whole sample of patients and controls (table R78) and between the subsample of

hallucinatory patients and controls (table R79). The main finding is the association of rs663303 (a SNP located upstream of *PLEKHB1)* with psychosis. As it can be seen in table R78, the T allele is more frequent in the patients group. However, this significance did not survive sequential Bonferroni correction. Moreover, despite there were not significant differences when hallucinatory patients were compared to controls (table R79), rs663303 still shows a trend toward association (p = 0.0593 uncorrected).

Table R78. Comparison of the allelic frequencies of *PLEKHB1-RAB6A* polymorphisms in psychotic patients and healthy controls from the Spanish sample.

Polymorphism	Allele	Patients frequency	Controls frequency	OR (95% CI)	χ^2	P[a]
rs663303	C	0.8624	0.9077	1	5.083	**0.02416** (0.145)
	T	0.1376	0.09225	1.569 (1.058 - 2.328)		
rs940828	G	0.2171	0.2058	1	0.1875	0.665 (1)
	T	0.7829	0.7942	0.9343 (0.6867 - 1.271)		
rs3741147	G	0.152	0.1954	1	3.281	0.07007 (0.350)
	T	0.848	0.8046	1.355 (0.9747 - 1.884)		
rs12274970	C	0.3013	0.319	1	0.3722	0.5418 (1)
	T	0.6987	0.681	1.086 (0.833 - 1.416)		
rs11235876	A	0.4847	0.5	1	0.2373	0.6261 (1)
	G	0.5153	0.5	1.063 (0.8312 - 1.36)		
rs7127066	C	0.2402	0.2238	1	0.377	0.5392 (1)
	G	0.7598	0.7762	0.9123 (0.6806 – 1.223)		

Significant *P* values ($P < 0.05$) are indicated in bold.
a. The corrected *P* value is indicated in brackets.
Abbreviations: OR, Odds Ratio; CI, confidence intervals.

Table R79. Comparison of the allelic frequencies of *PLEKHB1-RAB6A* polymorphisms in hallucinatory psychotic patients and healthy controls from the Spanish sample.

Polymorphism	Allele	Hallucinatory patients frequency	Controls frequency	OR (95% CI)	χ^2	P[a]
rs663303	C	0.8683	0.9077	1	3.558	0.0593 (0.356)
	T	0.1317	0.09225	1.493 (0.9825 – 2.268)		
rs940828	G	0.2108	0.2058	1	0.03337	0.8551 (1)
	T	0.7892	0.7942	0.9699 (0.6986 – 1.346)		
rs3741147	G	0.1522	0.1954	1	2.85	0.0914 (0.457)
	T	0.8478	0.8046	1.353 (0.9517 – 1.924)		
rs12274970	C	0.2957	0.319	1	0.5737	0.4488 (1)
	T	0.7043	0.681	1.116 (0.8406 – 1.48)		
rs11235876	A	0.4866	0.5	1	0.1627	0.6867 (1)
	G	0.5134	0.5	1.055 (0.8126 – 1.37)		
rs7127066	C	0.2231	0.2238	1	0.00064	0.9798 (1)
	G	0.7769	0.7762	1.004 (0.7325 – 1.376)		

a. The corrected *P* value is indicated in brackets.
Abbreviations: OR, Odds Ratio; CI, confidence intervals.

Interestingly, when other subsets of patients were considered, the T allele of SNP rs663303 was significantly associated with those disease traits, as it can be seen in table R80.

Table R80. Significant results from the remaining case-control association analysis which compared the allelic frequencies of *PLEKHB1-RAB6A* polymorphisms between different groups from the Spanish sample.

Comparison	Polymorphism	*P* Value[a]
Schizophrenic patients vs controls	rs663303	**0.02817** (0.169)
Patients without AH vs controls	rs663303	**0.04932** (0.296)
Schizophrenic patients with AH vs controls	rs663303	**0.04557** (0.273)

Significant *P* values (P < 0.05) are indicated in bold.
a. The corrected *P* value is indicated in brackets.
Abbreviation: vs, versus; AH, auditory hallucinations.

3.9.1.2. German Sample

In contrast to the findings obtained with the Spanish individuals, no differences in the allelic frequencies were found when the controls and patients of German origin were compared to each other (table R81). Results also remained not significant when only the subset of schizophrenics or the group of bipolar patients were compared to controls (data not shown).

Table R81. Comparison of the allelic frequencies of *PLEKHB1-RAB6A* polymorphisms in psychiatric patients and healthy controls from the German sample.

Polymorphism	Allele	Patients frequency	Controls frequency	OR (95% CI)	χ^2	P[a]
rs663303	C	0.8807	0.8736	1	0.1884	0.6642 (1)
	T	0.1193	0.1264	0.9365 (0.6963 - 1.259)		
rs4944850	A	0.8571	0.846	1	0.332	0.5645 (1)
	C	0.1429	0.154	0.9159 (0.6793 - 1.235)		
rs591804	A	0.6864	0.6746	1	0.2159	0.6422 (1)
	G	0.3136	0.3254	0.9475 (0.7548 - 1.189)		
rs6592527	C	0.247	0.2183	1	1.576	0.2093 (1)
	G	0.753	0.7817	0.8515 (0.6625 - 1.095)		
rs940828	G	0.234	0.2137	1	0.9704	0.3246 (1)
	T	0.766	0.7863	0.8897 (0.7051 - 1.123)		
rs3741147	G	0.1136	0.1105	1	0.04078	0.84 (1)
	T	0.8864	0.8895	0.9688 (0.7126 - 1.317)		
rs12274970	C	0.2205	0.2341	1	0.4244	0.5148 (1)
	T	0.7795	0.7659	1.08 (0.8565 - 1.362)		
rs10736793	A	0.1627	0.1704	1	0.1492	0.6993 (1)
	C	0.8373	0.8296	1.058 (0.796 - 1.405)		
rs11235876	A	0.4335	0.4204	1	0.2879	0.5916 (1)
	G	0.5665	0.5796	0.9478 (0.7791 - 1.153)		
rs11235880	A	0.1934	0.1784	1	0.5095	0.4754 (1)
	C	0.8066	0.8216	0.9057 (0.69 - 1.189)		
rs7127066	C	0.2553	0.2378	1	0.6744	0.4115 (1)
	G	0.7447	0.7622	0.9103 (0.7273 - 1.139)		

a. The corrected *P* value is indicated in brackets.
Abbreviations: OR, Odds Ratio; CI, confidence intervals.

3.9.2. Genotypic frequencies.

3.9.2.1. Spanish Sample

The results from the main comparisons of the genotypic frequencies can be found at tables R82 (all patients against controls) and R83 (hallucinatory patients against controls). According to the findings explained in the previous section, SNP rs663303, located upstream of the *PLEKHB1* gene, was associated with psychosis when all the samples of patients were considered (table R82). According to an additive model, genotypes including the T allele were found to be more frequent in the psychotic group, although the significance was lost after the correction for multiple testing.

However, when the subsample of patients with AH was compared to the control group (table R83), the significance of rs663303 disappeared and only became a trend (p = 0.06).

Table R82. Comparison of the genotypic distributions of *PLEKHB1-RAB6A* SNPs in psychotic patients and healthy controls from the Spanish sample.

SNP	Genotype	Controls frequency	Patients frequency	OR (95% CI)	*P* Value[b]	AIC	Model
rs663303	C/C	223 (82.3%)	171 (74.3%)	1.57 (1.05-2.34)[a]	**0.025** (0.15)	690.2	Additive
	T/C	46 (17%)	55 (23.9%)				
	T/T	2 (0.7%)	4 (1.7%)				
rs940828	T/T	161 (61.9%)	138 (60.3%)	1.07 (0.78-1.47)[a]	0.68 (1)	679.8	Additive
	G/T	91 (35%)	83 (36.2%)				
	G/G	8 (3.1%)	8 (3.5%)				
rs3741147	T/T	187 (65.8%)	163 (71.5%)	0.75 (0.55-1.04)[a]	0.085 (0.425)	704.7	Additive
	G/T	83 (29.2%)	60 (26.3%)				
	G/G	14 (4.9%)	5 (2.2%)				
rs12274970	T/T - T/C	254 (87.6%)	205 (89.1%)	1.00	0.59 (1)	717.6	Recessive
	C/C	36 (12.4%)	25 (10.9%)	0.86 (0.50-1.48)			
rs11235876	G/G - G/A	209 (73.3%)	177 (77%)	1.00	0.34 (1)	711.2	Recessive
	A/A	76 (26.7%)	53 (23%)	0.82 (0.55-1.23)			
rs7127066	G/G	167 (60.3%)	133 (57.8%)	1.10 (0.82-1.47)[a]	0.51 (1)	702.1	Additive
	C/G	96 (34.7%)	83 (36.1%)				
	C/C	14 (5%)	14 (6.1%)				

Significant *P* values (P < 0.05) are indicated in bold.
a. The indicated value corresponds to the difference which can be attributed to each allele in the additive model.
b. The corrected *P* value is indicated in brackets.
Abbreviations: OR, Odds Ratio; CI, confidence intervals.

Table R83. Comparison of the genotypic distributions of *PLEKHB1-RAB6A* SNPs in hallucinatory patients and healthy controls from the Spanish sample.

SNP	Genotype	Controls frequency	Hallucinatory patients frequency	OR (95% CI)	*P* Value[b]	AIC	Model
rs663303	C/C	223 (82.3%)	140 (75.3%)	1.50 (0.98-2.29)[a]	0.06 (0.36)	618.1	Additive
	T/C	46 (17%)	43 (23.1%)				
	T/T	2 (0.7%)	3 (1.6%)				
rs940828	T/T	161 (61.9%)	114 (61.6%)	1.03 (0.74-1.45)[a]	0.85 (1)	608.2	Additive
	G/T	91 (35%)	64 (34.6%)				
	G/G	8 (3.1%)	7 (3.8%)				
rs3741147	T/T	187 (65.8%)	133 (72.3%)	0.75 (0.53-1.06)[a]	0.098 (0.49)	628.5	Additive
	G/T	83 (29.2%)	46 (25%)				
	G/G	14 (4.9%)	5 (2.7%)				
rs12274970	T/T - T/C	254 (87.6%)	168 (90.3%)	1.00	0.35 (1)	640.1	Recessive
	C/C	36 (12.4%)	18 (9.7%)	0.76 (0.42-1.38)			
rs11235876	G/G - G/A	209 (73.3%)	144 (77.4%)	1.00	0.32 (1)	635	Recessive
	A/A	76 (26.7%)	42 (22.6%)	0.80 (0.52-1.24)			
rs7127066	G/G - C/G	263 (95%)	175 (94.1%)	1.00	0.69 (1)	627.7	Recessive
	C/C	14 (5%)	11 (5.9%)	1.18 (0.52-2.66)			

a. The indicated value corresponds to the difference which can be attributed to each allele in the additive model.
b. The corrected *P* value is indicated in brackets
Abbreviations: OR, Odds Ratio; CI, confidence intervals; N/A, not applicable.

Moreover, there were some significant associations in the remaining comparisons which also involved the SNP rs663303. A summary of these positive results can be found at table R84.

Table R84. Significant results from the case-control association analysis which compared the genotypic distribution of *PLEKHB1-RAB6A* polymorphisms between different groups from the Spanish sample.

Comparison	Polymorphism	*P* Value[a]	Model
Schizophrenic patients vs controls	rs663303	**0.027** (0.162)	Additive
Patients without AH vs controls	rs663303	**0.048** (0.282)	Additive
Schizophrenic patients with AH vs controls	rs663303	**0.045** (0.270)	Additive

a. The corrected *P* value is indicated in brackets.
Abbreviations: vs, versus; AH, auditory hallucinations.

3.9.2.2. German Sample

The results from the case-control analysis of the genotypic frequencies can be found at tables R85 (all patients against controls), R86 (schizophrenic patients compared to controls) and R87 (bipolar patients against controls). In the three comparisons some significant associations have been found:

- There are significant differences in the genotypic distribution of rs6592527 (*PLEKHB1* gene) between the whole sample of patients and controls (table R85) and between the subset of bipolar patients and controls (table R87). In both cases the genotypic

distribution fitted a recessive inheritance model, where the C/C genotype was found to be more frequent in the affected group.

- SNP rs940828 (*PLEKHB1* gene) gave significant findings in the three comparisons (tables R85-R87). In summary, individuals who were homozygotes for the G allele were more frequent in the affected group.

- Regarding to rs7127066 (*RAB6A* gene), there were significant differences when all patients were compared to controls (table R85) and when the subsample of schizophrenic subjects was compared to the control group (table R86). In both situations, the C/C genotype was more frequent in the affected group than in the control group. However, when bipolar patients were compared to control subjects (table R87) this significant result became only a trend.

- Finally, the SNP rs4944850 was found to be associated to bipolar disorder (table R87), since the C/C genotype was found to be more frequent in the bipolar group than in the control group.

However, all these findings should be taken cautiously due to the low frequency of the four risk genotypes. Moreover, neither of the *P* values resisted the sequential Bonferroni correction for multiple testing.

Table R85. Comparison of the genotypic distributions of *PLEKHB1-RAB6A* SNPs in psychiatric patients and healthy controls from the German sample.

SNP	Genotype	Controls frequency	Patients frequency	OR (95% CI)	*P* Value[a]	AIC	Model
rs663303	C/C	408 (76.4%)	260 (78.5%)	1.00	0.46 (1)	1154.5	Dominant
	T/C - T/T	126 (23.6%)	71 (21.4%)	0.88 (0.64-1.23)			
	C/C - T/C	525 (98.3%)	323 (97.6%)	1.00	0.46 (1)	1154.5	Recessive
	T/T	9 (1.7%)	8 (2.4%)	1.44 (0.55-3.78)			
rs4944850	A/A	255 (72%)	248 (75.4%)	1.00	0.32 (1)	948.9	Dominant
	C/A - C/C	99 (28%)	81 (24.6%)	0.84 (0.60-1.18)			
rs591804	A/A	162 (45.6%)	157 (47.6%)	1.00	0.61 (1)	952.4	Dominant
	A/G - G/G	193 (54.4%)	173 (52.4%)	0.92 (0.68-1.25)			
rs6592527	G/G - C/G	341 (96.1%)	304 (92.1%)	1.00	**0.027** (0.243)	947.8	Recessive
	C/C	14 (3.9%)	26 (7.9%)	2.08 (1.07-4.06)			
rs940828	T/T - G/T	510 (96%)	302 (91.8%)	1.00	**0.0094** (0.103)	1141.6	Recessive
	G/G	21 (4%)	27 (8.2%)	2.17 (1.21-3.91)			
rs3741147	T/T	422 (79%)	258 (78.2%)	1.00	0.77 (1)	1153	Dominant
	G/T - G/G	112 (21%)	72 (21.8%)	1.05 (0.75-1.47)			
	T/T - G/T	528 (98.9%)	327 (99.1%)	1.00	0.76 (1)	1153	Recessive
	G/G	6 (1.1%)	3 (0.9%)	0.81 (0.20-3.25)			
rs12274970	T/T	315 (59%)	204 (61.6%)	1.00	0.44 (1)	1154.5	Dominant
	T/C - C/C	219 (41%)	127 (38.4%)	0.90 (0.68-1.19)			
rs10736793	C/C - C/A	346 (97.5%)	326 (98.2%)	1.00	0.51 (1)	955.2	Recessive
	A/A	9 (2.5%)	6 (1.8%)	0.71 (0.25-2.01)			

Table R85 (continuation). Comparison of the genotypic distributions of *PLEKHB1-RAB6A* SNPs in psychiatric patients and healthy controls from the German sample.

SNP	Genotype	Controls frequency	Patients frequency	OR (95% CI)	*P* Value[a]	AIC	Model
rs11235876	G/G - G/A	447 (83.7%)	267 (80.7%)	1.00	0.25 (1)	1153.8	Recessive
	A/A	87 (16.3%)	64 (19.3%)	1.23 (0.86-1.76)			
rs11235880	C/C - C/A	343 (96.3%)	311 (94%)	1.00	0.14 (1)	953.3	Recessive
	A/A	13 (3.6%)	20 (6%)	1.70 (0.83-3.47)			
rs7127066	G/G - C/G	506 (94.8%)	300 (90.6%)	1.00	**0.021** (0.21)	1149.8	Recessive
	C/C	28 (5.2%)	31 (9.4%)	1.87 (1.10-3.17)			

Significant *P* values (P < 0.05) are indicated in bold.
a. The corrected *P* value is indicated in brackets.
Abbreviations: OR, Odds Ratio; CI, confidence intervals.

Table R86. Comparison of the genotypic distributions of *PLEKHB1* and *RAB6A* SNPs in schizophrenic patients and healthy controls from the German sample.

SNP	Genotype	Controls frequency	Schizophrenic patients frequency	OR (95% CI)	*P* Value[b]	AIC	Model
rs663303	C/C	408 (76.4%)	191 (78.6%)	1.00	0.5 (1)	969	Dominant
	T/C - T/T	126 (23.6%)	52 (21.4%)	0.88 (0.61-1.27)			
rs4944850	A/A	255 (72%)	188 (78%)	1.00	0.099 (0.891)	804.5	Dominant
	C/A - C/C	99 (28%)	53 (22%)	0.73 (0.50-1.07)			
rs591804	A/A	162 (45.6%)	119 (49%)	0.87 (0.68-1.12)[a]	0.28 (1)	810.8	Additive
	A/G	155 (43.7%)	104 (42.8%)				
	G/G	38 (10.7%)	20 (8.2%)				
rs6592527	G/G - C/G	341 (96.1%)	226 (93%)	1.00	0.1 (0.891)	809.2	Recessive
	C/C	14 (3.9%)	17 (7%)	1.83 (0.89-3.79)			
rs940828	T/T - G/T	510 (96%)	223 (92.5%)	1.00	**0.045** (0.495)	958.6	Recessive
	G/G	21 (4%)	18 (7.5%)	1.96 (1.02-3.75)			
rs3741147	T/T	422 (79%)	196 (80.7%)	0.90 (0.63-1.28)[a]	0.56 (1)	969.1	Additive
	G/T	106 (19.9%)	45 (18.5%)				
	G/G	6 (1.1%)	2 (0.8%)				
rs12274970	T/T	315 (59%)	153 (63%)	1.00	0.29 (1)	968.4	Dominant
	T/C - C/C	219 (41%)	90 (37%)	0.85 (0.62-1.16)			
rs10736793	C/C - C/A	346 (97.5%)	240 (98.4%)	1.00	0.45 (1)	813.1	Recessive
	A/A	9 (2.5%)	4 (1.6%)	0.64 (0.20-2.10)			
rs11235876	G/G - G/A	447 (83.7%)	199 (81.9%)	1.00	0.53 (1)	969.1	Recessive
	A/A	87 (16.3%)	44 (18.1%)	1.14 (0.76-1.69)			
rs11235880	C/C - C/A	343 (96.3%)	229 (93.8%)	1.00	0.16 (1)	812.8	Recessive
	A/A	13 (3.6%)	15 (6.2%)	1.73 (0.81-3.70)			
rs7127066	G/G - C/G	506 (94.8%)	221 (91%)	1.00	**0.051** (0.51)	965.6	Recessive
	C/C	28 (5.2%)	22 (9.1%)	1.80 (1.01-3.21)			

Significant *P* values (P < 0.05) are indicated in bold.
a. The indicated value corresponds to the difference which can be attributed to each allele in the additive model.
b. The corrected *P* value is indicated in brackets
Abbreviations: OR, Odds Ratio; CI, confidence interval.

Table R87. Comparison of the genotypic distributions of *PLEKHB1* and *RAB6A* SNPs in bipolar patients and healthy controls from the German sample.

SNP	Genotype	Controls frequency	Bipolar patients frequency	OR (95% CI)	*P* Value[b]	AIC	Model
rs663303	C/C - T/C	525 (98.3%)	85 (96.6%)	1.00	0.32 (1)	510.1	Recessive
	T/T	9 (1.7%)	3 (3.4%)	0.49 (0.13-1.83)			
rs4944850	A/A - C/A	344 (97.2%)	81 (92%)	1.00	**0.04** (0.36)	441	Recessive
	C/C	10 (2.8%)	7 (8%)	0.34 (0.12-0.91)			
rs591804	A/A - A/G	317 (89.3%)	73 (83.9%)	1.00	0.18 (0.9)	440.6	Recessive
	G/G	38 (10.7%)	14 (16.1%)	0.63 (0.32-1.21)			
rs6592527	G/G - C/G	341 (96.1%)	78 (89.7%)	1.00	**0.027** (0.27)	437.5	Recessive
	C/C	14 (3.9%)	9 (10.3%)	0.36 (0.15-0.85)			
rs940828	T/T	325 (61.2%)	57 (64.8%)	1.00	**0.024** (0.264)	504.8	Codominant
	G/T	185 (34.8%)	22 (25%)	1.47 (0.87-2.49)			
	G/G	21 (4%)	9 (10.2%)	0.41 (0.18-0.94)			
rs3741147	T/T	422 (79%)	62 (71.3%)	1.00	0.11 (0.77)	504.7	Dominant
	G/T - G/G	112 (21%)	25 (28.7%)	0.66 (0.40-1.09)			
rs12274970	T/T	315 (59%)	51 (58%)	1.00	0.86 (1)	511.1	Dominant
	T/C - C/C	219 (41%)	37 (42%)	0.96 (0.61-1.51)			
	T/T - T/C	503 (94.2%)	83 (94.3%)	1.00	0.96 (1)	511.1	Recessive
	C/C	31 (5.8%)	5 (5.7%)	1.02 (0.39-2.71)			
	T/T	315 (59%)	51 (58%)	0.98 (0.67-1.41)[a]	0.9 (1)	511.1	Additive
	T/C	188 (35.2%)	32 (36.4%)				
	C/C	31 (5.8%)	5 (5.7%)				
rs10736793	C/C	243 (68.5%)	58 (65.9%)	1.00	0.65 (1)	445.5	Dominant
	C/A - A/A	112 (31.6%)	30 (34.1%)	0.89 (0.54-1.46)			
rs11235876	G/G - G/A	447 (83.7%)	68 (77.3%)	1.00	0.15 (0.9)	509	Recessive
	A/A	87 (16.3%)	20 (22.7%)	0.66 (0.38-1.15)			
rs11235880	C/C - C/A	343 (96.3%)	82 (94.2%)	1.00	0.4 (1)	442.2	Recessive
	A/A	13 (3.6%)	5 (5.8%)	0.62 (0.22-1.79)			
rs7127066	G/G - C/G	506 (94.8%)	79 (89.8%)	1.00	0.089 (0.712)	508.2	Recessive
	C/C	28 (5.2%)	9 (10.2%)	0.49 (0.22-1.07)			

Significant *P* values (P < 0.05) are indicated in bold.
a. The indicated value corresponds to the difference which can be attributed to each allele in the additive model.
b. The corrected *P* value is indicated in brackets.
Abbreviations: OR, Odds Ratio; CI, confidence interval.

Unfortunately, when comparing the results obtained in the Spanish and the German sample for the SNPs which had been genotyped in both samples, no similarities have been found, since the positive SNPs are completely different between both samples.

3.9.3. Haplotype analysis.

3.9.3.1. Spanish Sample

The seven common 6-marker haplotypes from the Spanish sample, inferred from the genotypic data, can be found at table R88. Frequencies of these seven common haplotypes did not differ between patients and controls.

Table R88. Overview of the most common *PLEKHB1-RAB6A* haplotypes observed in the Spanish sample.

HAPLOTYPE							
rs663303	**rs940828**	**rs3741147**	**rs12274970**	**rs11235876**	**rs7127066**	**Patients frequency**	**Controls frequency**
C	T	T	T	G	G	0.5057	0.5095
C	T	G	C	A	G	0.1349	0.1719
C	T	T	C	A	C	0.06742	0.07122
C	G	T	T	A	C	0.06643	0.06333
T	G	T	T	A	G	0.05211	0.04293
T	G	T	C	A	C	0.08056	0.0414
C	T	T	T	A	G	0.04314	0.03793
All rare (freq < 0.03)						0.04974	0.06179

Note: the global *P* value (uncorrected) was 0.2093 (not significant). The patients' data refer to the whole sample of psychotic patients.
Abbreviations: freq, frequency; OR, Odds Ratio; CI, confidence intervals; N/A, not applicable.

A case-control analysis of the 2, 3 and 4-marker haplotype frequencies was finally performed with the 6 SNPs, according to the methodology explained in the Materials and Methods section. As it is shown in table R89, two risk haplotypes were found, implicating SNPs rs663303, rs940828 and rs3741147. Haplotype C-G-G is especially interesting because it remained significant after a 1000-permutation run. Curiously, despite both haplotypes have a higher frequency in patients, they contain different alleles for rs663303 and rs3741147, suggesting that the real risk allele would be allele G from rs940828, as it was detected in the analysis of the allelic and genotypic frequencies. Moreover, the same risk haplotypes were found when the subsets of hallucinatory patients or schizophrenic patients were considered (data not shown).

Table R89. *PLEKHB1-RAB6A* haplotypes significantly associated with psychosis in the Spanish sample.

HAPLOTYPE										
rs663303	**rs940828**	**rs3741147**	**rs12274970**	**rs11235876**	**rs7127066**	**Patients frequency**	**Controls frequency**	**df**	**Global *P* value**	**Haplotype *P* value[a]**
C	G	G				0.007113	0.03069	4	**0.0235**	**0.00785 (0.04795)**
T	G	T				0.1394	0.09426	4	**0.0235**	**0.03088** (> 0.05)

Significant *P* values (P < 0.05) are indicated in bold.
a. The corrected *P* value after 1000 permutations is indicated in brackets. Abbreviation: df, degrees of freedom.

3.9.3.2. German Sample

Eight common 11-marker haplotypes (frequency > 0.03) were inferred from the genotype data in the German samples. Frequencies of those haplotypes in both patients and healthy subjects can be found at table R90. However, no significant differences were found between both groups in the frequencies of 2, 3 and 4-marker haplotypes (data not shown).

Table R90. Overview of the most common *PLEKHB1-RAB6A* haplotypes observed in the German sample.

HAPLOTYPE												
rs663303	**rs4944850**	**rs591804**	**rs6592527**	**rs940828**	**rs3741147**	**rs12274970**	**rs10736793**	**rs11235876**	**rs11235880**	**rs7127066**	**Patients frequency**	**Controls frequency**
C	A	A	G	T	T	T	C	G	C	G	0.5138	0.5252
C	C	G	G	T	G	C	A	A	C	G	0.08073	0.09569
T	A	G	C	G	T	C	C	A	A	C	0.07609	0.07428
C	A	A	C	G	T	T	C	A	C	C	0.05745	0.04607
C	A	G	G	T	T	C	C	A	A	C	0.04658	0.04713
C	C	G	G	T	T	T	C	G	C	G	0.04348	0.04579
T	A	G	C	G	T	T	A	A	C	G	0.0372	0.03851
C	A	A	C	G	T	T	C	A	A	C	0.03106	0.01849
All rare (freq < 0.03)											0.11361	0.10884

Note: the global *P* value (uncorrected) was 0.7793 (not significant). The patients' data refer to the whole sample of psychiatric patients.
Abbreviations: freq, frequency; OR, Odds Ratio; CI, confidence intervals; N/A, not applicable.

3.9.4. Pooled case-control association analysis.

The German and the Spanish samples were combined to perform an association analysis of the genotypic frequencies according to the methodology explained in the Materials and Methods section. All control subjects, as well as those patients diagnosed as schizophrenics, were included in this part of the study.

The six SNPs from *PLEKHB1* and *RAB6A* genes which had been genotyped in both the Spanish and the German sample were included.

The results from the analysis with the pooled sample of patients and controls are shown at table R91. As it is shown, the distribution of three SNPs (rs3741147, rs12235876 and rs11235876) showed highly significant differences depending on the country of origin (the corrected *P* value from the Cochran-Mantel-Haenszel test for IxJxK stratified tables was also significant for these SNPs). These differences can make it difficult to perform and interpret a study with a pooled sample of individuals from different origin.
In any case, no differences were found between patients or controls for any of the six SNPs.

Table R91. Results from the case-control association analysis between *PLEKHB1-RAB6A* SNPs and schizophrenia when studying the pooled sample of German and Spanish schizophrenic patients.

SNP	Genotype	Schizophrenic patients frequency	Controls frequency	Heterogeneity *P* value[a,b]	$\chi^{2\,c}$	Caco *P* value[b]	OR (95% CI)
rs663303	C/C	326 (76.53%)	631 (78.39%)	0.284 (0.584)	0.847	0.357 (1)	1.132 (0.871 - 1.47)
	T/C	92 (21.60%)	163 (20.25%)				
	T/T	8 (1.88%)	11 (1.37%)				
rs940828	T/T	253 (59.81%)	486 (61.44%)	0.509 (0.589)	1.313	0.252 (1)	1.128 (0.918 - 1.384)
	G/T	145 (34.38%)	276 (34.89%)				
	G/G	25 (5.91%)	29 (3.67%)				
rs3741147	T/T	326 (76.71%)	609 (74.45%)	**0.000143 (0.00086)**	2.747	0.0974 (0.585)	0.808 (0.627 - 1.041)
	G/T	93 (21.88%)	189 (23.11%)				
	G/G	6 (1.41%)	20 (2.44%)				
rs12274970	T/T	246 (57.75%)	456 (55.34%)	**0.00478 (0.019)**	1.936	0.164 (0.821)	0.870 (0.715 - 1.058)
	T/C	150 (35.21%)	301 (36.53%)				
	C/C	30 (7.04%)	67 (8.13%)				
rs11235876	G/G	123 (28.87%)	248 (30.28%)	**0.00107 (0.0053)**	0.0088	0.925 (1)	1.008 (0.851 - 1.195)
	G/A	219 (51.41%)	408 (49.82%)				
	A/A	84 (19.72%)	163 (19.90%)				
rs7127066	G/G	249 (58.45%)	475 (58.57%)	0.195 (0.584)	0.933	0.334 (1)	1.103 (0.904 - 1.345)
	C/G	144 (33.80%)	294 (36.25%)				
	C/C	33 (7.75%)	42 (5.18%)				

Significant *P* values ($P < 0.05$) are indicated in bold.
a. Statistic for the Cochran-Mantel-Haenszel test for IxJxK stratified tables, which evaluates whether the *PLEKHB1-RAB6A* SNPs vary between the German and Spanish clusters.
b. The corrected *P* value is indicated in brackets.
c. Statistic for the Cochran-Mantel-Haenszel test for 2x2xK stratified tables, which evaluates the existence of associations with the disease.
Abbreviations: OR, Odds Ratio; CI, confidence interval; Caco, case-control.

4. Analysis of quantitative variables and other disease traits.

We used the clinical information available for the Spanish patients to investigate the effects of genetic variation on the scores of several clinical scales (BPRS, KGV, PANSS, PSYRATS for AH and delusions) as well as other disease traits (age of onset and response to treatment against AH). Furthermore, information about seven cognitive factors derived from different neuropsychological tests (as it is explained in the Materials and Methods section) was available for a subset of individuals (including both control subjects and schizophrenic patients) from the American sample and it was used to test the effect of *ASPM* gene on the cognitive performance of those individuals.

4.1. Estimation of the statistical power

Before the association analysis, the statistical power to detect association between genetic polymorphisms and quantitative variables was also calculated (table R92). This power was low when we considered that the proportion of variation explained by each polymorphism (R_g^2) was

around 1%. However, for percentages of explained variance around 30% the power to detect association was found to be very high. However, it is less probable than one SNP explains such a high percentage of the variance of a quantitative trait.

Table R92. Estimation of the statistical power for association studies with quantitative variables.

Type of study	Sample	N	Statistical Power	
			$R_g^2 = 0.01$	$R_g^2 = 0.3$
Analysis of quantitative variables: KGV scale	Spanish	270 patients	0.3774	0.9999
Analysis of quantitative variables: BPRS and PANSS scales	Spanish	130 patients	0.2079	0.9999
Analysis of quantitative variables: PSYRATS for AH	Spanish	210 patients	0.3063	0.9999
Analysis of quantitative variables: PSYRATS for delusions	Spanish	155 patients	0.2328	0.9999
Analysis of quantitative variables: Cognitive factors	American	430 Controls	0.5473	0.9999
		350 Patients	0.4664	0.9999

R_g^2: proportion of variation in the quantitative variable explained by the effect of certain polymorphism.

4.2. *SLC6A4* gene

The following tables show the significant findings from the lineal regression analyses between *SLC6A4* polymorphisms and different quantitative variables corresponding to different clinical scales.

4.2.1. KGV scale.

Table R93 shows the significant results for the KGV scale items. In summary, 5-HTTLPR promoter polymorphism was associated with 3 parameters (Total Score, Anxiety item and Delusions item) and fitted an additive or dominant model, whereas other polymorphisms where associated with other items (rs2066713 with Total Score, rs2020942 and STin2 with Hallucinations item and both rs12945042 and rs2020936 with Incoherence of Speech). Unfortunately, any result remained significant after correction for multiple testing.

Table R93. Significant results from the association analysis of *SLC6A4* polymorphisms and KGV scale parameters.

Polymorphism	Genotypic distribution	Average response (SE)	Difference (95% CI)	Model	*P* Value[b]	BIC
KGV total score						
5-HTTLPR	high/high : 78	9.77 (0.66)	-1.06 (-2.02 - -0.10)[a]	Additive	**0.031** (0.341)	1689.7
	high/low : 135	8.76 (0.48)				
	low/low : 54	7.78 (0.68)				
rs2066713	C/C - C/T : 236	8.61 (0.35)	0.00	Recessive	**0.046** (0.46)	1690.4
	T/T : 31	10.68 (1.14)	2.12 (0.04 - 4.19)			
KGV - hallucinations						
rs2020942	G/G -A/G : 231	1.29 (0.11)	0.00	Recessive	**0.025** (0.275)	1062.3
	A/A : 36	1.97 (0.31)	0.69 (0.09 - 1.29)			
STin2	12/12 - 12/10 : 222	1.3 (0.11)	0.00	Recessive	**0.03** (0.3)	1018.1
	10/10:34	1.97 (0.32)	0.69 (0.07 - 1.30)			

Table R93 (continuation). Significant results from the association analysis of *SLC6A4* polymorphisms and KGV scale parameters.

Polymorphism	Genotypic distribution	Average response (SE)	Difference (95% CI)	Model	*P* Value[b]	BIC
KGV - anxiety						
5-HTTLPR	low/low : 78	1.72 (0.119)	0.00	Dominant	**0.04** (0.44)	789
	high/low-high/high: 135	1.44 (0.08)	-0.29 (-0.57 - -0.01)			
KGV - delusions						
5-HTTLPR	low/low : 78	1.6 (0.18)	-0.36 (-0.62 - -0.09)[a]	Additive	**0.008** (0.088)	994.5
	high/low : 135	1.43 (0.13)				
	high/high : 54	0.91 (0.17)				
KGV - incoherence of speech						
rs12945042	G/G : 119	0.87 (0.11)	0.00	Dominant	**0.037** (0.37)	741.4
	G/A -G/G : 120	0.57 (0.09)	-0.30 (-0.58 - -0.02)			
rs2020936	T/T : 190	0.75 (0.08)	-0.26 (-0.49 - -0.03)[a]	Additive	**0.025** (0.275)	799.5
	T/C : 66	0.48 (0.1)				
	C/C : 11	0.27 (0.19)				

Significant *P* values (P < 0.05) are indicated in bold.
a. The indicated value corresponds to the difference which can be attributed to each allele in the additive model.
b. The corrected *P* value is indicated in brackets
Abbreviations: SE, standard error; CI, confidence interval.

4.2.2. BPRS scale.

Regarding BPRS scale (see table R94), two polymorphisms (5-HTTLPR, located in the promoter region, and rs4251417, in intron 1A) were found to be associated with the BPRS total score. However, after Bonferroni sequential correction, *P* values are not significant.

Table R94. Significant results from the association analysis of *SLC6A4* polymorphisms and BPRS parameters.

Polymorphism	Genotypic distribution	Average response (SE)	Difference (95% CI)	Model	*P* Value[b]	BIC
BPRS total score						
rs4251417	G/G : 88	46.1 (1.28)	0.00	N/A	**0.011** (0.121)	842.4
	A/G : 20	39.6 (2.13)	-7.17 (-12.58 - -1.76)			
5-HTTLPR	low/low : 37	47.16 (2.08)	-3.24 (-5.99 - -0.50)[a]	Additive	**0.022** (0.22)	1012.9
	high/low : 61	43.9 (1.6)				
	high/high : 31	40.68 (1.83)				

Significant *P* values (P < 0.05) are indicated in bold.
a. The indicated value corresponds to the difference which can be attributed to each allele in the additive model.
b. The corrected *P* value is indicated in brackets
Abbreviations: SE, standard error; CI, confidence interval; N/A, not applicable.

4.2.3. PANSS.

Table R95 shows those polymorphisms which were significantly associated with the different PANSS scale parameters. Given the high number of items and polymorphisms, the number of positive findings is also high. However, some of these results are not reliable, since the number of individuals in one of the genotypic groups is low. This can be applied to the associations between rs2020942, STin2 and rs2066713 with Guilt item (G3); rs12945042 and item N6 (Spontaneity and flow of conversation); rs2066713 and item P6 (Suspiciousness); and finally, all associations implicating SNP rs140700. By contrast, the most interesting findings are the associations of 5-HTTLPR with three parameters from de the positive subscale: PANSS positive score (p = 0.0079, not significant after correction but showing a trend), P4 – Excitement (p = 0.015, corrected value) and P7 – Hostility (p = 0.0044, corrected value).

Table R95. Significant results from the association analysis of *SLC6A4* polymorphisms and PANSS parameters.

Polymorphism	Genotypic distribution	Average response (SE)	Difference (95% CI)	Model	*P* Value[b]	BIC
PANSS total score						
rs4251417	G/G : 88 A/G : 20	62.07 (1.84) 54.25 (3.01)	0.00 -8.77 (-16.56 - -0.99)	N/A	**0.029** (0.319)	920.9
PANSS General score						
rs4251417	G/G : 88 A/G : 20	31.72 (0.89) 27.9 (1.7)	0.00 -4.14 (-8.06 - -0.23)	N/A	**0.041** (0.451)	772.6
PANSS G3 - Guilt feelings						
rs2020942	G/G - A/G: 116 A/A : 13	1.65 (0.1) 2.77 (0.39)	0.00 1.15 (0.48 - 1.81)	Recessive	**0.0004** (**0.004**)	418.1
STin2	12/12-10/12: 110 10/10 : 14	1.68 (0.11) 2.64 (0.39)	0.00 0.98 (0.32 - 1.63)	Recessive	**0.0041** (**0.041**)	407.6
rs2066713	C/C - C/T : 119 T/T : 10	1.69 (0.11) 2.6 (0.45)	0.00 0.96 (0.20 - 1.73)	Recessive	**0.015** (0.135)	423.4
PANSS G4 - Tension						
rs140700	G/G : 96 A/G : 12	1.83 (0.1) 3 (0.46)	0.00 1.17 (0.55 - 1.80)	N/A	**0.0004** (**0.044**)	331.1
rs2020936	T/T : 92 T/C - C/C : 37	1.83 (0.09) 2.3 (0.23)	0.00 0.48 (0.08 - 0.88)	Dominant	**0.021** (0.21)	393.3
PANSS G7 -Motor retardation						
rs12945042	G/G : 54 G/A : 50 A/A :4	1.74 (0.12) 2.02 (0.19) 3 (0.71)	0.40 (0.02 - 0.79)[a]	Additive	**0.044** (0.484)	353.6
PANSS G9 -Unusual thoughts						
rs140700	G/G : 96 A/G : 12	1.43 (0.1) 2.42 (0.57)	0.00 0.99 (0.31 - 1.67)	N/A	**0.0051** (0.0561)	349.3
PANSS G10 -Disorientation						
rs2020936	T/T : 92 T/C - C/C : 37	1.61 (0.09) 1.27 (0.08)	0.00 -0.35 (-0.64 - -0.05)	Dominant	**0.025** (0.275)	318.7

Table R95 (continuation). Significant results from the association analysis of *SLC6A4* polymorphisms and PANSS parameters.

Polymorphism	Genotypic distribution	Average response (SE)	Difference (95% CI)	Model	*P* Value[b]	BIC
PANSS G14 - Poor impulse control						
rs140700	G/G : 96	1.7 (0.11)	0.00	N/A	**0.037** (0.333)	345.8
	A/G : 12	2.42 (0.36)	0.72 (0.05 - 1.39)			
rs2020939	C/C : 33	2 (0.2)	0.00	Codominant	**0.023** (0.231)	352.6
	C/T : 56	1.46 (0.12)	-0.53 (-1.00 - -0.06)			
	T/T : 21	2.14 (0.3)	0.13 (-0.47 - 0.74)			
5-HTTLPR	low/low : 37	2.11 (0.21)	0.00	Dominant	**0.021** (0.231)	398.9
	high/low-high/high:92	1.63 (0.1)	-0.48 (-0.89 - -0.08)			
PANSS N5 - Difficulty in abstract thinking						
rs3813034	T/T : 40	3.33 (0.31)	0.00	Dominant	**0.04** (0.4)	532.4
	G/T - G/G : 89	2.62 (0.18)	-0.70 (-1.37 - -0.04)			
STin2	12rep/12rep : 48	2.46 (0.24)		Additive	**0.049** (0.441)	508.6
	10rep/12rep : 62	2.84 (0.22)	0.48 (0.01 - 0.96)[a]			
	10rep/10rep : 14	3.5 (0.56)				
rs2066713	C/C : 51	2.43 (0.23)		Additive	**0.029** (0.319)	531.8
	C/T : 68	3.06 (0.23)	0.57 (0.06 - 1.08)[a]			
	T/T : 10	3.4 (0.54)				
PANSS N6 - Spontaneity and flow of conversation						
rs2020939	C/C - C/T : 89	1.71 (0.1)	0.00	Recessive	**0.044** (0.440)	348.6
	T/T : 21	2.29 (0.34)	0.55 (0.02 - 1.07)			
rs12945042	G/G - A/G : 104	1.77 (0.1)	0.00	Recessive	**0.007** (0.077)	341.2
	A/A : 4	3.25 (0.95)	1.53 (0.44 - 2.63)			
PANSS Positive score						
rs4251417	G/G :88	14.59 (0.68)	0.00	N/A	**0.031** (0.31)	704.3
	A/G : 20	11.8 (1.11)	-3.18 (-6.04 - -0.32)			
5-HTTLPR	low/low : 37	15.54		Additive	**0.0079** (0.0869)	843.9
	high/low : 61	13.97	-1.96 (-3.39 - -0.54)[a]			
	high/high : 31	11.58				
PANSS P4 - Excitement						
5-HTTLPR	low/low : 37	2.43 (0.18)	0.00	Dominant	**0.0014** **(0.015)**	394.3
	high/low-high/high:92	1.77 (0.11)	-0.67 (-1.07 - -0.27)			
PANSS P6 - Suspiciousness						
rs3813034	T/T : 40	2.67 (0.25)		Additive	**0.016** (0.176)	468.5
	G/T : 61	2.16 (0.19)	-0.42 (-0.75 - -0.08)[a]			
	G/G :28	1.86 (0.24)				
rs1042173	T/T : 37	2.7 (0.27)		Additive	**0.018** (0.180)	468.7
	T/G: 63	2.14 (0.18)	-0.42 (-0.75 - -0.08)[a]			
	G/G:29	1.93 (0.24)				
rs2066713	C/C - C/T : 119	2.2 (0.13)	0.00	Recessive	**0.035** (0.280)	469.9
	T/T : 10	2.9 (0.6)	1.00 (0.08 - 1.92)			

Table R95 (continuation). Significant results from the association analysis of *SLC6A4* polymorphisms and PANSS parameters.

Polymorphism	Genotypic distribution	Average response (SE)	Difference (95% CI)	Model	*P* Value[b]	BIC
PANSS P6 - Suspiciousness						
5-HTTLPR	low/low : 37	2.57 (0.27)	-0.41 (-0.74 - -0.07)[a]	Additive	**0.018** (0.180)	468.8
	high/low : 61	2.33 (0.2)				
	high/high : 31	1.74 (0.18)				
PANSS P7 - Hostility						
5-HTTLPR	low/low : 37	2.19 (0.2)	0.00	Dominant	**0.0004** (**0.0044**)	364.9
	high/low - high/high : 92	1.53 (0.09)	-0.67 (-1.03 - -0.32)			

Significant *P* values ($P < 0.05$) are indicated in bold.
a. The indicated value corresponds to the difference which can be attributed to each allele in the additive model.
b. The corrected *P* value is indicated in brackets.
Abbreviations: SE, standard error; CI, confidence interval; N/A, not applicable.

4.2.4. PSYRATS subscale for auditory hallucinations.

Finally, the significant results from the association analysis between the 11 *SLC6A4* polymorphisms and PSYRATS scale for AH can be found at table R96. In this case, three polymorphisms gave significant results with the subscale for AH:

- SNP rs2020939, located in intron 1A, was found to be associated with three different items (Location, p = 0.04; Amount of Distress, p = 0.025; and Intensity of Distress, p = 0.046). However, the corrected *P* values were not significant.
- SNP rs12945042 was associated with the amount of distress caused by AH (p = 0.036, not significant after correction for multiple testing).
- The most interesting results are the associations of the functional polymorphism 5-HTTLPR and the three emotional items from PSYRATS scale: Amount of Distress (p = 0.011), Intensity of Distress (p = 0.0061) and Disruption to Life (p = 0.012). In all cases, those patients with a low expression genotype scored higher than the other patients and this finding is in agreement with the positive associations found with PANSS, BPRS and KGV scale. However, although the three tests became not significant after the correction, the association with the Intensity of Distress item still showed a trend (p = 0.0671).

Table R96. Significant results from the association analysis of *SLC6A4* polymorphisms and PSYRATS scale parameters for auditory hallucinations.

Polymorphism	Genotypic distribution	Average response (SE)	Difference (95% CI)	Model	*P* Value[b]	BIC
PSYRATS AH - Location						
rs2020939	C/C : 62	1.92 (0.22)	0.00	Dominant	**0.04** (0.44)	760.7
	C/T - T/T : 132	1.39 (0.14)	-0.52 (-1.02 - -0.03)			
PSYRATS AH - Amount of distress						
rs2020939	C/C - C/T : 151	1.28 (0.13)	0.00	Recessive	**0.025** (0.25)	753.4
	T/T : 43	1.88 (0.27)	0.63 (0.08 - 1.18)			
5-HTTLPR	low/low : 68	1.78 (0.21)	0.00	Dominant	**0.011** (0.121)	799.5
	high/low-high/high:140	1.21 (0.13)	-0.61 (-1.08 - -0.14)			
rs12945042	G/G : 103	1.68 (0.17)		Additive	**0.036** (0.324)	749.2
	G/A : 76	1.24 (0.18)	-0.39(-0.75 - -0.03)[a]			
	A/A : 14	1 (0.36)				
PSYRATS AH - Intensity of distress						
rs2020939	C/C - C/T : 151	1.28 (0.13)	0.00	Recessive	**0.046** (0.46)	745.8
	T/T : 43	1.81 (0.26)	0.55 (0.01 - 1.09)			
5-HTTLPR	low/low : 68	1.79 (0.2)	0.00	Dominant	**0.0061** (0.0671)	790.1
	high/low-high/high:140	1.18 (0.13)	-0.65 (-1.11 - -0.19)			
PSYRATS AH - Disruption to life						
5-HTTLPR	low/low : 68	1.84 (0.18)	0.00	Dominant	**0.012** (0.132)	765.3
	high/low-high/high:140	1.28 (0.12)	-0.56 (-0.99 - -0.13)			

Significant *P* values (P < 0.05) are indicated in bold.
a. The indicated value corresponds to the difference which can be attributed to each allele in the additive model.
b. The corrected *P* value is indicated in brackets
Abbreviations: SE, standard error; CI, confidence interval; AH, auditory hallucinations.

4.3. *HTR2A* gene

The effects of the *HTR2A* SNPs on the scores of several clinical scales (BPRS, KGV, PANSS, PSYRATS for AH and delusions) as well as other disease traits (age of onset and response to treatment against AH) were tested. The significant results can be consulted at tables R97 to R99.

4.3.1. KGV and BPRS scales.

The significant results for KGV and BPRS scales are summarized in table R97. As it can be seen, both SNPs were associated with three items of KGV scale (Poverty of Speech, Depression and Anxiety) as well as one item of BPRS scale (Anxiety). Interestingly, the association with the KGV parameters Anxiety and Depression remained significant after sequential Bonferroni correction.

Table R97. Significant results from the association analysis of *HTR2A* polymorphisms and parameters from KGV and BPRS scales.

SNP	Genotypic distribution	Average response (SE)	Difference (95% CI)	Model	*P* Value[b]	BIC
KGV - poverty of speech						
rs6313	C/C - T/C : 217	0.86 (0.07)	0.00	Recessive	**0.034** (0.052)	785.6
	T/T : 50	0.52 (0.11)	-0.34 (-0.66 - -0.03)			
rs6311	G/G - A/G : 216	0.87 (0.07)	0.00	Recessive	**0.026** (0.052)	785.1
	A/A : 51	0.51 (0.11)	-0.36 (-0.67 - -0.04)			
KGV - anxiety						
rs6313	C/C : 87	1.75 (0.11)	0.00	Dominant	**0.014 (0.014)**	781.6
	T/C - T/T : 180	1.42 (0.08)	-0.33 (-0.59 - -0.07)			
rs6311	G/G : 72	1.82 (0.12)	0.00	Dominant	**0.0041 (0.0082)**	779.4
	A/G - A/A : 195	1.42 (0.07)	-0.40 (-0.68 - -0.13)			
KGV - depression						
rs6313	C/C : 87	1.09 (0.11)	-0.22 (-0.38 - -0.05)[a]	Additive	**0.011 (0.022)**	756.3
	T/C : 130	0.93 (0.09)				
	T/T : 50	0.64 (0.11)				
rs6311	G/G - A/G : 216	1 (0.07)	0.00	Recessive	**0.022 (0.022)**	757.6
	A/A : 51	0.65 (0.11)	-0.35 (-0.65 - -0.05)			
BPRS - anxiety						
rs6313	C/C : 52	3.38 (0.21)	-0.32 (-0.63 - -0.02)[a]	Additive	**0.041** (0.06)	652
	T/C : 93	2.96 (0.14)				
	T/T : 34	2.76 (0.27)				
rs6311	G/G : 53	3.4 (0.2)	-0.34 (-0.64 - -0.04)[a]	Additive	**0.03** (0.06)	651.5
	A/G : 91	2.96 (0.14)				
	A/A : 35	2.74 (0.26)				

Significant *P* values (P < 0.05) are indicated in bold.
a. The indicated value corresponds to the difference which can be attributed to each allele in the additive model.
b. The corrected *P* value is indicated in brackets
Abbreviations: SE, standard error; CI, confidence interval.

4.3.2. PANSS scale.

Table R98 show those PANSS scale parameters which were significantly influenced by the *HTR2A* SNPs. In summary, both SNPs were associated with several items from the General Subscale: General score, Anxiety, Disturbing of Volition and Preoccupation. Moreover, after applying the correction for multiple testing, the association with the PANSS General Score, as well as the items Anxiety and Preoccupation, were still significant.

Table R98. Significant results from the association analysis of *HTR2A* SNPs and PANSS parameters.

SNP	Genotypic distribution	Average response (SE)	Difference (95% CI)	Model	*P* Value[b]	BIC
PANSS General score						
rs6313	C/C : 39	33.26 (1.53)	-2.81 (-4.94 - -0.68)[a]	Additive	**0.011 (0.0138)**	928.5
	T/C : 67	30.24 (0.96)				
	T/T : 23	27.7 (1.67)				

Table R98 (continuation). Significant results from the association analysis of *HTR2A* SNPs and PANSS parameters.

SNP	Genotypic distribution	Average response (SE)	Difference (95% CI)	Model	*P* Value[b]	BIC
PANSS General score						
	C/C : 39	33.26 (1.53)				
rs6313	T/C : 67	30.24 (0.96)	-2.81 (-4.94 - -0.68)[a]	Additive	**0.011 (0.0138)**	928.5
	T/T : 23	27.7 (1.67)				
	G/G : 39	33.26 (1.53)				
rs6311	A/G : 66	30.41 (0.96)	-2.95 (-5.05 - -0.84)[a]	Additive	**0.0069 (0.0138)**	927.7
	A/A : 24	27.33 (1.64)				
PANSS G2 – Anxiety						
	C/C : 39	3.18 (0.22)				
rs6313	T/C : 67	2.6 (0.14)	-0.50 (-0.80 - -0.19)[a]	Additive	**0.0021 (0.0038)**	430.7
	T/T : 23	2.22 (0.25)				
	G/G : 39	3.18 (0.22)				
rs6311	A/G : 66	2.61 (0.14)	-0.50 (-0.80 - -0.19)[a]	Additive	**0.0019 (0.0038)**	430.4
	A/A : 24	2.21 (0.24)				
PANSS G13 - Disturbing of volition						
	C/C : 39	2.54 (0.21)				
rs6313	T/C : 67	2.12 (0.15)	-0.31 (-0.62 - -0.00)[a]	Additive	0.052 (0.102)	429.4
	T/T : 23	1.96 (0.24)				
	G/G : 39	2.54 (0.21)				
rs6311	A/G : 66	2.12 (0.15)	-0.31 (-0.61 - -0.00)[a]	Additive	0.051 (0.102)	429.4
	A/A : 24	1.96 (0.23)				
PANSS G15 - Preoccupation						
rs6313	C/C : 39	3.08 (0.23)	0.00	Dominant	**0.012 (0.024)**	457.8
	T/C - T/T : 90	2.41 (0.14)	-0.67 (-1.18 - -0.16)			
rs6311	G/G : 39	3.08 (0.23)	0.00	Dominant	**0.012 (0.024)**	457.8
	A/G - A/A : 90	2.41 (0.14)	-0.67 (-1.18 - -0.16)			

Significant *P* values (P < 0.05) are indicated in bold.
a. The indicated value corresponds to the difference which can be attributed to each allele in the additive model.
b. The corrected *P* value is indicated in brackets
Abbreviations: SE, standard error; CI, confidence interval.

4.3.3. PSYRATS scale.

Finally, rs6311 was associated with one item from the PSYRATS subscale for AH (see table R99). This item was Intensity of Distress to AH. However, the *P* value did not survive sequential Bonferroni correction.

Table R99. Significant results from the association analysis of *HTR2A* SNPs and PSYRATS scale parameters for auditory hallucinations.

SNP	Genotypic distribution	Average response (SE)	Difference (95% CI)	Model	*P* Value[b]	BIC
			PSYRATS AH - Intensity of distress			
	G/G : 53	1.74 (0.24)				
rs6311	A/G : 112	1.32 (0.14)	-0.33 (-0.64 - -0.01)[a]	Additive	**0.042** (0.084)	788.2
	A/A : 43	1.09 (0.23)				

Significant *P* values (P < 0.05) are indicated in bold.
a. The indicated value corresponds to the difference which can be attributed to each allele in the additive model.
b. The corrected *P* value is indicated in brackets
Abbreviations: SE, standard error; CI, confidence interval; AH, auditory hallucinations.

As a final observation, it is remarkable that these two SNPs located in the *HTR2A* gene have been associated in this study with several items (of different scales: KGV, BPRS, PANSS, PSYRATS) which evaluate alterations in the emotional response of psychotic patients.

4.4. *TPH2* gene

The association analysis of the SNP rs4570625 with several disease traits mainly gave negative results. Only for some items of PSYRATS scale for AH, there was a trend toward association, which turned into significant when only a more restricted sample (which only included those hallucinatory patients diagnosed as schizophrenics) was tested. In that case (see table R100), the SNP rs4570625 was found to be associated with the total score as well as the items Duration, Location, Intensity, Degree of Negative Content and Amount of Distress. The model which best adjusted to the results was always the codominant one, according to the BIC scores, and in all cases the heterozygotes scored higher than the homozygotes G/G.

Table R100. Significant results from the association analysis of the *TPH2* SNP and PSYRATS scale parameters for auditory hallucinations.

Polymorphism	Genotypic distribution	Average response (SE)	Difference (95% CI)	Model	*P* Value	BIC
			PSYRATS AH - Total score			
	G/G : 124	16.94 (1.38)	1.00			
rs4570625	G/T : 43	22.98 (2.41)	6.03 (0.69 - 11.38)	Codominant	**0.037**	1446.5
	T/T : 5	9 (5.51)	-7.94 (-21.72 - 5.83)			
			PSYRATS AH - Duration			
	G/G : 124	1.62 (0.15)	1.00			
rs4570625	G/T : 43	2.35 (0.26)	0.73 (0.15 - 1.31)	Codominant	**0.031**	684.1
	T/T : 5	1 (0.63)	-0.62 (-2.12 - 0.88)			
			PSYRATS AH - Location			
	G/G : 124	1.47 (0.14)	1.00			
rs4570625	G/T : 43	2.07 (0.26)	0.60 (0.05 - 1.16)	Codominant	**0.042**	668.2
	T/T : 5	0.6 (0.4)	-0.87 (-2.30 - 0.57)			

Table R100 (continuation). Significant results from the association analysis of the *TPH2* SNP and PSYRATS scale parameters for auditory hallucinations.

Polymorphism	Genotypic distribution	Average response (SE)	Difference (95% CI)	Model	*P* Value	BIC
PSYRATS AH - Intensity						
rs4570625	G/G : 124	1.24 (0.11)	1.00	Codominant	**0.043**	588.4
	G/T : 43	1.67 (0.21)	0.43 (-0.01 - 0.87)			
	T/T : 5	0.4 (0.24)	-0.84 (-1.98 - 0.30)			
PSYRATS AH - Degree of negative content						
rs4570625	G/G : 124	1.49 (0.15)	1.00	Codominant	**0.037**	675.5
	G/T : 43	2.16 (0.26)	0.67 (0.10 - 1.24)			
	T/T : 5	0.8 (0.49)	-0.69 (-2.16 - 0.77)			
PSYRATS AH - Amount of distress						
rs4570625	G/G : 124	1.33 (0.14)	1.00	Codominant	**0.02**	665.8
	G/T : 43	2 (0.25)	0.67 (0.12 - 1.22)			
	T/T : 5	0.4 (0.4)	-0.93 (-2.35 - 0.49)			

The results correspond to the subsample of schizophrenic hallucinatory patients.
Significant *P* values (P < 0.05) are indicated in bold.
Abbreviations: SE, standard error; CI, confidence interval; AH, auditory hallucinations.

4.5. *NOS1* gene

Results were mainly negative for the majority of scales, with the exception of PANSS. As it is shown in table R101, the VNTR was associated with some items: General score, Anxiety (G2), Guilt Feelings (G3), Preoccupation (G15), Lack of Spontaneity and Flow of Conversation (N6), Suspiciousness/persecution (P6) and Hostility (P7). The most significant associations were found with items Guilt Feelings (P = 0.0012) and Suspiciousness/persecution (P = 0.0018).

In all cases, individuals with short alleles obtained the highest scores for all items. However, the sample size was extremely low, so these findings should be taken with precaution.

Table R101. Significant results from the association analysis of the *NOS1* polymorphism and PANSS parameters.

Polymorphism	Genotypic distribution	Average response (SE)	Difference (95% CI)	Model	*P* Value	BIC
PANSS General score						
***NOS1* Ex1f-VNTR**	L/L - S/L : 78	30.14 (1.04)	0.00	Recessive	**0.048**	750.7
	S/S : 24	34.38 (1.74)	4.23 (0.09 - 8.38)			
PANSS G2 - Anxiety						
***NOS1* Ex1f-VNTR**	L/L - S/L : 78	2.55 (0.15)	0.00	Recessive	**0.044**	353.4
	S/S : 24	3.17 (0.26)	0.62 (0.02 - 1.21)			
PANSS G3 - Guilt feelings						
***NOS1* Ex1f-VNTR**	L/L - S/L : 78	1.54 (0.11)	0.00	Recessive	**0.0012**	325.9
	S/S : 24	2.42 (0.32)	0.88 (0.36 - 1.39)			

Table R101 (continuation). Significant results from the association analysis of the *NOS1* polymorphism and PANSS parameters.

Polymorphism	Genotypic distribution	Average response (SE)	Difference (95% CI)	Model	*P* Value	BIC
PANSS G15 - Preoccupation						
***NOS1* Ex1f-VNTR**	L/L : 35	2.2 (0.2)	0.46 (0.11 - 0.82)[a]	Additive	**0.012**	365.5
	S/L : 43	2.67 (0.23)				
	S/S : 24	3.12 (0.28)				
PANSS N6 - Lack of spontaneity and flow of conversation						
***NOS1* Ex1f-VNTR**	L/L : 35	2.09 (0.23)	0.00	Dominant	**0.032**	309.8
	S/L - S/S : 67	1.61 (0.1)	-0.47 (-0.90 - -0.05)			
PANSS P6 - Suspiciousness / persecution						
***NOS1* Ex1f-VNTR**	L/L - S/L : 78	2.08 (0.15)	0.00	Recessive	**0.0018**	378.4
	S/S : 24	3.17 (0.37)	1.09 (0.42 - 1.76)			
PANSS P7 - Hostility						
***NOS1* Ex1f-VNTR**	L/L : 35	1.51 (0.16)	0.30 (0.03 - 0.56)[a]	Additive	**0.029**	304.1
	S/L : 43	1.7 (0.15)				
	S/S : 24	2.12 (0.23)				

Significant *P* values (P < 0.05) are indicated in bold.
a. The indicated value corresponds to the difference which can be attributed to each allele in the additive model.
Abbreviations: SE, standard error; CI, confidence interval.

4.6. *STMN1* gene

We tested the influence of both *STMN1* markers on several disease traits (items from BPRS, KGV, PANSS and PSYRATS for AH and delusions, as well as age of onset and response to treatment against AH). Positive associations can be found at tables R102 to R104.

4.6.1. KGV scale.

SNP rs182455 was found to be associated with two items of KGV scale (table R102): Flattened affect and Incoherence of speech. In those cases, T/T homozygotes scored less than C/C homozygotes, although the model which best adjusted to the data (according to BIC parameter) was different on each case. Significant *p* values did not survive sequential Bonferroni correction.

Table R102. Significant results from the association analysis of *STMN1* SNPs and items from KGV scale.

SNP	Genotypic distribution	Average response (SE)	Difference (95% CI)	Model	*P* Value[a]	BIC
KGV - Flattened Affect						
rs182455	C/C - T/C : 222	1.29 (0.07)	0.00	Recessive	**0.038** (0.076)	813.1
	T/T : 45	0.93 (0.15)	-0.37 (-0.71 - -0.02)			
KGV - Incoherence of Speech						
rs182455	C/C : 85	0.87 (0.13)	0.00	Dominant	**0.027** (0.054)	799.6
	T/C - T/T : 182	0.57 (0.07)	-0.31 (-0.58 - -0.04)			

Significant *P* values (P < 0.05) are indicated in bold.
a. The corrected *P* value is indicated in brackets. Abbreviations: SE, standard error; CI, confidence interval.

4.6.2. PANSS scale.

Furthermore, table R103 summarizes the positive associations between *STMN1* markers and PANSS items. In this case, rs182455 was significantly associated with 4 parameters (G16 - Active Social Avoidance, Negative score, N6 - Lack of spontaneity and flow of conversation and P6 - Suspiciousness / persecution). As in table R104 with KGV, individuals who were T/T homozygotes got lower mean scores than C/C homozygotes. Furthermore, the other marker, rs12037513, was associated with G1 – Somatic Concern, N1 – Blunted Affect and P3 – Hallucinatory Behavior. However, the association with Hallucinatory cannot be considered a reliable finding, since the number of C/C homozygotes is only 2. In summary, the most interesting results of table R103 are the associations of a) rs12037513 with Blunted affect and b) rs182455 with Suspiciousness / Persecution, since they survived the correction for multiple testing.

Table R103. Significant results from the association analysis of *STMN1 SNPs* and PANSS parameters.

SNP	Genotypic distribution	Average response (SE)	Difference (95% CI)	Model	*P* Value[a]	BIC
PANSS G1 - Somatic concern						
rs12037513	T/T : 63	1.49 (0.12)	0.00	Dominant	**0.029** (0.058)	345.8
	T/C - C/C : 45	1.98 (0.19)	0.48 (0.06 - 0.91)			
PANSS G16 - Active social avoidance						
rs182455	C/C - T/C : 106	1.96 (0.15)	0.00	Recessive	**0.027** (0.054)	471.4
	T/T : 23	1.26 (0.16)	-0.73 (-1.36 - -0.09)			
PANSS Negative score						
rs182455	C/C - T/C : 106	15.57 (0.61)	0.00	Recessive	**0.05** (0.1)	840.7
	T/T : 23	12.96 (0.94)	-2.70 (-5.36 - -0.03)			
PANSS N1 - Blunted affect						
rs12037513	T/T : 63	2.43 (0.16)	0.00	Dominant	**0.015 (0.03)**	352
	T/C - C/C : 45	1.91 (0.15)	-0.55 (-0.99 - -0.11)			
PANSS N6 - Lack of spontaneity and flow of conversation						
rs182455	C/C - T/C : 106	1.83 (0.11)	0.00	Recessive	**0.029** (0.058)	395.7
	T/T : 23	1.3 (0.12)	-0.54 (-1.01 - -0.06)			
PANSS P3 - Hallucinatory behavior						
rs12037513	T/T - T/C : 106	2.32 (0.2)	0.00	Recessive	**0.023 (0.046)**	480.7
	C/C : 2	6 (0)	3.45 (0.52 - 6.38)			
PANSS P6 - Suspiciousness / persecution						
rs182455	C/C : 38	1.76 (0.17)	0.00	Codominant	**0.0013 (0.0026)**	465.6
	T/C : 68	2.68 (0.19)	0.91 (0.37 - 1.45)			
	T/T : 23	1.83 (0.32)	0.03 (-0.68 - 0.73)			

Significant *P* values (P < 0.05) are indicated in bold.
a. The corrected *P* value is indicated in brackets.
Abbreviations: SE, standard error; CI, confidence interval.

4.6.3. PSYRATS scale.

Finally, SNP rs182455 was also associated with two items of PSYRATS scale for AH (table R104): Beliefs about Origin and Intensity of Distress, although the significance was lost after the correction for multiple testing. Once again, individuals with at least one copy of T allele scored higher than C/C hallucinatory patients.

Table R104. Significant results from the association analysis of *STMN1* SNPs and PSYRATS scale parameters for auditory hallucinations.

SNP	Genotypic distribution	Average response (SE)	Difference (95% CI)	Model	*P* Value[a]	BIC
PSYRATS AH - Beliefs about origin						
rs182455	C/C : 70	1.43 (0.2)	0.00	Dominant	**0.041** (0.082)	832
	T/C - T/T : 138	1.94 (0.15)	0.52 (0.02 - 1.01)			
PSYRATS AH - Intensity of distress						
rs182455	C/C : 70	1.07 (0.17)	0.00	Dominant	**0.044** (0.088)	793.6
	T/C - T/T : 138	1.54 (0.14)	0.46 (0.02 - 0.91)			

Significant *P* values (P < 0.05) are indicated in bold.
a. The corrected *P* value is indicated in brackets.
Abbreviations: SE, standard error; CI, confidence interval; AH, auditory hallucinations.

4.7. *ASPM* gene

4.7.1. Spanish Sample.

The exhaustive clinical information available for the Spanish patients was used to study the effect of *ASPM* variation on several disease traits. The significant findings can be found in the following tables.

4.7.1.1. Age of onset

As it is shown in table R105, four SNPs (located in the region between intron 17 and intron 19) were associated with the age of onset of the disease, although any results survived the sequential Bonferroni correction.

Table R105. Significant results from the association analysis of *ASPM* SNPs with the age of onset of the disease.

SNP	Genotype	Early-onset group frequency	Late-onset group frequency	OR (95% CI)	*P* Value[a]	AIC	Model
rs6700180	C/C- T/C	85 (85%)	128 (74.8%)	1.00	**0.021** (0.168)	354.1	Recessive
	T/T	15 (15%)	43 (25.1%)	2.12 (1.09 - 4.10)			
rs3762271	C/C	31 (28.7%)	75 (39.5%)	1.00	**0.036** (0.245)	389.1	Codominant
	A/C	31 (28.7%)	83 (43.7%)	0.53 (0.31 - 0.90)			
	A/A	13 (12%)	32 (16.8%)	0.95 (0.44 - 2.06)			

Table R105 (continuation). Significant results from the association analysis of *ASPM* SNPs with the age of onset of the disease.

SNP	Genotype	Early-onset group frequency	Late-onset group frequency	OR (95% CI)	*P* Value[a]	AIC	Model
rs41310927	A/A	32 (29.6%)	74 (39%)	1.00	**0.035** (0.245)	389	Codominant
	A/G	64 (59.3%)	83 (43.7%)	0.55 (0.33 - 0.94)			
	G/G	12 (11.1%)	33 (17.4%)	1.13 (0.51 - 2.47)			
rs10922163	A/A - A/G	84 (84.8%)	128 (75.3%)	1.00	**0.035** (0.245)	351.4	Recessive
	G/G	15 (15.2%)	42 (24.7%)	1.99 (1.03 - 3.85)			

Significant *P* values (P < 0.05) are indicated in bold.
a. The corrected *P* value is indicated in brackets.
Abbreviations: CI, confidence interval.

4.7.1.2. KGV and BPRS scales

The significant associations with BPRS and KGV items are shown at table R106. In this case, two SNPs in high LD (rs10922163 and rs6700180) were found to be slightly associated with the item Flattened Affect from KGV scale, where the homozygotes for the minor allele got lower scores. Moreover, SNP rs4915337 (exon 14) had a small but significant effect on the scores for the Anxiety item (BPRS scale) and those individuals who were homozygotes for the A allele scored higher than the other patients.

Table R106. Significant results from the association analysis of *ASPM* SNPs and KGV scale parameters.

Polymorphism	Genotypic distribution	Average response (SE)	Difference (95% CI)	Model	*P* Value[a]	BIC
KGV - flattened affect						
rs6700180	C/C - T/C : 189	1.38 (0.08)	0.00	Recessive	**0.031** (0.248)	739.2
	T/T : 51	1.04 (0.14)	-0.38 (-0.71 - -0.04)			
rs10922163	A/A - G/A : 189	1.36 (0.08)	0.00	Recessive	**0.049** (0.343)	721.3
	G/G : 50	1.04 (0.14)	-0.34 (-0.68 - -0.00)			
BPRS - anxiety						
rs4915337	A/A : 136	3.17 (0.12)	0.00	Dominant	**0.033** (0.264)	656.5
	A/T - T/T : 43	2.65 (0.22)	-0.55 (-1.05 - -0.05)			

Significant *P* values (P < 0.05) are indicated in bold.
a. The corrected *P* value is indicated in brackets.
Abbreviations: SE, standard error; CI, confidence interval.

4.7.1.3. PANSS scale

Table R107 shows all the significant associations between *ASPM* polymorphisms and PANSS items. Due to the high number of items, the number of positive associations is high and all SNPs were associated with at least one item. Nevertheless, the majority of positive findings did not resist the correction for multiple testing, with three exceptions:

- The association of rs9726778 (located in the putative promoter region of the *ASPM* gene) with the General Score. In this case, G/G homozygotes scored higher on average than C/G - C/C group.
- Two SNPs in almost complete LD (rs67008180 and rs10922163) were associated with the negative item Blunted Affect (P = 0.042 and P = 0.49, corrected values). In this case, homozygotes for the minor allele obtained lower scores than the other genotypes.

Table R107. Significant results from the association analysis of *ASPM* SNPs and PANSS items.

Polymorphism	Genotypic distribution	Average response (SE)	Difference (95% CI)	Model	*P* Value[b]	BIC
PANSS total score						
rs4915337	A/A : 95	61.83 (1.87)	0.00	Dominant	**0.03** (0.24)	1102.9
	A/T - T/T : 34	53.53 (2.16)	-7.20 (-13.62 - -0.77)			
rs9726778	G/G : 94	61.94 (1.89)	0.00	Dominant	**0.031** (0.24)	1103
	C/G - C/C:35	53.49 (2.1)	-7.11 (-13.50 - -0.73)			
PANSS general score						
rs4915337	A/A : 95	31.96 (0.94)	0.00	Dominant	**0.0089** (0.0623)	926.4
	A/T - T/T:34	27.18 (0.96)	-4.39 (-7.64 - -1.15)			
rs9726778	G/G : 94	32.07 (0.94)	0.00	Dominant	**0.0056** **(0.0448)**	925.5
	C/G - C/C:35	27 (0.95)	-4.62 (-7.83 - -1.41)			
PANSS G2 - Anxiety						
rs12138336	G/G : 107	2.81 (0.12)	-0.59 (-1.10 - -0.09)[a]	Additive	**0.023** (0.264)	439.9
	C/G : 20	2.25 (0.27)				
	C/C : 2	1.5 (0.5)				
PANSS G5 - Mannerism and posturing						
rs6700180	C/C - T/C:87	1.55 (0.08)	0.00	Recessive	**0.017** (0.136)	255.8
	T/T : 23	1.17 (0.1)	-0.41 (-0.75 - -0.08)			
rs3762271	C/C : 49	1.22 (0.07)	0.00	Dominant	**0.017** (0.136)	286.1
	A/C - A/A:80	1.52 (0.09)	0.30 (0.06 - 0.55)			
rs41310827	A/A : 49	1.22 (0.07)	0.00	Dominant	**0.019** (0.136)	286.3
	A/G - G/G:80	1.52 (0.09)	0.30 (0.05 - 0.54)			
rs10922163	A/A - G/A:84	1.55 (0.09)	0.00	Recessive	**0.022** (0.136)	252.1
	G/G : 24	1.17 (0.1)	-0.39 (-0.72 - -0.06)			
PANSS G7 - Motor retardation						
rs3762271	C/C : 49	1.65 (0.12)	0.29 (0.00 - 0.57)[a]	Additive	**0.05** (0.4)	417.9
	A/C : 60	1.97 (0.16)				
	A/A : 20	2.2 (0.33)				
PANSS G11 - Poor attention						
rs12138336	G/G : 107	1.91 (0.1)	0.45 (0.03 - 0.86)[a]	Additive	**0.036** (0.288)	389.4
	C/G : 20	2.2 (0.28)				
	C/C : 2	3.5 (0.5)				
PANSS G12 - Lack of judgment and insight						
rs4915337	A/A : 95	2.67 (0.16)	0.00	Codominant	**0.014** (0.098)	485.3
	A/T : 33	1.97 (0.27)	-0.60 (-1.19 - -0.02)			
	T/T : 1	6 (0)	3.07 (0.17 - 5.96)			

Table R107 (continuation). Significant results from the association analysis of *ASPM* SNPs and PANSS.

Polymorphism	Genotypic distribution	Average response (SE)	Difference (95% CI)	Model	*P* Value[b]	BIC
PANSS G12 - Lack of judgment and insight						
rs9726778	G/G : 94	2.69 (0.16)	0.00	Codominant	**0.012** (0.096)	484.8
	C/G : 34	1.94 (0.26)	-0.63 (-1.21 - -0.05)			
	C/C : 1	6 (0)	3.06 (0.17 - 5.95)			
PANSS G16 - Active social avoidance						
rs4915337	A/A : 95	2 (0.16)	-0.54 (-1.07 - -0.01)[a]	Additive	**0.048** (0.352)	472.4
	A/T : 33	1.39 (0.19)				
	T/T : 1	1 (0)				
rs6677082	T/T : 81	1.98 (0.17)	0.00	N/A	**0.046** (0.352)	397.9
	T/C : 27	1.3 (0.19)	-0.64 (-1.26 - -0.02)			
rs9726778	G/G : 94	2.01 (0.16)	-0.55 (-1.08 - -0.02)[a]	Additive	**0.044** (0.352)	472.3
	C/G : 34	1.38 (0.19)				
	C/C : 1	1 (0)				
PANSS negative score						
rs3762271	C/C : 49	13.84 (0.73)	1.63 (0.16 - 3.09)[a]	Additive	**0.031** (0.217)	839.9
	A/C : 60	15.58 (0.81)				
	A/A : 20	16.75 (1.59)				
rs41310927	A/A : 49	13.69 (0.74)	1.77 (0.31 - 3.22)[a]	Additive	**0.019** (0.152)	839
	A/G : 60	15.58 (0.81)				
	G/G : 20	17.1 (1.54)				
PANSS N1 - Blunted affect						
rs6700180	C/C - T/C: 87	2.36 (0.13)	0.00	Recessive	**0.0053 (0.0424)**	354.6
	T/T : 23	1.7 (0.16)	-0.76 (-1.28 - -0.24)			
rs41310927	A/A : 49	1.88 (0.14)	0.32 (0.04 - 0.59)[a]	Additive	**0.026** (0.156)	410.1
	A/G : 60	2.28 (0.15)				
	G/G : 20	2.45 (0.31)				
rs10922163	A/A - G/A:84	2.37 (0.14)	0.00	Recessive	**0.007 (0.049)**	350.6
	G/G : 24	1.67 (0.16)	-0.73 (-1.24 - -0.21)			
PANSS P6 - Suspiciousness / persecution						
rs10922163	A/A - G/A:84	2.07 (0.14)	0.00	Recessive	**0.04** (0.24)	386.7
	G/G : 24	2.75 (0.34)	0.65 (0.04 - 1.26)			
PANSS P7 - Hostility						
rs6700180	C/C : 29	1.28 (0.11)	0.00	Dominant	**0.02** (0.16)	327.2
	T/C - T/T : 81	1.88 (0.12)	0.52 (0.09 - 0.95)			
rs10922163	A/A : 27	1.33 (0.12)	0.00	Dominant	**0.039** (0.273)	323.5
	G/A - G/G : 81	1.88 (0.12)	0.47 (0.03 - 0.92)			
rs4915337	A/A : 95	1.84 (0.11)	0.00	Dominant	**0.041** (0.273)	373.9
	A/T - T/T : 34	1.38 (0.13)	-0.40 (-0.78 - -0.02)			

Significant *P* values (P < 0.05) are indicated in bold.
a. The indicated value corresponds to the difference which can be attributed to each allele in the additive model.
b. The corrected *P* value is indicated in brackets.
Abbreviations: SE, standard error; CI, confidence interval; N/A, not applicable.

4.7.1.4. PSYRATS subscale for AH

When the effect of *ASPM* SNPs on PSYRATS subscales for AH and delusions was analyzed, there were two positive findings: the SNP rs3762271 (non-synonymous SNP located in exon 18) was slightly associated with the item Duration of AH, while the SNP rs41310927, also a non-synonymous change from exon 18, was related with the item Frequency of AH (table R108). In both cases, the homozygotes for the minor allele presented hallucinations with a lower duration and frequency than the other genotypic groups. However, both associations were very weak and possibly represent a false positive.

Table R108. Significant results from the association analysis of *ASPM* polymorphisms and PSYRATS scale parameters for auditory hallucinations.

Polymorphism	Genotypic distribution	Average response (SE)	Difference (95% CI)	Model	*P* Value[a]	BIC
PSYRATS AH - Duration						
rs3762271	C/C - A/C : 177	1.79 (0.13)	0.00	Recessive	**0.05** (0.4)	824.7
	A/A : 31	1.13 (0.26)	-0.65 (-1.30 - -0.00)			
PSYRATS AH - Frequency						
rs41310827	A/A - A/G : 176	1.83 (0.13)	0.00	Recessive	**0.05** (0.4)	829.8
	G/G : 32	1.19 (0.26)	-0.65 (-1.30 - -0.01)			

Significant *P* values (P < 0.05) are indicated in bold.
a. The corrected *P* value is indicated in brackets.
Abbreviations: SE, standard error; CI, confidence interval; AH, auditory hallucinations.

4.7.2. American Sample.

We studied the effects of the 6 *ASPM* polymorphisms on the performance of the seven cognitive factors derived from different neuropsychological tests (as it is explained in the Materials and Methods section). Table R109 shows the results for the three positive phenotypes, which can be considered as measures of working memory. The three non-synonymous SNPs located in exon 18 were associated with different cognitive measures: rs3762271 and rs41310927, which were in high LD, were associated with the Wisconsin Card Sorting Test (WCST) factor in controls. In detail, the homozygotes for the major allele scored higher for the WCST factor than heterozygotes (P = 0.017 uncorrected for rs3762271 and P = 0.048 corrected for rs41310927). Moreover, rs12138336 had an effect on other measures in cases and controls; for N Back, heterozygotes for the SNP rs12138336 scored higher than G/G homozygotes in the control sample (P = 0.011 uncorrected), while, in patients, homozygotes G/G had a better performance for Digit Span when compared to the heterozygotes (P = 0.023 uncorrected).

Table R109. Effect of *ASPM* SNPs in schizophrenic patients and normal controls on several cognitive factors.

Polymorphism	Contrast	Regression Coeff.	*P* Value[a]	Subset
		N Back		
rs12138336	C/C vs C/A	-1.748	**0.011** (0.066)	Controls
		WCST		
rs3762271	C/C vs C/A	-1.609	**0.017** (0.085)	Controls
rs41310927	A/A vs A/G	-1.834	**0.008 (0.048)**	Controls
		Digit Span		
rs12138336	G/G vs G/A	-1.616	**0.023** (0.138)	Patients

Significant *P* values (P < 0.05) are indicated in bold.
a. The corrected *P* value is indicated in brackets.
Abbreviations: WCST, Wisconsin Card Sorting Test; Coef, coefficient for the linear regression analysis; vs, versus.

4.8. *PDE4D* gene

We used the clinical information available for the Spanish sample to investigate the effect of *PDE4D* SNPs on several disease traits. Tables R110 to R113 show all the positive associations detected during the analysis.

4.8.1. Age of onset.

As it can be seen in table R110, two SNPs were associated with the age of onset of the psychotic symptoms. Genotype A/A for rs17291089 was more frequent in those patients with early-onset psychosis, while, regarding SNP rs7713345, those individuals with at least one copy of the G allele were more frequent in the early-onset group. However, after sequential Bonferroni correction, the significance was lost. Moreover, another factor which should be taken into account when valuating these findings is the small size of the early-onset psychosis sample.

Table R110. Significant results from the association analysis of *PDE4D* polymorphisms with the age of onset of the disease.

SNP	Genotype	Early-onset group frequency	Late-onset group frequency	OR (95% CI)	*P* Value[b]	AIC	Model
rs17291089	A/A	81 (93.1%)	115 (81.6%)	3.03 (1.21 - 7.58)[a]	**0.0093** (0.0744)	300.4	Additive
	C/A	6 (6.9 %)	25 (17.7%)				
	C/C	0 (0%)	1 (0.7%)				
rs7713345	C/C	56 (64.4%)	110 (78%)	1.00	**0.026** (0.182)	302.2	Dominant
	G/C - G/G	31 (35.6%)	31 (22%)	0.51 (0.28 - 0.92)			

Significant *P* values (P < 0.05) are indicated in bold.
a. The indicated value corresponds to the difference which can be attributed to each allele in the additive model.
b. The corrected *P* value is indicated in brackets.
Abbreviations: CI, confidence interval.

4.8.2. KGV and BPRS scales.

Table R111 shows the significant results for the KGV and BPRS items. In summary, SNP rs17719378 appeared to be associated with 2 parameters: Incoherence of Speech, from the KGV scale, an Anxiety, from the BPRS scale. In both cases, G/G genotype was associated with higher scores of both items. However, the model which best adjusted to the results was different in each case. Unfortunately, any result remained significant after correction for multiple testing.

Table R111. Significant results from the association analysis of *PDE4D* polymorphisms and parameters from KGV and BPRS scales.

SNP	Genotypic distribution	Average response (SE)	Difference (95% CI)	Model	*P* Value[b]	BIC
KGV - incoherence of speech						
rs17719378	A/A : 80	0.55 (0.1)	0.30 (0.08 - 0.52)[a]	Additive	**0.0089** (0.0712)	589.2
	A/G : 93	0.77 (0.12)				
	G/G : 25	1.2 (0.26)				
BPRS - anxiety						
rs17719378	A/A - A/G : 109	3.09 (0.14)	0.00	Recessive	**0.025** (0.2)	435.2
	G/G : 12	4.08 (0.36)	0.99 (0.13 - 1.85)			

Significant *P* values (P < 0.05) are indicated in bold.
a. The indicated value corresponds to the difference which can be attributed to each allele in the additive model.
b. The corrected *P* value is indicated in brackets.
Abbreviations: SE, standard error; CI, confidence interval.

4.8.3. PANSS scale.

Table R112 shows those polymorphisms which were significantly associated with the different PANSS scale parameters. However, some of these results are not reliable, since the number of individuals in one of the genotypic groups is low. This can be applied to the associations between rs10461656 and rs7714708 with Uncooperativeness item (G8), and rs17719378 and item G11 (Poor Attention). In these three cases, the only significant model was the recessive one but the size of the group homozygote for the recessive allele was very low.

By contrast, the most interesting findings are the associations of polymorphism rs17719378 with different items from the positive subscale: PANSS positive score (p = 0.045 corrected), P1 – Delusions (p = 0.037, corrected value), P3 – Hallucinations (p = 0.024 uncorrected) and P5 – Grandiosity (p = 0.026, uncorrected value). In all cases, the individuals with the A/A genotype got the lowest mean scores.

Table R112. Significant results from the association analysis of *PDE4D SNPs* and PANSS items.

SNP	Genotypic distribution	Average response (SE)	Difference (95% CI)	Model	*P* Value[b]	BIC
PANSS total score						
rs17719378	A/A : 37	59.32 (2.9)	0.00	Dominant	**0.034** (0.272)	676.8
	A/G - G/G : 42	67.26 (2.33)	7.94 (0.71 - 15.16)			
PANSS G8 - Uncooperativeness						
rs10461656	G/G - A/G : 72	1.11 (0.04)	0.00	Recessive	**0.0042 (0.0294)**	130.9
	A/A : 7	1.71 (0.57)	0.60 (0.20 - 1.00)			
rs4700316	C/C : 49	1.08 (0.04)	0.22 (0.01 - 0.43)[a]	Additive	**0.04** (0.24)	135
	C/G : 27	1.26 (0.16)				
	G/G : 3	1.67 (0.33)				
rs7714708	C/C - T/C : 71	1.1 (0.04)	0.00	Recessive	**0.0023 (0.0184)**	130.5
	T/T : 9	1.67 (0.44)	0.57 (0.21 - 0.92)			
PANSS G11 - Poor attention						
rs17719378	A/A -A/G : 74	2.03 (0.13)	0.00	Recessive	**0.002 (0.016)**	245.1
	G/G : 5	3.6 (0.24)	1.57 (0.61 - 2.54)			
PANSS G14 - Poor impulse control						
rs4700316	C/C : 49	2.1 (0.17)	0.00	Dominant	**0.028** (0.224)	249.3
	C/G - G/G : 30	1.53 (0.18)	-0.57 (-1.07 - -0.07)			
rs7714708	C/C : 34	2.18 (0.2)	0.00	Dominant	**0.037** (0.259)	252.5
	T/C - T/T: 46	1.65 (0.15)	-0.52 (-1.01 - -0.04)			
PANSS G16 - Active social avoidance						
rs7714708	C/C : 34	2.59 (0.35)	0.00	Dominant	**0.021** (0.168)	316.4
	T/C - T/T: 46	1.72 (0.19)	-0.87 (-1.59 - -0.15)			
PANSS N7 - Stereotyped thinking						
rs829259	T/T : 30	1.87 (0.21)	0.00	Codominant	**0.016** (0.128)	230.6
	A/T : 36	1.28 (0.09)	-0.59 (-1.05 - -0.13)			
	A/A : 13	2 (0.36)	0.13 (-0.49 - 0.75)			
rs10461656	G/G : 38	1.76 (0.18)	0.00	Codominant	**0.028** (0.196)	231.8
	A/G : 34	1.32 (0.1)	-0.44 (-0.88 - 0.00)			
	A/A : 7	2.29 (0.57)	0.52 (-0.25 - 1.29)			
PANSS Positive score						
rs17719378	A/A : 37	13.49 (0.87)	0.00	Codominant	**0.0057 (0.0456)**	516.4
	A/G : 37	17.38 (1.07)	3.89 (1.25 - 6.54)			
	G/G : 5	11 (1.05)	-2.49 (-7.90 - 2.93)			
PANSS P1 - Delusions						
rs17719378	A/A : 37	2.03 (0.27)	0.00	Codominant	**0.0046 (0.0368)**	336.2
	A/G : 37	3.3 (0.35)	1.27 (0.42 - 2.12)			
	G/G : 5	1.2 (0.2)	-0.83 (-2.56 - 0.91)			
PANSS P3 - Hallucinatory behaviour						
rs17719378	A/A : 37	2.16 (0.33)	0.00	Codominant	**0.024** (0.192)	360.2
	A/G : 37	3.27 (0.39)	1.11 (0.12 - 2.09)			
	G/G : 5	1 (0)	-1.16 (-3.18 - 0.85)			
PANSS P5 - Grandiosity						
rs17719378	A/A : 37	1.54 (0.2)	0.00	Dominant	**0.026** (0.208)	304.3
	A/G - G/G : 42	2.33 (0.28)	0.79 (0.11 - 1.48)			

Significant *P* values (P < 0.05) are indicated in bold.
a. The indicated value corresponds to the difference which can be attributed to each allele in the additive model.
b. The corrected *P* value is indicated in brackets.
Abbreviations: SE, standard error; CI, confidence interval.

4.8.4. PSYRATS subscales for AH and delusions.

Finally, the significant results from the association analysis between the 9 *PDE4D* SNPs and PSYRATS subscales for AH and delusions can be found at table R113. In this case, only one SNP (rs17719378) gave significant results. This SNP was found to be associated with one item of the AH subscale (Controllability of Voices) and 6 items from the delusions subscale. Regarding the delusions subscale, the A/A genotype was associated with a lower score in all cases. Nevertheless, neither of the values survived the sequential Bonferroni correction.

Table R113. Significant results from the association analysis of *PDE4D* SNPs and parameters from PSYRATS subscales for auditory hallucinations and delusions.

SNP	Genotypic distribution	Average response (SE)	Difference (95% CI)	Model	*P* Value[a]	BIC
PSYRATS AH - Grade of control						
rs17719378	A/A - A/G : 154 G/G : 22	2.09 (0.14) 1.27 (0.37)	0.00 -0.82 (-1.62 - -0.02)	Recessive	**0.046** (0.414)	717.1
PSYRATS Delusions - Total score						
rs17719378	A/A : 44 A/G - G/G : 54	4.14 (1.01) 7.69 (1.2)	0.00 3.55 (0.39 - 6.71)	Dominant	**0.03** (0.24)	695.8
PSYRATS Delusions - Conviction						
rs17719378	A/A : 44 A/G - G/G : 54	0.84 (0.21) 1.52 (0.23)	0.00 0.68 (0.06 - 1.30)	Dominant	**0.035** (0.28)	376.9
PSYRATS Delusions – Duration of preoccupation						
rs17719378	A/A : 44 A/G - G/G : 54	0.77 (0.19) 1.37 (0.22)	0.00 0.60 (0.01 - 1.18)	Dominant	**0.048** (0.384)	364.8
PSYRATS Delusions - Disruption						
rs17719378	A/A : 44 A/G - G/G : 54	0.59 (0.15) 1.22 (0.19)	0.00 0.63 (0.14 - 1.12)	Dominant	**0.013** (0.104)	330.9
PSYRATS Delusions – Amount of preoccupation						
rs17719378	A/A : 44 A/G - G/G : 54	0.73 (0.18) 1.3 (0.22)	0.00 0.57 (-0.01 - 1.14)	Dominant	0.055, ns (0.44)	361.8
PSYRATS Delusions – Intensity of distress						
rs17719378	A/A : 44 A/G - G/G : 54	0.57 (0.15) 1.17 (0.19)	0.00 0.60 (0.11 - 1.09)	Dominant	**0.018** (0.144)	329.8

Significant *P* values (P < 0.05) are indicated in bold.
a. The corrected *P* value is indicated in brackets.
Abbreviations: SE, standard error; CI, confidence interval; ns, not significant; AH, auditory hallucinations.

4.9. *PLEKHB1* and *RAB6A* genes

We investigated the effect of *PLEKHB1* and *RAB6A* SNPs on several disease traits which had been assessed on the Spanish patients. Tables R114 to R116 show all the significant associations found during the analysis.

4.9.1. KGV and BPRS scales.

The positive associations with BPRS and KGV scales can be found at table R114. As it can be seen, the SNP rs7127066 was found to be associated with three items, Hallucinations and Psychomotor Retardation from KGV scale and the BPRS total score. However, only the association with Hallucinations should be taken into account, since the two other findings are based on the existence of genotypic groups of very small size (8 and 4 individuals with C/C genotype, respectively).

Table R114. Significant results from the association analysis of *PLEKHB1-RAB6A* polymorphisms and parameters from KGV and BPRS scales.

Polymorphism	Genotypic distribution	Average response (SE)	Difference (95% CI)	Model	*P* Value[a]	BIC
KGV - hallucinations						
rs7127066	G/G : 116	1.84 (0.16)	0.00	Dominant	**0.02 (0.12)**	789.2
	C/G - C/C : 82	1.26 (0.18)	-0.58 (-1.07 - -0.09)			
KGV - anxiety						
rs940828	T/T : 121	1.73 (0.09)	0.00	Dominant	0.051, ns (0.306)	580.5
	G/T - G/G : 76	1.43 (0.11)	-0.29 (-0.59 - -0.00)			
KGV - psychomotor retardation						
rs7127066	G/G - C/G : 190	0.95 (0.08)	0.00	Recessive	**0.038** (0.228)	596.9
	C/C : 8	1.75 (0.49)	0.80 (0.05 - 1.54)			
BPRS - total score						
rs7127066	G/G - C/G : 75	46.2 (1.18)	0.00	Recessive	**0.038** (0.228)	616.1
	C/C : 4	58.25 (12.16)	12.05 (0.85 - 23.25)			
BPRS - anxiety						
rs3741147	T/T : 83	2.95 (0.16)	0.00	Dominant	**0.014** (0.084)	435.4
	G/T - G/G : 36	3.67 (0.24)	0.71 (0.15 - 1.27)			

Significant *P* values ($P < 0.05$) are indicated in bold.
a. The corrected *P* value is indicated in brackets.
Abbreviations: SE, standard error; CI, confidence interval; ns, not significant.

4.9.2. PANSS scale.

Table R115 shows those polymorphisms which were significantly associated with the different PANSS scale parameters. Due to the high number of different items, there are an important number of positive associations. However, only some of these results should be considered, because many of the positive associations are not reliable due to the low number of individuals in one of the groups.

In summary, the most interesting findings are the following:

- The polymorphism rs11235876, located in intron 3 from the *RAB6A* gene, was associated with 6 different items (two negative items and the negative global score, two positive items and one item from the general subscale). In all cases, individuals who were A/A homozygotes scored higher than G/G homozygotes.

- The SNP rs663303, located 5' upstream of *PLEKHB1*, was found to be associated with one positive item, three negative items and two general items. Interestingly, the T/T genotype always scored higher than the other genotypes.
- The SNP rs7127066 (intron 1 from *RAB6A* gene) was associated with two positive items: Conceptual Disorganization and Hallucinatory Behaviour. The association with the Hallucinations item is very interesting since it is in coherence with the association with the Hallucinations item from KGV scale. Moreover, in the two cases the G/G homozygotes scored higher in average than the other genotypic groups.
- rs940828 (intron 5 from *PLEKHB1* gene) was associated with item P6-Suspiciousness/Persecution, and fitted a dominant model where the individuals with at least one copy of the G allele obtained higher scores.
- The polymorphism rs12274970 (located in the intergenic region between *PLEKHB1* and *RAB6A* genes) was associated with the General item Disturbing of Volition, and followed a dominant model.

However, despite the high number of findings, only four of them survived sequential Bonferroni correction (table R115), as expected. These results were the three associations of rs11235876 with Hostility, Passive/Apathetic Social Withdrawal and Disturbing of Volition; and the association of rs12274970 with the General item Disturbing of Volition.

Another handicap to be taken into account is the low number of subjects with genotype information who have been evaluated for the PANSS.

Table R115. Significant results from the association analysis of *PLEKHB1-RAB6A SNPs* and PANSS parameters.

Polymorphism	Genotypic distribution	Average response (SE)	Difference (95% CI)	Model	*P* Value[b]	BIC
PANSS negative score						
rs11235876	G/G : 18 G/A : 42 A/A :19	14.5 (1.2) 15.83 (0.94) 18.84 (1.43)	2.18 (0.29 - 4.08)[a]	Additive	**0.027** (0.135)	515.4
PANSS P2 - Conceptual disorganization						
rs663303	C/C : 56 T/C : 21 T/T : 2	1.54 (0.12) 2.1 (0.3) 2.5 (1.5)	0.53 (0.08 - 0.99)[a]	Additive	**0.023** (0.138)	244.3
PANSS P3 - Hallucinatory behavior						
rs7127066	G/G : 39 C/G : 36 C/C : 4	3.03 (0.37) 1.97 (0.32) 4.25 (1.25)	0.00 -1.05 (-2.04 - -0.07) 1.22 (-1.01 - 3.46)	Codominant	**0.038** (0.228)	361.2
PANSS P6 - Suspiciousness / persecution						
rs940828	T/T : 44 G/T - G/G : 35	2.07 (0.19) 2.71 (0.25)	0.00 0.65 (0.04 - 1.25)	Dominant	0.04 (0.16)	284.7
rs11235876	G/G : 18 G/A - A/A : 61	1.67 (0.27) 2.56 (0.18)	0.00 0.89 (0.18 - 1.60)	Dominant	**0.016** (0.096)	283.1

Table R115 (continuation). Significant results from the association analysis of *PLEKHB1-RAB6A SNPs* and PANSS parameters.

Polymorphism	Genotypic distribution	Average response (SE)	Difference (95% CI)	Model	*P* Value[b]	BIC
PANSS P6 - Suspiciousness / persecution						
rs7127066	G/G : 39 C/G : 36 C/C : 4	2.05 (0.19) 2.56 (0.25) 3.5 (0.87)	0.60 (0.09 - 1.10)[a]	Additive	**0.024 (0.12)**	283.8
PANSS P7 - Hostility						
rs11235876	G/G - G/A : 60 A/A :19	1.33 (0.18) 2.08 (0.14)	0.00 0.75 (0.20 - 1.30)	Dominant	**0.0094 (0.047)**	242.7
PANSS N2 - Emotional withdrawal						
rs663303	C/C : 56 T/C : 21 T/T : 2	2.54 (0.16) 3.14 (0.36) 3.5 (0.5)	0.57 (0.01 - 1.12)[a]	Additive	**0.049** (0.205)	277.3
rs11235876	G/G : 18 G/A - A/A : 61	2.55 (0.16) 3.26 (0.35)	0.00 0.71 (0.04 - 1.38)	Dominant	**0.037** (0.205)	277
PANSS N3 - Poor rapport						
r663303	C/C : 56 T/C : 21 T/T : 2	1.73 (0.13) 2.19 (0.25) 3 (0)	0.51 (0.08 - 0.95)[a]	Additive	**0.022** (0.132)	236.7
PANSS N4 - Passive / apathetic social withdrawal						
rs663303	C/C : 56 T/C - T/T : 23	2.62 (0.2) 3.57 (0.37)	0.00 0.94 (0.18 - 1.70)	Dominant	**0.017** (0.068)	305.3
rs11235876	G/G : 18 G/A : 42 A/A :19	2.06 (0.33) 3.02 (0.25) 3.42 (0.34)	0.68 (0.18 - 1.18)[a]	Additive	**0.0093 (0.0465)**	304.2
PANSS G8 - Uncooperativeness						
rs663303	C/C : 56 T/C : 21 T/T : 2	1.09 (0.04) 1.33 (0.2) 1.5 (0.5)	0.23 (0.00 - 0.46)[a]	Additive	**0.049** (0.245)	135.3
PANSS G9 - Unusual thought content						
rs663303	C/C : 56 T/C : 21 T/T : 2	1.46 (0.15) 2.1 (0.33) 3 (2)	0.67 (0.12 - 1.22)[a]	Additive	**0.019** (0.114)	275.3
PANSS G13 - Disturbing of volition						
rs12274970	T/T : 67 C/C : 12	1.83 (0.19) 2.65 (0.21)	0.00 0.82 (0.25 - 1.39)	Dominant	**0.0065 (0.0325)**	275.9
rs11235876	G/G : 18 G/A : 42 A/A :19	1.67 (0.21) 2.21 (0.21) 3 (0.31)	0.67 (0.26 - 1.08)[a]	Additive	**0.0021 (0.0126)**	273.8

a. The indicated value corresponds to the difference which can be attributed to each allele in the additive model.
b. The corrected *P* value is indicated in brackets.
Abbreviations: SE, standard error; CI, confidence interval.

4.9.3. PSYRATS subscales for AH and delusions.

Regarding PSYRATS for AH and delusions, there was only one positive finding: the SNP rs7127066 (located in intron 1 from *RAB6A* gene) appeared to be associated with the item Intensity of distress to AH (table R116). The results fitted a recessive inheritance model where the recessive homozygote genotype significantly scored higher than the other genotypes. However, this significant finding did not survive the correction for multiple testing.

Table R116. Significant results from the association analysis of *PLEKHB1-RAB6A* SNPs and PSYRATS scale parameters for auditory hallucinations and delusions.

Polymorphism	Genotypic distribution	Average response (SE)	Difference (95% CI)	Model	*P* Value[a]	BIC
PSYRATS AH - Intensity of distress						
rs7127066	G/G - C/G : 166	1.42 (0.12)	0.00	Recessive	**0.036** (0.216)	673.3
	C/C : 58	2.5 (0.56)	1.08 (0.08 - 2.09)			

Significant *P* values (P < 0.05) are indicated in bold.
a. The corrected *P* value is indicated in brackets.
Abbreviations: SE, standard error; CI, confidence interval; AH, auditory hallucinations.

5. Gene-gene interaction analyses.

5.1. Emotional variables.

We performed a gene-gene interaction analysis to study the epistatic effects of several polymorphisms which had been associated to emotional variables in this study and/or in other independent studies. The selected polymorphisms were:

- 5-HTTLPR (including rs25531), from the serotonin transporter gene: this polymorphism was associated in our study with several emotional items from the PSYRATS scale for auditory hallucinations, as well as the Anxiety item from KGV scale. Moreover, there are previous studies which clearly relate this functional polymorphism with the neural mechanisms of the emotional response.

- The SNP rs6313, located in the first exon from the *HTR2A* gene. In the present study, this polymorphism (as well as the SNP rs6311, in high LD with rs6313) was repeatedly associated with the emotional items from different scales (KGV, BPRS, PANSS, PSYRATS…); moreover, the *HTR2A* gene is also an important component of the serotonergic system, together with the serotonin transporter gene.

- The SNP rs4570625 from the *TPH2* gene. Although our results were mainly negative (with the exception of a slight association with the item Amount of Distress from PSYRATS scale for AH), other authors (Herrmann *et al.,* 2006) have found an association of this SNP with the modulation of emotional processing. Moreover, this polymorphism is also a key element of the serotonergic system.

- The polymorphism rs182455 from the *STMN1* gene. This polymorphism was also considered for the interaction analysis because of the previous findings from other

authors (Shumyatsky *et al.,* 2005) who demonstrated its high expression in limbic areas and its essential role in the regulation of both innate and learned fear.

- Finally, the ability of the NO system to affect the serotonin transporter activity (Kiss and Vizi, 2001; Kilic *et al.*, 2003) led us to include the functional VNTR from the *NOS1* in the gene-gene interaction analysis. Particularly, a *NOS1* x *SLC6A4* interaction analysis was performed for the variables described below.

Variables which were included in the analysis of pair wise polymorphism interactions were:
1. The comparison of the genotypic frequencies among patients and controls.
2. The Anxiety and Depression items from KGV, BPRS and PANSS.
3. Three emotional items from PSYRATS subscale for AH: Amount of Distress, Intensity of Distress and Disruption.
4. Two items from PSYRATS subscale for delusions: Disruption and Amount of Distress.

Table R117 shows all the significant findings from the pair wise gene-gene interaction analysis. According to this information, there was an interaction between 5-HTTLPR and rs6313 which had an impact on several measures (the three items from the PSYRATS subscale for AH, as well as the Anxiety items from BPRS and KGV, see figure R10 for a graphical representation of the mean scores). Moreover, the distribution of the genotypic frequencies among patients and controls also seemed to be affected by this interaction. Furthermore, an interaction between rs4570625 from *TPH2* and rs6313 from *HTR2A* was also detected. This interaction affected the mean scores for both items from the PSYRATS subscale for delusions, as well as the Depression item from PANSS (see table R117 and figure R11). However, the only significant interactions which survived the correction for multiple testing were those affecting the items Intensity of Distress and Amount of Distress to AH, from the PSYRATS scale.

Table R117. Significant results from the pair wise interaction analysis among four polymorphisms related to emotionality.

Polymorphism 1 (gene)	Polymorphism 2 (gene)	Response Variable	OR or β[a]	Statistic	P[b]
rs6313 *(HTR2A)*	5-HTTLPR (*SLC6A4*)	All cases versus controls	1.506	5.426	**0.0198** (0.119)
rs6313 *(HTR2A)*	5-HTTLPR (*SLC6A4*)	PSYRATS for AH - Intensity of distress	0.6877	9.921	**0.0016 (0.0098)**
rs6313 *(HTR2A)*	5-HTTLPR (*SLC6A4*)	PSYRATS for AH - Amount of distress	0.6726	9.058	**0.0026 (0.0156)**
rs6313 *(HTR2A)*	5-HTTLPR (*SLC6A4*)	PSYRATS for AH - Disruption	0.4607	4.94	**0.0263** (0.1575)
rs6313 *(HTR2A)*	5-HTTLPR (*SLC6A4*)	BPRS - Anxiety	0.4756	4.184	**0.0408** (0.2448)
rs6313 *(HTR2A)*	5-HTTLPR (*SLC6A4*)	KGV - Anxiety	0.2709	4.127	**0.0422** (0.2533)
rs4570625 *(TPH2)*	rs6313 *(HTR2A)*	PSYRATS for delusions - Disruption	0.6679	5.62	**0.0178** (0.1065)
rs4570625 *(TPH2)*	rs6313 *(HTR2A)*	PSYRATS for delusions – Amount of distress	0.6692	5.462	**0.0194** (0.1166)
rs4570625 *(TPH2)*	rs6313 *(HTR2A)*	PANSS G6 - Depression	0.6451	3.86	**0.0495** (0.2967)

Significant *P* values ($P < 0.05$) are indicated in bold.
a. This value refers to the pair wise odds ratios (case-control variables) or β values (quantitative traits) which represent the magnitude of the epistasis.
b. The corrected *P* value is indicated in brackets.
Abbreviation: OR, Odds ratio.

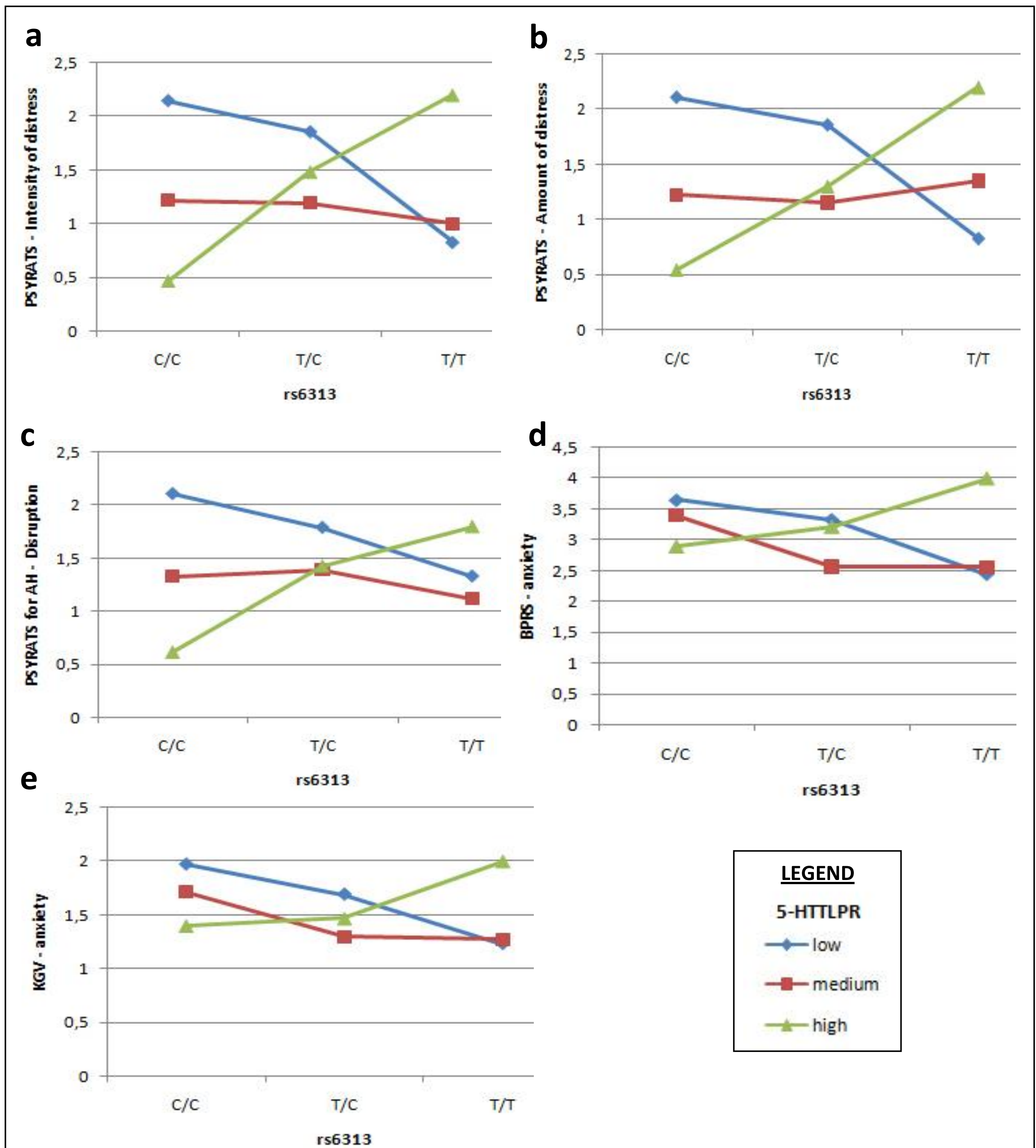


Figure R10. Effect of *SLC6A4-HTR2A* interactions on the mean scores for different clinical measures. Graphs represent all the gene-gene interaction analyses with a significant result. Graphs **a** to **c** correspond to the effect on three emotional items of the PSYRATS subscale for AH, while graphs **d** and **e** refer to the influence of a genetic interaction on the Anxiety items from BPRS and KGV scales, respectively.

Abbreviations: AH, auditory hallucinations.

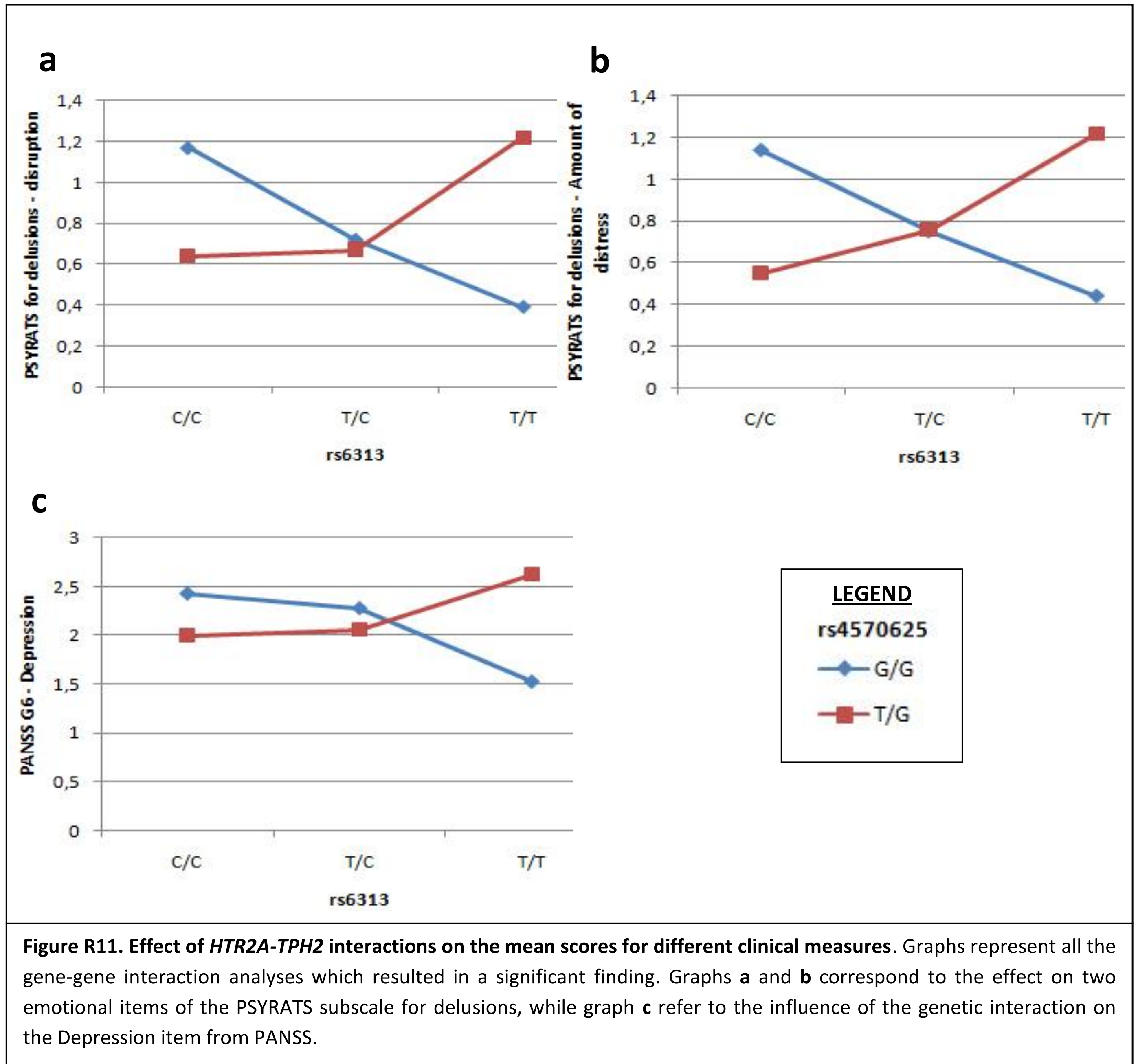


Figure R11. Effect of *HTR2A-TPH2* interactions on the mean scores for different clinical measures. Graphs represent all the gene-gene interaction analyses which resulted in a significant finding. Graphs **a** and **b** correspond to the effect on two emotional items of the PSYRATS subscale for delusions, while graph **c** refer to the influence of the genetic interaction on the Depression item from PANSS.

5.2. Other gene-gene interaction analyses.

Other polymorphisms and variables were also considered to be interesting. Particularly, when two or more polymorphisms were significantly associated (after the correction for multiple testing) with certain variable (quantitative or binary), a pair wise gene-gene interaction analysis was performed for those SNPs and variables. The selected polymorphisms and variables were the following:

- The single SNP analysis showed that three SNPs (rs2020936, from the *SLC6A4* gene, as well as rs6700180 and rs10922163, from the *ASPM* gene) were significantly associated (after Bonferroni sequential correction) with the risk for schizophrenia. For this reason, an analysis was performed to evaluate whether the genotypic distributions among cases

and controls were influenced by an *ASPM* x *SLC6A4* interaction. It should be remarked that both SNPs located in *ASPM* (rs6700180 and rs10922163) are in perfect LD (r^2 = 1). Therefore, only rs10922163 was selected for the interaction analysis, together with the serotonin transporter gene polymorphism.

- We also studied the likely effect of a *NOS1* x 5-HTTLPR interaction on the PANSS variable Hostility, a positive item from the PANSS.
- The effect of an interaction between the SNPs rs9726778 (*ASPM* gene) and rs6313 (*HTR2A* gene) on the PANSS general score was also analyzed.
- Both the *NOS1* ex1-VNTR and the SNP rs182455 from the *STMN1* gene were found to be associated with the positive PANSS item Suspiciousness-Persecution. For this reason, the effect of an interaction of both genes on this parameter was also evaluated.
- Finally, we studied the existence of pair wise gene-gene interactions which could affect the score for one negative item from the PANSS: Blunted Affect. The selected SNPs were: rs12037513 (*STMN1*), rs6700180 (*ASPM*) and rs10922163 (*ASPM*).

Table R118 shows a summary of the significant findings resulting from these gene-gene interaction analyses. An interaction of *ASPM* x *SLC6A4* appears to affect the genotypic distributions in cases and controls. Unfortunately, this significant value did not resist the correction for multiple testing. Moreover, a trend towards association (P = 0.07 uncorrected) was also found for the interaction *STMN1* x *NOS1* on the score for the PANSS item "Blunted Affect".

Table R118. Significant results from the pair wise interaction analysis among those polymorphisms which were associated with several response variables during the single SNP association analysis.

Polymorphism 1 (gene)	Polymorphism 2 (gene)	Response Variable	OR or β[a]	Statistic	P[b]
rs10922163 (*ASPM*)	rs2020936 (*SLC6A4*)	All cases versus controls	1.615	4.733	**0.0296** (0.139)
rs182455 (*STMN1*)	Ex1f-VNTR (*NOS1*)	PANSS P6 – Suspiciousness/Persecution	0.6451	3.281	0.0701 (0.252)

Significant *P* values (P < 0.05) are indicated in bold.
a. This value refers to the pair wise odds ratios (case-control variables) or β values (quantitative traits) which represent the magnitude of the epistasis.
b. The corrected *P* value is indicated in brackets.
Abbreviation: OR, Odds ratio.

The genotypic distributions in patients and controls for the SNP rs2020936 (intron 1A of the *SLC6A4* gene) depending on the genotype for the SNP rs10922163 (*ASPM* gene) can be also observed in table R119.

Table R119. Genotypic frequencies in patients and controls for the SNP rs2020936 (*SLC6A4* gene), depending on the genotype for the SNP rs10922163 (*ASPM* gene).

rs2020936	rs10922163	CONTROLS	PATIENTS	OR (95% CI)
T/T	G/G	60	44	1.00
	A/G	81	112	1.89 (1.16-3.06)
	A/A	49	32	0.89 (0.49-1.61)
T/C	G/G	41	14	1.00
	A/G	54	35	1.90 (0.90-3.98)
	A/A	22	20	2.66 (1.13-6.27)
C/C	G/G	3	2	1.00
	A/G	2	4	3 (0.25-35.34)
	A/A	3	5	2.50 (0.25-24.72)

The ORs for each rs2020936 genotypic group are indicated.
Abbreviation: OR, Odds ratio. CI, confidence interval.

6. Correlation with neuroimaging variables

6.1. *SLC6A4* gene

6.1.1. Effect of 5-HTTLPR polymorphism on brain morphometry.

We explored the influence of the promoter polymorphism in brain structure measured by voxel-based morphometry (VBM). The effect of 5-HTTLPR on both control subjects and schizophrenic patients was studied. However, it should be noted that, for this analysis, the variation of SNP rs25531 was not taken into account. Therefore, subjects were only classified as *l/l*, *l/s* or *s/s* and grouped in two categories: on the one hand, *l/l* homozygotes and, on the other hand, the *s*-carriers (*l/s* and *s/s* subjects).

A region of interest (ROI) around the limbic system was defined. This region included the cingulate cortex, fornix, hippocampus, parahippocampal gyrus and amygdala.

Table R120 shows information about the main clinical, genetic and demographic variables for the 30 controls and 24 patients included in this neuroimaging analysis. There were no significant differences in age between control subjects with the *l/l* genotype and *s*-carriers (F=0.94, p=0.55). Moreover, no differences were found between schizophrenic patients with *l/l* genotype and *s*-carriers in age (F=0.18, p=0.67), age of first hallucinations (F=0.78, p=0.78), illness duration (F=0.12, p=0.72), BPRS general score (F=0.82, p=0.34), PSYRATS general score (F=1.07, p=0.31), PANSS total (F=0.59, p=0.45), PANSS positive (F=1.27, p=0.27), PANSS negative (F=0.34, p=0.56) and PANSS general (F=0.05, p=0.81).

Table R120. Clinical and demographic data from control subjects and schizophrenic patients included in the correlation study between 5-HTTLPR and VBM data from limbic areas.

	Control subjects	
	l/l group (N = 9)	**l/s + s/s group (N = 21)**
Age (years)	29.78 ± 11.68	32.05 ± 7.21
	Schizophrenic patients	
	l/l group (N = 6)	**l/s + s/s group (N = 18)**
Age (years)	40.67 ± 6.92	38.89 ± 9.20
Age first hallucinations	20.33 ± 11.57	21.78 ± 10.81
Illness duration	15.67 ± 7.91	14.47 ± 6.85
BPRS total score	47.00 ± 12.34	51.61 ± 9.44
PSYRATS for AH total score	32.50 ± 1.73	29.93 ± 4.77
PANSS total score	64.00 ± 20.14	70.00 ± 15.34
PANSS positive score	15.33 ± 5.31	18.33 ± 5.71
PANSS negative score	16.00 ± 7.53	18.11 ± 7.60
PANSS general score	32.67 ± 9.30	33.56 ± 7.61

Data are displayed as mean ± SD.
Abbreviations: SD, standard deviation; BPRS, Brief Psychiatric Rating Scale; PSYRATS, Psychotic Symptom Rating Scale; PANSS, Positive and Negative Syndrome Scale.

6.1.1.1. Effect of 5-HTTLPR in healthy subjects

The results from the VBM analysis in healthy subjects can be found at table R121 and figure R12. Significant (p<0.05 FDR corrected, k=34) gray matter (GM) differences were found between control subjects with *l/l* genotype and *s*-carriers, revealing that healthy subjects with at least one copy of the short allele showed GM density reductions in hippocampus (bilateral, Brodmann areas 20, 36 and 35) and left parahippocampus (BA 30) when compared with *l/l* homozygote subjects. By contrast, there were no significant areas where the *s*-carriers showed greater GM density values than subjects with *l/l* genotype.

Table R121. Significant areas with GM density reduction in healthy subjects who were *s*-carriers (*l/s* + *s/s*) when compared to healthy subjects with *l/l* genotype.

Coordinates (mm) *X*	*Y*	*Z*	*Label*	*Hemisphere*	*t value*	*P* (corrected)	*BA*
30	-7	-24	Hippocampus	R	5.56	0.00	20, 36
-25	-10	-21	Hippocampus	L	5.32	0.00	35
-24	-26	-17	Parahippocampus	L	4.14	0.01	30

p < 0.05 FDR corrected *k*=34. Corrected *P* values are given at cluster level.
Abbreviations: R, right; L, left; GM, gray matter; FDR, false discovery rate; BA, Brodmann area.

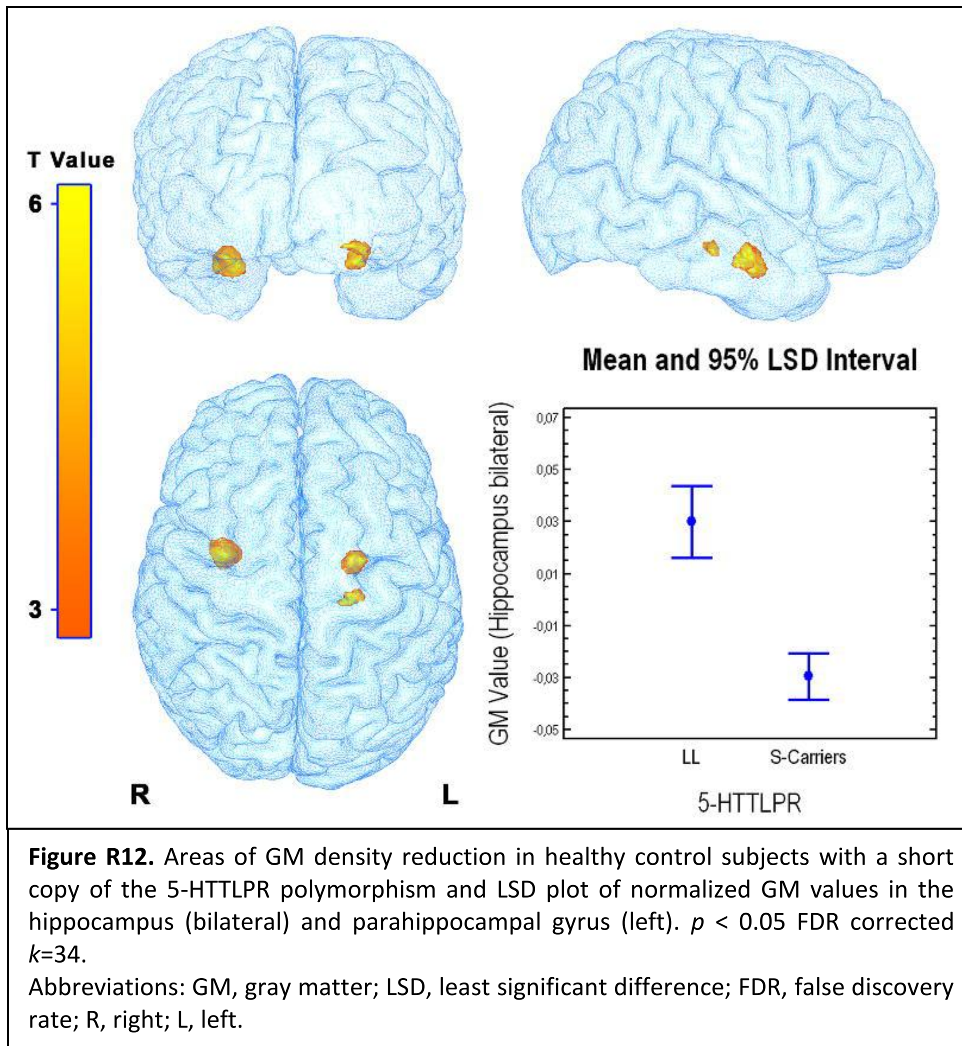


Figure R12. Areas of GM density reduction in healthy control subjects with a short copy of the 5-HTTLPR polymorphism and LSD plot of normalized GM values in the hippocampus (bilateral) and parahippocampal gyrus (left). p < 0.05 FDR corrected k=34.
Abbreviations: GM, gray matter; LSD, least significant difference; FDR, false discovery rate; R, right; L, left.

6.1.1.2. Effect of 5-HTTLPR in schizophrenic patients

GM density reduction was found in patients who had at least one copy of the short allele (table R122, figure R13). The most relevant area that showed significant reduction of GM density was the anterior cingulate gyrus, specifically Brodmann areas 24 and 32 (p<0.05 FDR corrected, k=34). Analogously to the control group, the schizophrenic *s* group did not show any regions of greater GM values, compared with the *l/l* group.

Table R122. Significant areas with GM density reduction in schizophrenic patients with at least one short allele when compared to healthy subjects with *l/l* genotype.

Coordinates (mm) X	*Y*	*Z*	*Label*	*Hemisphere*	*t value*	*P* (corrected)	*BA*
-3	42	14	Anterior cingulate gyrus	L	4.72	0.01	24,32

p < 0.05 FDR corrected k=34. Corrected P values are given at cluster level.
Abbreviations: L, left; GM, gray matter; FDR, false discovery rate; BA, Brodmann area.

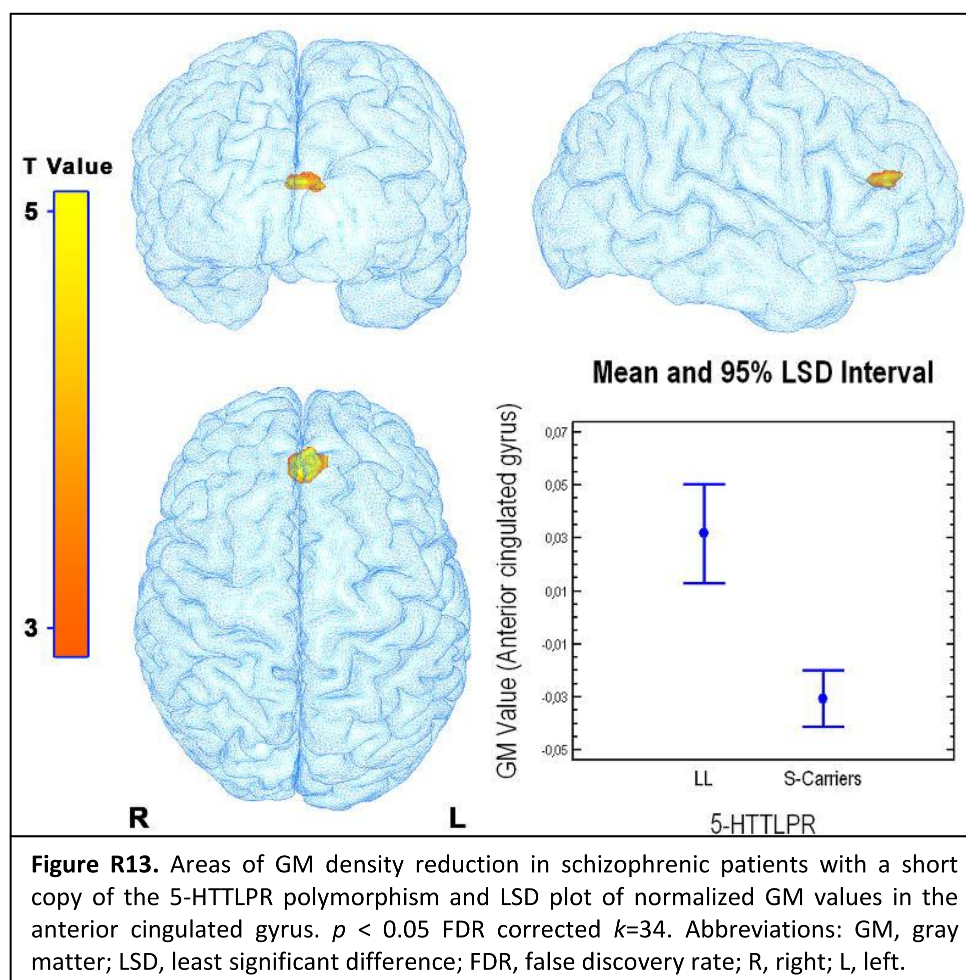


Figure R13. Areas of GM density reduction in schizophrenic patients with a short copy of the 5-HTTLPR polymorphism and LSD plot of normalized GM values in the anterior cingulated gyrus. $p < 0.05$ FDR corrected k=34. Abbreviations: GM, gray matter; LSD, least significant difference; FDR, false discovery rate; R, right; L, left.

6.1.2. Effect of 5-HTTLPR polymorphism on a functional neuroimaging phenotype.

An exploratory and preliminary analysis to study the effect of the promoter polymorphism on the emotional response to voices (evaluated through an emotional-auditory functional neuroimaging paradigm) was performed on a group of 26 male right-handed schizophrenic subjects (DSM-IV criteria) from the Spanish sample. All patients were under antipsychotic treatment in the moment of evaluation. Some demographic and clinical characteristics from these patients (depending on their genotype) can be found at table R123.

As in the VBM study, SNP rs25531 was not considered for the fMRI analysis and patients were only characterized as *l/l*, *l/s* or *s/s*. Particularly, the genotypic distribution was 6 homozygotes *l/l*, 12 heterozygotes (*l/s*) and 8 homozygotes *s/s*. No differences were found between schizophrenic patients depending on the genotype in age (F=0.43, p=0.66), age of first hallucinations (F=1.78, p=0.21), illness duration (F=0.33, p=0.72), PANSS positive (F=0.78, p=0.47), PANSS negative (F=2.23, p=0.13) and PSYRATS general score (F=0.54, p=0.59). By contrast, significant differences, depending on genotype for 5-HTTLPR, were found in BPRS general score (F=3.53, p=0.047), PANSS total (F=4.77, p=0.019) and PANSS general (F=7.09, p=0.0042), probably due to the small sample size of *l/l* and *s/s* groups.

Table R123. Clinical and demographic data from the schizophrenic patients included in the correlation study between 5-HTTLPR and fMRI data.

	l/l group (N = 6)	l/s group (N = 15)	s/s group (N = 4)
Age (years)	44.3±6.28	46.4±13.08	51.25±11.81
Age first hallucinations	25.75±9.39	17.1±13.03	31.33±14.01
Illness duration	17.5±4.97	17.6±8.71	14.25±4.03
BPRS total score	42.8±13.8	46.7±9.12	59.75±7.54
PSYRATS for AH total score	15.66±17.23	17.4±15.15	25.75±17.48
PANSS total score	55.16±17.2	60.66±10.97	82±19.91
PANSS positive score	14.66±7.52	16.8±6.16	19.75±4.5
PANSS negative score	11.83±4.16	13.26±4.07	18.25±8.3
PANSS general score	28.66±8.40	30.6±5.97	44±7.87

Data are displayed as mean ± SD.
Abbreviations: SD, standard deviation; BPRS, Brief Psychiatric Rating Scale; PSYRATS, Psychotic Symptom Rating Scale; PANSS, Positive and Negative Syndrome Scale.

A region of interest (ROI) which included the left and the right amygdala was considered.

The most interesting finding (figure R14) was the greater right amygdala activation to emotional words in the *s/s* group, compared to the *l/s* and *l/l* genotypes (P < 0.0001 uncorrected, MNI coordinates 32 -2 -12). However, there was no significant effect of 5-HTTLPR on the activation of the left amygdala.

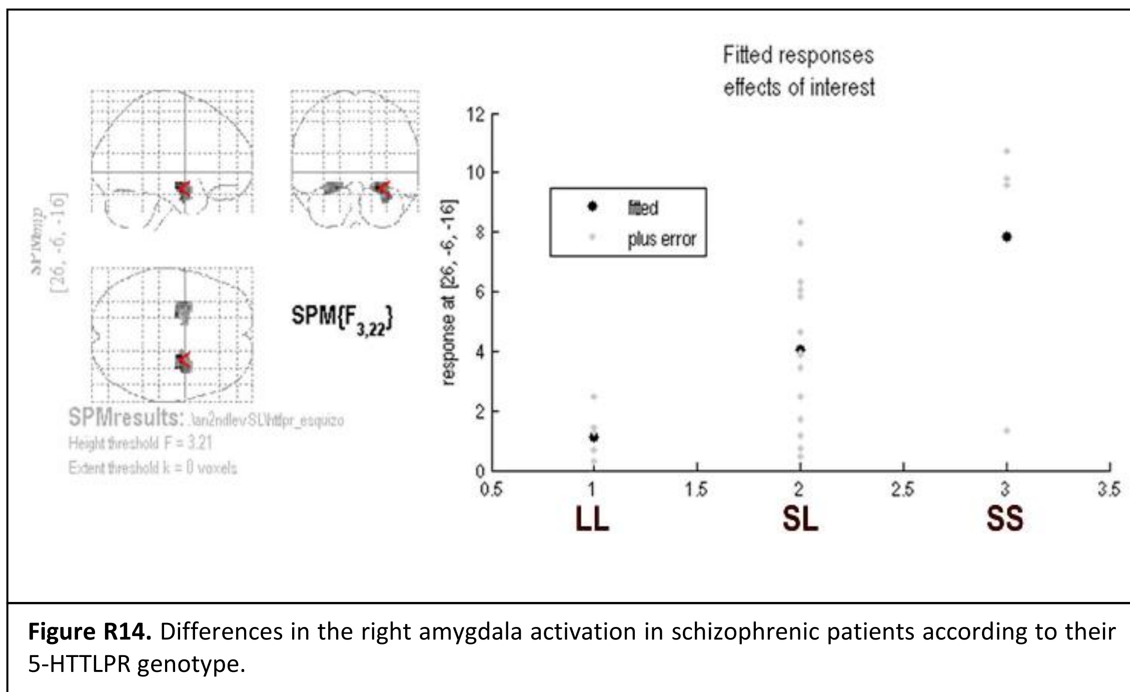


Figure R14. Differences in the right amygdala activation in schizophrenic patients according to their 5-HTTLPR genotype.

6.2. *ASPM* gene

6.2.1. Effect of *ASPM* gene on brain volume.

To analyse the effect of *ASPM* variation on brain morphology, we considered to be interesting those polymorphisms which were associated with risk for schizophrenia, but also the SNPs significantly associated with cognitive performance. Moreover, in order to get more reliable results, only those significant SNPs with a balanced genotypic distribution were studied: on one hand, rs10922163 (located in intron 17 and slightly associated with schizophrenia in both case-control and family-based association analyses) and, on the other hand, rs41310927 and rs3762271 (located in exon 18 and associated with WCST performance).

Table R124 shows information about the main clinical, genetic and demographic variables for the 153 controls and 169 patients from the American sample included in this neuroimaging analysis.

Table R124. Clinical and demographic data from the control subjects and schizophrenic patients included in the correlation study between *ASPM* polymorphisms and VBM data.

Control subjects (N = 153)	
Age (years)	32.78 ± 9.61
Gender	78 males, 75 females
Education years	16.98 ± 3.21
WAIS-R IQ	106.43 ± 15.09
Genotypic distribution	rs41310927: 49 A/A; 67 A/G; 26 G/G rs3762271: 51 C/C; 65 C/A; 24 A/A rs10922163: 43 A/A; 75 A/G; 35 G/G
Schizophrenic patients (N = 169)	
Age (years)	37.00 ± 9.70
Gender	131 males, 38 females
Education years	14.38 ± 2.20
Diagnosis	146 schizophrenics, 21 schizoaffective disorder, 2 other psychoses
WAIS-R IQ	94.89 ± 11.29
Genotypic distribution	rs41310927: 61 A/A; 82 A/G; 23 G/G rs3762271: 61 C/C; 80 C/A; 25 A/A rs10922163: 37 A/A; 91 A/G; 41 G/G

Data are displayed as mean ± SD.
Abbreviations: SD, standard deviation; WAIS-R, Wechsler Adults Intelligence Scale revised; IQ, intelligence quotient.

We did not find any effect of genotype on total intracranial volume from patients or controls for any SNP. By contrast, we found a significant effect of the three SNPs on regional volumes in healthy subjects and schizophrenic patients:

- **rs41310927 (table R125)**: With regard to control subjects, allele G carriers showed a reduced volume in prefrontal cortex (Brodmann Area 9) when compared to A/A homozygotes as well as an increased volume in the subgenual cingulate cortex (Brodmann Area 25). By contrast,

schizophrenic patients who were allele G carriers had greater cerebellum volumes as well as a reduced gray matter volume in prefrontal cortex (Brodmann area 9) compared with A/A homozygote patients.

Table R125. Significant areas with GM density differences depending on the genotype for rs41310927.

Coordinates (mm) X	Y	Z	*Label (Brodmann area)*	*Contrast*	*Sample*	*Hemisphere*	*Z*	*K*
-10	14	-2	Subgenual Cingulate cortex (BA25)	A/A < A/G + G/G	Controls	L	3.99	1822
-18	11	-5					3.50	
-11	3	12					3.49	
56	24	31	Prefrontal cortex (BA9)	A/A > A/G + G/G	Controls	R	3.47	134
55	-63	-32	Cerebellum	A/A < A/G + G/G	Patients	R	4.02	1446
52	-61	-44					3.98	
-55	8	33	Prefrontal cortex (BA9)	A/A > A/G + G/G	Patients	L	3.51	239

P = 0.000 uncorrected.
Abbreviations: L, left; R, right; GM, gray matter; BA, Brodmann area.

- **rs3762271:** The analysis performed on the control sample (table R126; figure R15) showed that allele A carriers had a reduced volume in prefrontal cortex (Brodmann Area 9) when compared to C/C homozygotes, as well as an increased volume in the subgenual cingulate cortex (Brodmann Area 25). However, the differences in regional volumes observed in schizophrenic patients were different from those in healthy subjects (table R126, figure R16): those patients with at least one copy of allele A had greater cerebellum volumes as well as a reduced gray matter volume in prefrontal cortex (Brodmann area 9) compared with C/C homozygote patients.

Table R126. Significant areas with GM density differences depending on the genotype for rs3762271.

Coordinates (mm) X	Y	Z	*Label (Brodmann area)*	*Contrast*	*Sample*	*Hemisphere*	*Z*	*K*
-9	16	-3	Subgenual Cingulate cortex (BA25)	C/C < C/A + A/A	Controls	L	3.84	1403
-16	12	-5					3.61	
-6	5	-8					3.20	
56	26	33	Prefrontal cortex (BA9)	C/C > C/A + A/A	Controls	R	3.69	265
55	-65	-30	Cerebellum	C/C < C/A + A/A	Patients	R	3.88	1097
52	-61	-44					3.70	
-55	8	33	Prefrontal cortex (BA9)	C/C > C/A + A/A	Patients	L	3.63	360
-62	1	31					3.18	

P = 0.000 uncorrected.
Abbreviations: L, left; R, right; GM, gray matter; BA, Brodmann area.

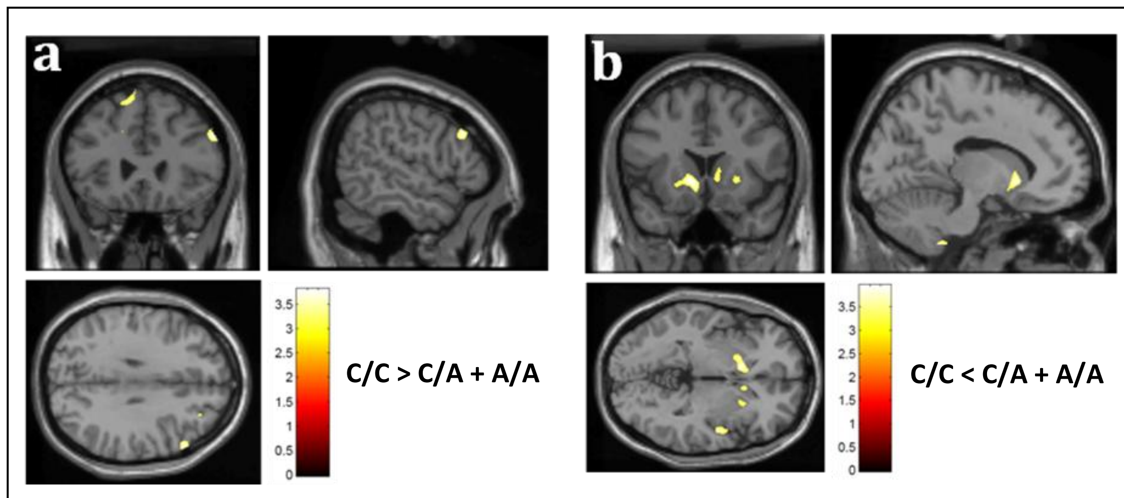


Figure R15. Correlation between VBM data and SNP rs3762271 in healthy subjects. Allele A carriers (**a**) showed a reduced volume in prefrontal cortex (Brodmann area 9) compared with C/C homozygotes, as well as an increased volume (**b**) in the subgenual cingulate cortex (Brodmann area 25).
Map threshold: P < 0.001 uncorrected.

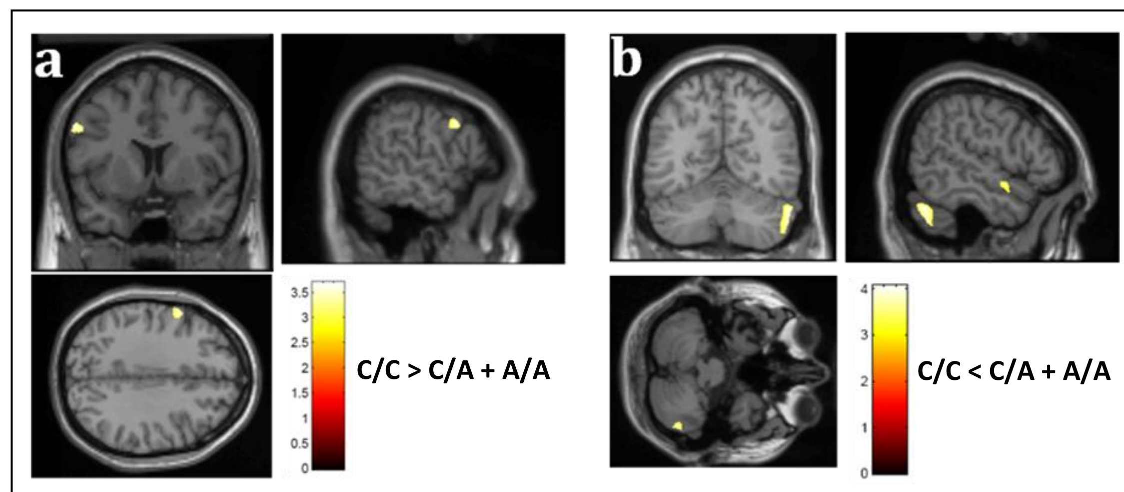


Figure R16. Correlation between VBM data and SNP rs3762271 in schizophrenic patients. Allele A carriers (**a**) have a reduced volume in prefrontal cortex (Brodmann area 9) compared with C/C homozygotes, as well as an increased volume (**b**) in the cerebellum.
Map threshold: P < 0.001 uncorrected.

- We also found a significant effect of SNP rs10922163 on regional brain volumes. Those control subjects with at least one copy of allele G (G-carriers) showed significant reductions in basal ganglia (caudate nucleus) and subgenual cingulate cortex (Brodmann area 25) when compared to A/A homozygotes (table R127). Moreover, we also detected an effect of rs10922163 in schizophrenic patients. In this case, allele G carriers had a significant reduction in cerebellum (table R127).

Table R127. Significant areas with GM density differences depending on the genotype for rs10922163.

Coordinates (mm) X Y Z			*Label (Brodmann area)*	*Contrast*	*Sample*	*Hemisphere*	*Z*	*K*
-5	19	-7	Basal ganglia (caudate) Subgenual cingulate cortex (BA25)	A/A > A/G + G/G	Controls	L	3.92	2525
-7	8	-8					3.70	
-10	2	3					3.67	
52	-61	-44	Cerebellum	A/A > A/G + G/G	Patients	R	3.64	454

P = 0.000 uncorrected.
Abbreviations: L, left; R, right; GM, gray matter; BA, Brodmann area.

6.2.2. Effect of *ASPM* gene on a functional neuroimaging phenotype.

Given the effect of *ASPM* genotype on brain morphology, we hypothesized that *ASPM* variation would also correlate with an effect on neural activation of prefrontal areas during N Back task in healthy subjects. For this purpose, 79 healthy subjects were included in the study. These subjects did not differ in N Back performance depending on their genotype (table R128).

Table R128. Performance of healthy subjects during the 2 back task associated to the fMRI procedure.

		rs41310927		
	A/A (N = 21)	**A/G** (N= 41)	**G/G** (N = 9)	***P*[a]**
% correct 2-back	0.99±0.002	0.99±0.002	0.99±0.003	0.71
Reaction Time (s)	0.57±0.04	0.60±0.030	0.58±0.06	0.81
		rs3762271		
	C/C (N = 31)	**C/A** (N= 41)	**A/A** (N = 7)	***P*[a]**
% correct 2-back	0.99±0.002	0.99±0.002	0.99±0.003	0.68
Reaction Time (s)	0.58±0.04	0.60±0.030	0.57±0.07	0.93
		rs10922163		
	A/A (N = 31)	**A/G** (N= 41)	**G/G** (N = 7)	***P*[a]**
% correct 2-back	0.99±0.002	0.99±0.002	0.99±0.002	0.52
Reaction Time (s)	0.54±0.04	0.62±0.030	0.56±0.07	0.23

Data are displayed as mean ± SE.
a. An Analysis of Variance (ANOVA) test was performed to compare the mean values of each genotypic group.

Consistent with our initial hypothesis, we found a significant effect of *ASPM* genotypic variation on working memory performance. The most significant effect was caused by SNP rs10922163. During the N Back working memory task, healthy subjects with two copies of the A allele showed greater activation in the anterior cingulate cortex (Brodmann area 32), compared with allele G carriers (table R129, figure R17).

Table R129. Significant differences in brain activation during a N Back task depending on the genotype for *ASPM* SNP rs10922163.

Coordinates (mm) X Y Z			*Label (Brodmann area)*	*Contrast*	*Hemisphere*	*Z*	*P* (uncorrected)	*K*
-8	19	30	Anterior cingulate cortex (BA32)	A/A > A/G > G/G	L	4.00	0.000	39

Abbreviations: L, left; BA, Brodmann area.

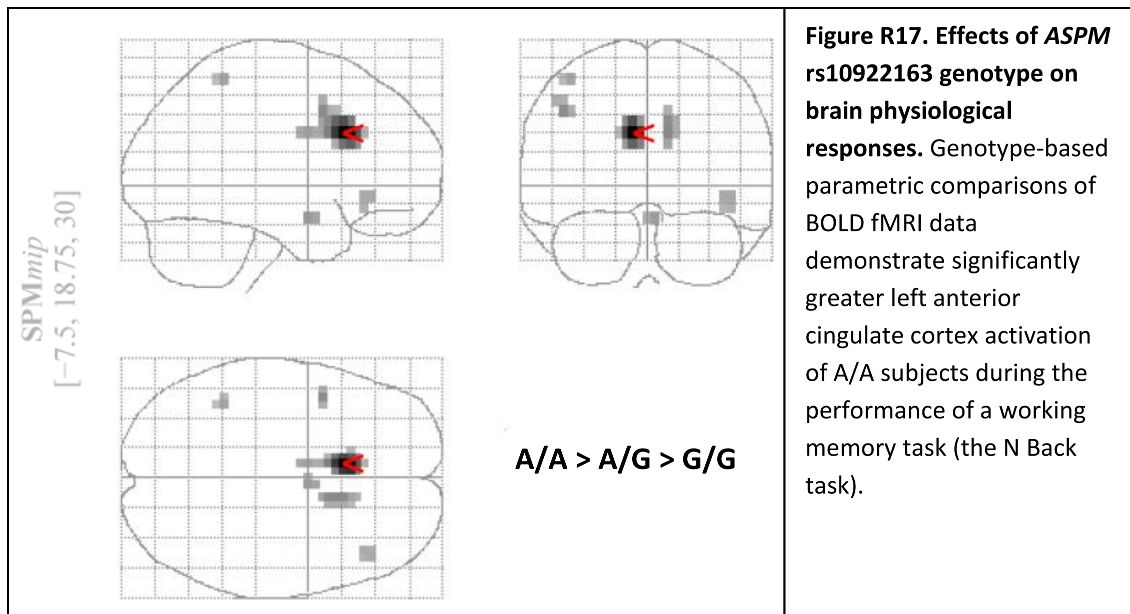


Figure R17. Effects of *ASPM* rs10922163 genotype on brain physiological responses. Genotype-based parametric comparisons of BOLD fMRI data demonstrate significantly greater left anterior cingulate cortex activation of A/A subjects during the performance of a working memory task (the N Back task).

Moreover, slightly significant results were also found for SNPs rs41310927 (table R130) and rs3762271 (table R131): those individuals who were A/A homozygotes for rs41310927 showed less activation in anterior cingulate cortex (Brodmann area 32) and prefrontal cortex (Brodmann area 9) when compared to allele G carriers (table R130). Moreover, a decrease in prefrontal cortex (Brodmann area 9) activation was also observed for those individuals who had at least one A allele from SNP rs3762271 (table R131). However, the reliability of these results is limited given the low *P* values, especially for the prefrontal cortex findings.

Table R130. Significant differences in brain activation during an N Back task depending on the genotype for *ASPM* SNP rs41310927.

Coordinates (mm) X	*Y*	*Z*	*Label (Brodmann area)*	*Contrast*	*Hemisphere*	*Z*	*P* (uncorrected)	*K*
-8	19	30	Anterior cingulate cortex (BA32)	A/A < A/G + G/G	L	3.24	0.001	21
45	8	18	Prefrontal cortex (BA9)	A/A < A/G + G/G	R	2.78	0.003	13

Abbreviations: L, left; R, right; BA, Brodmann area.

Table R131. Significant differences in brain activation during an N Back task depending on the genotype for *ASPM* SNP rs3762271.

Coordinates (mm) X	*Y*	*Z*	*Label (Brodmann area)*	*Contrast*	*Hemisphere*	*Z*	*P* (uncorrected)	*K*
45	11	24	Prefrontal cortex (BA9)	C/C < C/A + A/A	R	2.66	0.004	6

Abbreviations: R, right; BA, Brodmann area.

DISCUSSION

DISCUSSION

1. Initial considerations

This doctoral thesis project is part of a multidisciplinary research effort whose general objective is to better understand the underlying causes of schizophrenia and other related psychoses, with a special interest in the genetic causes of this disorder. Prior to the discussion of our results, there are several issues and limitations that need to be acknowledged and considered to fully understand the conclusions of this study. These limitations concern different aspects of the study, such as the alternative phenotypes and sample features, the clinical information available from each dataset, the genotyping process, marker coverage and statistical analysis.

1.1. About the samples and phenotypes considered in this study

The complexity and the probable lack of utility of the clinical definition of schizophrenia are unquestionable handicaps which had led to the use of complementary approaches in the study of the genetic bases of this disorder. Among these approaches, the use of alternative phenotypes can be especially useful. For this reason, several alternative phenotypes have been selected and several samples have been used in the present study.

Auditory hallucinations (AH) are one of the alternative phenotypes tackled in this study. As explained in the Introduction, AH are one of the hallmarks of psychoses and, despite they can be detected in other types of patients (neurological, drug abusers) or even in healthy subjects, the phenomenology of AH has particular features in psychotic patients. Given our interest in studying the genetics of AH in psychotic patients, a sample of Caucasian psychotic patients and control subjects from Valencia (Spain) began to be collected some years ago by our group at the Departments of Medicine and Genetics from the University of Valencia. The main interest of this sample is that the clinical characteristics of AH have been studied in great detail on every patient. We consider that auditory hallucinations are a multidimensional phenomenon and therefore it cannot be evaluated through a presence/absence item (as it happens in general scales such as PANSS, BPRS or KGV). For this reason, all hallucinatory patients were assessed through the PSYRATS scale for auditory hallucinations (Haddock *et al.*, 1999; González *et al.*, 2003b), which is composed of 11 different items which cover the different dimensions of auditory hallucinations, and also including three parameters which evaluate the emotional response to this positive symptom (Intensity of Distress, Amount of Distress and Disruption to life). Moreover, a small subset of these patients (as well as a group of healthy controls) was also subjected to magnetic resonance (MR) protocols, in an attempt to evaluate the presence of changes in brain volume or brain activity correlating with variation at the selected candidate genes. The fMRI emotional-auditory paradigm elaborated by our group (Sanjuán *et al.*, 2007) also provides a good way to evaluate how patients respond to auditory hallucinations. In conclusion, the sample of Spanish patients and controls allows the study of the genetics of auditory hallucinations from three different levels: a) case-control association studies, which evaluate the existence of different allelic, genotypic or haplotype distributions in hallucinatory patients compared to other groups (controls, patients without AH…); b) association analyses

between genetic polymorphisms and items from PSYRATS subscale for AH; and c) association analyses with neuroimaging MR phenotypes (sMRI-VBM and fMRI). However, an important disadvantage is that the number of patients without hallucinations is still low in our sample. Although this type of patients is necessary for comparison purposes, the high frequency of auditory hallucinations in psychotic patients makes the search for non-hallucinatory patients difficult. For this reason, the search for psychiatric patients without AH led us to include some patients with bipolar disorder. However, the homogeneity of the sample, with regard to the absence/presence of auditory hallucinations, is guaranteed.

Other Caucasian samples of different origin have also been included in this study. The **American sample collected at the Clinical Brain Disorders Branch** (National Institute of Mental Health) is of special interest because exhaustive clinical information on cognitive variables is available. Due to its high impact on patients' life quality, **cognitive impairment** is the second alternative phenotype considered of interest for this doctoral thesis project. Thus, it is possible to study the effect of genetic variation on several cognitive factors corresponding to different cognitive domains (Genderson *et al.*, 2007). Furthermore, an important number of individuals from the CBDB dataset were also subjected to several MR procedures, including morphometry as well as functional neuroimaging measures during the performance of several cognitive tasks. For the present study, several SNPs on *ASPM* gene were genotyped during my stay at the CBDB, thus allowing the study of how *ASPM* variation influences schizophrenia and cognition by using different approaches: case-control and family-based studies; linear regression studies with cognitive factors; and correlation studies with neuroimaging data.

The third sample which has been also included in this study is a **German case-control dataset from the Department of Psychiatry and Psychotherapy at the University of Würzburg**. In this case, we have no information regarding auditory hallucinations or cognition; however, the results from the case-control association studies can be compared to those obtained in the Spanish sample. Particularly, *PDE4D*, *PLEKHB1* and *RAB6A* genes were genotyped in this sample and included in the present study.

Although the study of AH and emotionality was our primary goal, other aspects were also analyzed in the Spanish sample. As it was explained before, Spanish patients underwent an exhaustive clinical examination through different clinical scales. Some of them were general (PANSS, BPRS, KGV), while others focused on specific disease traits, such as the PSYRATS subscales for auditory hallucinations and delusions. It is important to remark that all these variables provide information about the emotional state of the schizophrenic patients, and an effect of genotype on positive and negative symptoms can also be studied. However, although the search for association with these measures can provide us with a lot of information, this approach also presents several limitations. First, these traits are extraordinarily **variable and can change over time** without following a logical pattern (Cuesta and Peralta, 2005). Thus, our measures represent a photograph, a static capture of a complex process. As a result, it is particularly hard to define the effect of genetic variation on these disease traits. Second, it may be argued that these measures **do not have biological correlates and are away from the genetic etiology of psychoses** (Sanjuán, 2007). Although this may be true for some measures, many clinical items may help us to know if certain polymorphism has a role on general behavioral dimensions, such as impulsivity, emotionality or cognition. A clear example is the

HTR2A gene, which was found to be associated with an important number of measures from different scales related to emotionality (see tables R97 to R99). Third, the analysis of such a huge number of variables drastically **increases the chance of spurious associations (type I error)**. This scenario would explain the high number of nominal associations found with clinical scores. It is also likely that the sequential Bonferroni correction is too permissive. However, the number of positive findings after correction for multiple testing is small and may help us to know which measures are really influenced by the genotype.

We are also aware that our **sample size is relatively small** and the statistical power to detect associations is reduced. Thus, small effect sizes, or marginal effects in case interactions exist, may be difficult to detect, particularly for those polymorphisms with a low minor allele frequency. For example, the Spanish case-control data set provides 99% power to detect association if the OR is 2 for a polymorphism with a risk allele frequency of at least 0.1. However, this power drastically reduces to 0.33 if the OR is 1.3. This power is even more dramatically reduced (7.6%) for those polymorphisms with a very small frequency of the risk allele (0.01), if the OR that have been considered are small. This could be the case for polymorphisms such as rs12138336 (*ASPM*) or rs17291089 (*PDE4D*)

Particular cases are certain subgroups, such as the non-hallucinatory patients. This subset is extremely small (only 77 individuals). Given the high prevalence of AH among psychotic patients (Slade and Bentall, 1988; Andreasen and Flaum, 1991), it is extremely difficult to collect a group of non-hallucinatory patients with enough size for reliable association studies. Analogously, the number of patients who underwent clinical evaluation through certain scales, namely PANSS and PSYRATS subscale for delusions, is also relatively small. This small sample size could explain the high number of significant associations obtained for the PANSS items.

The difficulty to collect certain individuals or clinical measures emphasizes the importance of performing cooperative studies involving different research groups, in an attempt to gather larger samples (Levinson *et al.*, 2000; McInnis *et al.*, 2003; Vilella *et al.*, 2008). According to this idea, this study presents data from several Caucasian samples from Spain, Germany and the United States of America. The use of pooled samples can be useful to improve the power to detect associations. In our study, the use of a combined Spanish-American sample confirmed the association with SNP rs10922163 (from the *ASPM* gene) which had been detected in both samples separately (table R64). However, despite the use of larger samples in combination with GWA studies, there is still uncertainty into the field of psychiatric genetics, since each new large scale study has obtained a different result (Sullivan *et al.*, 2008; O'Donovan *et al.*, 2008; Shifman *et al.*, 2008b; Allen *et al.*, 2008). Nevertheless, it is necessary to remark that all GWA studies for psychiatric diseases such as schizophrenia and bipolar disorder (Lencz *et al.*, 2007; Sullivan *et al.*, 2008) have used modest sample sizes, in comparison with other diseases, such as type-2 diabetes mellitus (more than 38,000 cases and controls) or obesity (more than 30,000 subjects) (Burmeister *et al.*, 2008; Thorleifsson *et al.*, 2009;). Thus, it is likely that we are still far from obtaining coherent findings through this approach.

Population stratification is another central issue of population-based association studies (Langefeld and Fingerlin, 2007; Tian *et al.*, 2008). It may be due to the existence of subpopulations with different rates of disease or allele frequencies, or because of admixture,

when the sample is composed of individuals with differing fractions of ancestry from distinct populations, and the genetic admixture differs between patients and controls (Langefeld and Fingerlin, 2007). In the present study, affected and control subjects were carefully matched on ethnicity (all Caucasian), gender (with the exception of the American sample, where females were more abundant in the control group) and age (mean age was similar among cases and controls). However, the possibility of a potential effect of population stratification on the association results still exists, particularly due to the small sample size.

Unfortunately, the confounding effects of population stratification may be clearer when two samples of different geographical origin are combined. However, in order to minimize this effect in our combined association analyses, we performed a specific test which allows controlling potential confounding factors due to a different geographical origin.

1.2. Genotyping methodologies: advantages and disadvantages

Nowadays, researchers can count on a wide variety of genotyping techniques to fulfill the requirements and aims of each study design. Examples of these techniques are the RFLP-genotyping methods, single strand conformation polymorphisms (SSCPs), sequencing methods, Taqman SNP genotyping Assays, iPLEX-MassArray genotyping and Illumina GoldenGate Genotyping Assay, among others.

During this study, some of these methodologies were used to genotype the different samples. Particularly, three methods have been mainly used: RFLP-genotyping, Taqman SNP genotyping Assays and iPLEX-MassArray genotyping. This variability is due to the fact that different parts of the study were carried out at different laboratories. All these genotyping techniques are based on different processes and present different features (table D1) which should be considered when designing an association study.

Table D1. Features of the different genotyping methods used in the present study.

Method	Infrastructure	Simplicity of experiment design and optimization	Simplicity of the whole process	Speed	Number of SNPs or samples (throughput level)	Accuracy	Price
RFLPs	+	++	++	++	+	+++	variable
Taqman assays	+++	++++	+	+++	+++	+++	++
Iplex-MassArray	++++	+++	++	++++	++++	+++	+++

Each feature has been evaluated from + (low) to ++++ (very high).
It should be noted that the ratings may be subjective.

RFLP-based genotyping methods are based on the differential digestion of PCR products, depending on the genotype for the SNP of interest. In the present study, this method was used to genotype some polymorphisms from the *SCL6A4*, *HTR2A*, *TPH2*, *ASPM* and *STMN1* genes. One of the most important advantages of this traditional technique is that **it does not require any specific or expensive infrastructure**. Thus, it is eligible as an initial option to start out genotyping, as it was the case in the present study. The **optimization of each genotyping protocol** can take a long time, since the assay conditions (PCR conditions, restriction enzyme…) are generally specific to each polymorphism. However, nowadays there is a wide variety of computer utilities which may help in the election of the best primer sequences, PCR conditions or the most suitable restriction endonuclease.

By contrast, once the conditions are set, the genotyping process is **easy to perform** and does not require specific training. But compared to other techniques, it is a **slow process** with several steps (amplification, PCR testing, digestion and separation of digestion products). Therefore, it can take more time than other methods.

Despite its simplicity, this is a **low-throughput genotyping method** which does not allow handling a huge number of DNA samples at the same time. For this reason, it is not suitable for large sample sizes.

Our own experience suggests that the **accuracy** of RFLP-genotyping is more than 98% and the reliability of the results is high. Moreover, the number of fails is low, particularly when the amplification conditions have been perfectly established. However, it has been reported that the concentration of magnesium chloride in the PCR reaction mix can be of vital importance for the genotyping of VNTRs, such as 5-HTTLPR or STin2 (from the *SLC6A4* gene), since a preferential amplification of one of the alleles of heterozygous individuals can happen under certain $MgCl_2$ concentrations (Kaiser *et al.*, 2002; Yonan *et al.*, 2006). The use of different Mg^{2+} concentrations did not appear to affect the genotyping of those polymorphisms in our study, however. Other factors which could produce genotyping errors are the use of restriction endonucleases with star activity (relaxed specificity under certain incubation conditions) and the use of enzymes which are affected by DNA methylation.

Finally rare restriction endonucleases become particularly expensive and the final price of the entire process can increase spectacularly. In this latter case, this methodology would not be advisable.

In conclusion, this methodology would be appropriate for low to medium sample sizes and for cheap restriction enzymes. If both conditions are not fulfilled, other methods should be used.

Taqman SNP genotyping assays are based on the use of a 5' nuclease assay to discriminate between two alleles of a specific SNP (Livak *et al.*, 1995a). This methodology was used to genotype the *ASPM* SNPs in the American sample. The overall performance of this technique is pretty satisfactory. In contrast with the gel-based methods, this **high-throughput genotyping approach** needs **more infrastructures**, such as a Real-Time PCR System (compulsory) or automated systems to dispense DNA samples on 384-well plates or to prepare amplification reactions. These latter automated systems, although optional, can substantially reduce the preparation time, allowing the genotyping of a huge number of samples in one day (Livak *et al.*, 1995b).

The **simplicity of the protocol** (only reduced to two main steps: amplification reaction and plate read) is substantially important because it reduces the possibility of contamination.

Moreover, the entire process is also faster than other existing genotyping methods. Interestingly, this technology can be considered of high throughput and it is suitable for high sample sizes, since it has been designed to work with up to 384-well plates.
Compared to other genotyping approaches, one of the most important advantages of this technique is that there is **no need of optimization**, since each assay is functionally tested by the manufacturer. Moreover, the marker coverage of these assays is huge and includes over 3.5 million genome-wide SNPs; furthermore, other SNPs not included in the catalogue can be also genotyped with this methodology by using a custom assay service. Unfortunately, our own experience shows that, for a small percentage of SNPs, the assay quality is not as the manufacturer had declared, and the clusters can be hardly distinguished, thus making the assignment of genotypes particularly difficult (see figure D1). In those cases, genotyping accuracy can be compromised.

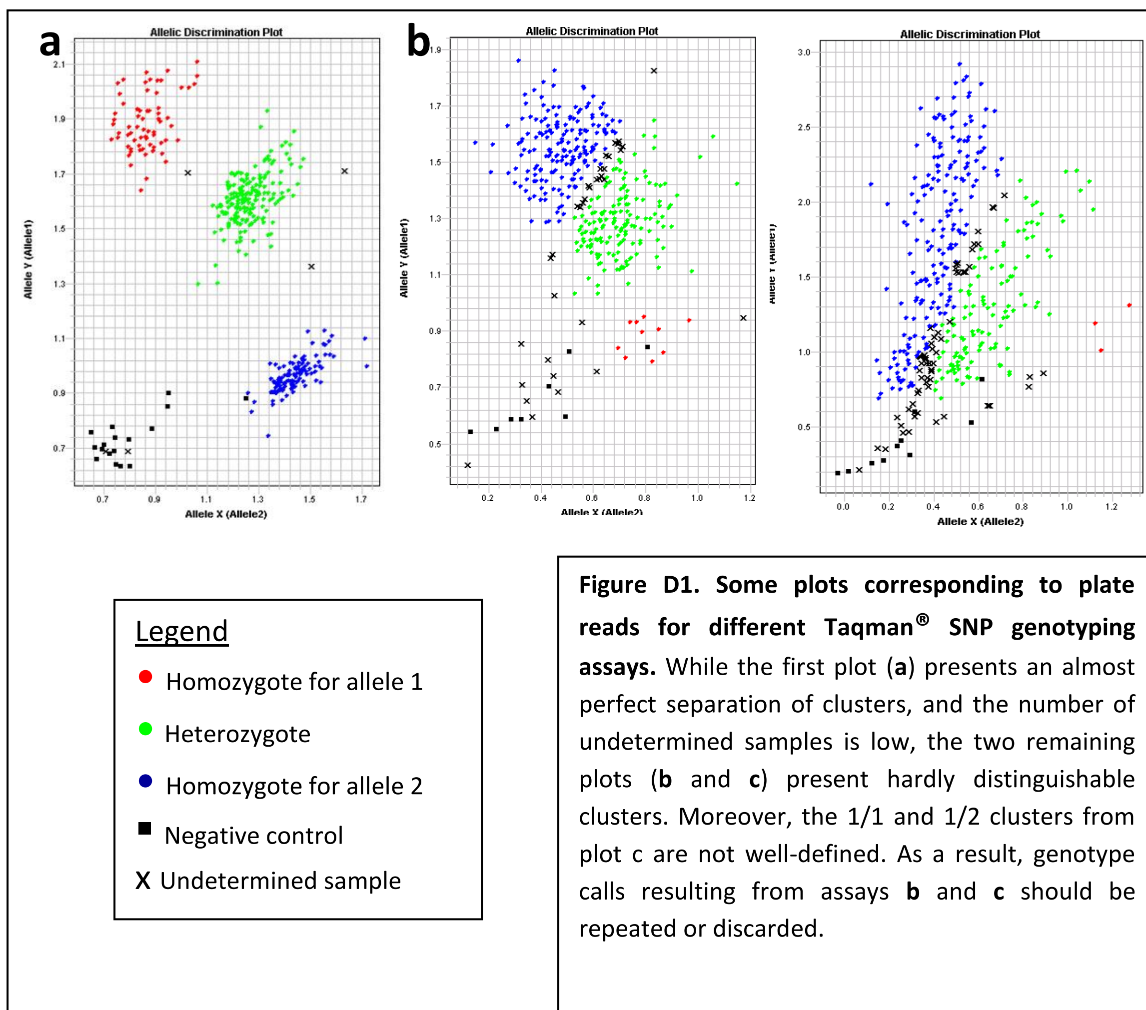


Figure D1. Some plots corresponding to plate reads for different Taqman® SNP genotyping assays. While the first plot (**a**) presents an almost perfect separation of clusters, and the number of undetermined samples is low, the two remaining plots (**b** and **c**) present hardly distinguishable clusters. Moreover, the 1/1 and 1/2 clusters from plot c are not well-defined. As a result, genotype calls resulting from assays **b** and **c** should be repeated or discarded.

Finally, the **price**, higher than in agarose-based methods, can be another inconvenient, particularly if the number of fails for certain assay is high and requires repeating the entire process. Moreover, if the number of fails exists but it is not very high, the regenotyping of fails may be not profitable. This can be a real problem if we are working with a small dataset, due

to the loss of genotypic information. In summary, Taqman SNP genotyping assays are an excellent alternative for large datasets.

The third technique which was used in the present study (for *PDE4D*, *PLEKHB1*, *RAB6A*, *STMN1* and *SLC6A4* genes) uses the **iPLEX-MassArray® technology** from Sequenom. It is based on the generation of single base extension (SBE) products, which can be subsequently read by MALDI-TOF mass spectrometry (Ross *et al.*, 1998). The most striking advantage of this genotyping technology is the possibility of genotyping up to 29 SNPs at the same time in a multiplex reaction protocol. However, one of the most important disadvantages of iPLEX assays is that **expensive equipment** (MALDI-TOF mass spectrometer, MassARRAY® Nanodispenser) is required. It is also highly recommendable the use of additional equipment, such as a PCR workstation to prepare the reaction mix and an automated system to dispense solutions onto 384-well plates.
However, despite the initial outlay, this approach can be considered of great utility. Its hallmark is the **possibility of performing up to 29-plex reactions in 384-well samples** (Oeth *et al.*, 2005). Thus, the number of DNA samples and SNPs which can be processed at the same time is hardly unbeatable by the preceding genotyping methods described above. This technique may be highly eligible for GWA studies.
The **assay design** requires specific software to ensure multiplexing efficiency. This software is easy to use and the resulting primers are usually good to achieve a **high accuracy**. However, the subsequent steps are not as easy to perform as the Taqman SNP genotyping assays: a previous training is highly recommendable to ensure that all the devices (particularly the MALDI-TOF mass spectrometer) are correctly calibrated and used. Finally, data are analyzed through intuitive and useful specific software. Unfortunately, it should be remarked that the use of this technology may be problematical to genotype polymorphisms located in highly repetitive or low complexity genomic regions.
Despite the disadvantages, iPLEX assays are one of the best alternatives to perform large scale association studies with medium to large samples.

1.3. About the association studies

One of the most important aims of an association study design is to ensure a high level of coverage for each gene. For this reason, an indirect approach (The International HapMap Consortium, 2003), based on the selection of tagSNPs, was combined with the selection of certain additional polymorphisms which appeared to have functional implications. However, there are three genes (*HTR2A*, *TPH2* and *NOS1*), which do not accomplish these criteria, since only some representative and/or functional polymorphisms were selected and analyzed. Undoubtedly, this fact can be a problem and we cannot rule out the possibility that other polymorphisms may have an effect on the vulnerability to schizophrenia or specific disease traits.

Moreover, despite using the exhaustive genotypic information publicly available at the HapMap Project database (http://www.hapmap.org/), it may be possible that the selected tags are not sufficient to capture all the variability of certain genomic region of interest, due to the existence of unknown polymorphisms with an important role on gene functioning. Moreover, another limitation is related to the use of CEPH-CEU samples as the reference population for

tag selection. Despite this population (Utah residents with ancestry from northern and western Europe) is of Caucasian origin, it is unclear whether these samples share a highly similar genetic structure and LD patterns with current European populations. Thus, this fact could also affect tag selection. However, this population was the best alternative for our study, although this issue should be taken into account.

Another important problem related to any association analysis lies in the **limitation of the current statistical tools** to cope with the complexity of genetic-environmental models of complex mental disorders (Balding, 2006). As much as possible, we have tried to use valid statistical tools, such as the SNPstats program (Solé *et al.*, 2006), which considers different inheritance models and allows the use of covariates by applying a logistic regression approach (for case-control comparisons) or a lineal regression approach (for quantitative disease traits). The possibility of testing multiple inheritance models (recessive, dominant, additive, codominant and overdominant) is extremely useful, because it prevents the loss of information when only a codominant model is tested. However, different inheritance models for the same significant SNP depending on the response variable have been observed in the present study. One example is the serotonin transporter promoter polymorphism 5-HTTLPR, which equally fitted to both additive and dominant models depending on the clinical variable associated with the polymorphism. However, this fact should not lead to confusion, particularly if all associations show the same trend (namely, if the same genotypes or alleles are always associated with an increase in scores, even though different inheritance models have been observed).

Haplotype analysis is also a particular case. Many approaches have been developed to deal with haplotypes (Zhao *et al.*, 2003; Balding, 2006; Langefeld and Fingerlin, 2007). The most important problem is that the phase is generally unknown. Thus, the first difficulty is to infer the haplotype phase from genotypic data. Several methods have been developed for this purpose, although their efficiency depends on several factors, such as the heterozigosity of each subject and the LD levels among polymorphisms. The second problem is haplotype complexity. There are several methods to reduce the number of haplotypes considered in association studies (Zhao *et al.*, 2003). For this study, a 2-, 3- and 4-marker sliding-window analysis was used. From our point of view, this was the more appropriate model, since each window has less haplotypes than the whole candidate region and the computation requirements are lower. Moreover, the statistical power to detect association is increased. However, we are aware that this methodology does not take into account the evolutionary history of the population and the linkage disequilibrium patterns. Moreover, the small sample size, together with the elimination of rare haplotypes (frequency < 0.03) from the case-control analysis, can lead to a loss of information.

Finally, the high number of polymorphisms and variables analyzed in this study can **increase type I errors (false positives) due to multiple testing**. Multiple testing is one of the most difficult issues in statistical genetics. There are different ways to control the increase of type I error. One possibility is the Bonferroni correction. However, this correction tends to be extremely conservative and can increase the number of false negatives (type II error), particularly when we are dealing with a high number of tests. By contrast, some research groups consider that it is not necessary to apply corrections. However, there is a practical

alternative, which consists in applying less conservative correction methods, such as the false discovery rate correction (FDR) (Storey and Tibshirani, 2003) or the sequential Bonferroni correction (Rice, 1989). With these corrections, the risk of a false positive is higher, although the power is higher too. In the present study, the sequential Bonferroni correction was applied. As a result, the original number of significant associations was drastically reduced. However, it may be argued that the other possibilities (Bonferroni or absence of correction) would be also recommendable.

Moreover, an important difficulty related to association studies is the **lack of replication among studies and populations**. As a result of this observation, two explanations arise: are there different risk variants depending on the population? or is the lack of replication a result of a bad study design? In the present study, we have considered different populations, all of them of Caucasian origin, in order to have more complete information about each candidate gene.

Despite the replication of the association or the SNP rs10922163 in both the American and the Spanish sample, results are usually enormously different. Several reasons can be invoked to explain these differences and some of them have been discussed previously: a) differences in the genetic structure of each population, which would lead to the existence of different risk alleles and genotypes on each group; b) increase in type I error (chance of positive associations) due to the reduced sample size. Moreover, a third reason could be the use of different statistical programs, which are based on different assumptions and algorithms. This situation would apply to the analysis of *ASPM* variation, since different programs were used for the Spanish and the American sample.

1.4. About the correlation with neuroimaging phenotypes

Some issues commented above can be also applied to our neuroimaging studies (Flint and Munafò, 2006; Glahn *et al.*, 2007; Pell *et al.*, 2008). These limitations are a) the small sample size in the Spanish dataset (particularly for control subjects); b) the likely effect of multiple comparisons; c) the limitations of current statistical utilities to work with such a complex combination of multimodal imaging parameters and genetic information.

Other variables, such as age, gender or education years, have been described to have an effect on neuroimaging measures (Pell *et al.*, 2008;). However, the subjects included in this study were carefully matched by all these variables and age and sex were used as covariates in the statistical analysis. Therefore, a small effect of these factors is expected. However, the only exception is the existence of an imbalanced sex ratio in the American dataset. The use of sex as a covariate is expected to minimize the effect of sex on our results.

However, the greatest problem is the small sample size. Although there is much debate on adequate sample sizes to evaluate the potential role of structural brain abnormalities as endophenotypes, a review of the relevant literature suggests that sample sizes of approximately 50–100 patients are sufficient to report consistent findings (Van Haren *et al.*, 2008). Thus, one of our goals is to increase this sample for future research projects.

2. Genes and polymorphisms related to emotionality: Serotonergic system genes, *NOS1* and *STMN1*.

2.1. *Serotonergic system genes: SLC6A4* gene

The hypothesis that schizophrenia and psychoses are related to dysfunctions in serotonergic neurotransmission was formulated by Wooley and Shaw in 1957. Since then, the serotonergic hypothesis of schizophrenia has been developed and many studies have been done to understand how the 5-HT system can be involved in the pathogenesis of schizophrenia.

For the present study, 12 polymorphisms in the serotonin transporter gene (*SLC6A4*) were selected, genotyped and analyzed in the Spanish case-control dataset. These polymorphisms were supposed to capture all the variability from this gene, according to the information available at the HapMap Project database (http://www.hapmap.org/). Several significant associations were found. The significance and relevance of these findings will be commented above.

Case-control single-SNP association analyses revealed that **two SNPs (rs140700 and rs2020936) were associated with psychosis and hallucinatory psychosis in the comparisons of allelic as well as genotypic frequencies** (tables R22, R23, R25 and R26). Moreover, other significant results were found when other comparisons were made. These findings also included SNP rs2020936 in almost all comparisons, as well as rs140700 and other polymorphisms. However, almost all these findings did not survive Bonferroni sequential correction. Curiously, **the promoter functional polymorphism 5-HTTLPR was not found to be associated with psychosis or AH in any comparison.** This finding is indeed in coherence with the previous literature, since the implication of this functional polymorphism in the vulnerability to schizophrenia is still unclear (for meta-analyses, see Fan and Sklar, 2005; and Levinson, 2005)

Although an important number of nominally significant associations have been found, many of these findings should be taken into account, since **the number of individuals in some subsamples is particularly limited**. This can be applied to patients without AH (N = 77) and patients with chronic AH (N = 81). In such cases, the statistical power to detect a true association is extremely low and therefore the results from these comparisons are subject to the effects of genotypic and allelic distributions clearly away from the real distribution of those polymorphisms in the population they are supposed to represent. Moreover, another issue is that **only a few significant values resisted Bonferroni sequential correction**. An increase in the number of comparisons probably leads to an increase in the type I error, which is directly related to the number of false positives, because it is highly likely that at least one null hypothesis will be rejected incorrectly.

Thus, according to these ideas, the most interesting finding from the case-control comparisons would be the **association of SNP rs2020936 with psychosis**. A different distribution of genotypic frequencies of SNP rs2020936 among the whole sample of patients and controls (P = 0.049 corrected) was detected. In this case, C-carriers were less abundant in the affected

group than in the control group (OR = 0.57). This finding was confirmed in the remaining comparisons, with a lower significance. However, the relationship among this SNP, located in intron 1A, and schizophrenia, had not been previously studied, although Kim *et al.* (2002) found a preferential transmission of the T allele in an autistic sample, which supports our results, since we found that the T allele was more abundant in the psychotic group compared to the control group. However, McCauley and colleagues (2004) did not replicate this initial finding (although they found a preferential transmission of allele G from rs140700 from parents to their autistic children, also in accordance with our findings). Nevertheless, our results regarding rs2020936 should be also considered cautiously, because this SNP was found to be slightly out of HWE in controls. Although this significant deviation from HWE did not survive the correction for multiple testing, further studies on bigger or additional samples would be necessary to confirm or discard this initial finding. There is still uncertainty about the functionality of this SNP, since our analysis with the online utility PupaSuite (Conde *et al.*, 2006) showed that this polymorphism did not seem to affect any regulatory sequence. However, we cannot discard the possibility that this SNP, or another variation in high LD with it, may alter the serotonin transporter expression.

Regarding haplotype analysis, **several 2-, 3- and 4-marker protective haplotypes were found**. These haplotypes were variable combinations of the same alleles from SNPs rs3813034, rs140700, rs2020942, rs2020939 and rs2020936 and they were around 50% more frequent in the control group. As expected, all these haplotypes included allele A from rs140700, and one haplotype also included allele C from rs2020936, in agreement with single-SNP analyses. However, after a 1000-permutation run, all haplotypes became not significant. Although a trend can still be observed, the results are not strong enough to completely ensure that these are really protective haplotypes. Up to now only a few studies have tackled the impact of haplotype variation at *SLC6A4* locus and schizophrenia, and most of them have been focused on the functional polymorphisms 5-HTTLPR and STin2 (Stöber *et al.*, 1998; Dubertret *et al.*, 2005), although some of them have studied other additional SNPs (Zaboli *et al.*, 2008). Interestingly, in the present study we have found some novel protective haplotypes which had not been previously described.

With regard to the **analysis of quantitative variables and other disease traits**, several associations were detected. Many SNPs were found to be associated with at least one item of KGV scale, being the nature of the items (positive, negative, related to language abilities) extremely variable. Remarkably, the functional promoter polymorphism 5-HTTLPR was found to be associated with the total KGV score, as well as with the Anxiety and Delusions scores. Unfortunately, all results remained not significant after correction for multiple testing. However, a trend toward association can be observed among 5-HTTLPR and the Delusions score. The results obtained for both **BPRS and PANSS** are also very similar: a high number of associations affecting different polymorphisms and items emerged. However, the number of individuals who underwent an evaluation through PANSS and BPRS scales was low (around 100-120) and some genotypic groups contained only a few individuals. As a result, many significant results appear unreliable. By contrast, some findings are especially interesting, namely the **associations of 5-HTTLPR with three parameters from the PANSS positive subscale**: PANSS positive score ($p = 0.0079$, not significant after correction but still showing a trend), P4 – Excitement ($p = 0.015$, corrected value) and P7 – Hostility ($p = 0.0044$, corrected

value). Particularly, **low expression alleles (S_A, S_G and L_G) were associated with higher scores in all cases**. It should be noted that Excitement and Hostility are two items from **the Excited Component from PANSS**, according to Kay and Sevy (1990).

Finally, we also found some nominal associations between three *SLC6A4* polymorphisms (rs2020939, rs12945042 and 5-HTTLPR) and several items from the **PSYRATS subscale for AH**. Among these positive associations, the most interesting findings were the **association of the 5-HTTLPR polymorphism with the three emotional items from this scale: Intensity of Distress** ($p = 0.0061$), **Amount of Distress** ($p = 0.011$), and **Disruption to Life** ($p = 0.012$). In all cases, those patients with a low expression genotype scored higher than the other patients and this fact is in agreement with the significant associations found with PANSS, BPRS and KGV scales. Moreover, although the three tests became not significant after the correction, the association with the Intensity of Distress item still showed a clear trend ($p = 0.0671$). Interestingly, similar results were also obtained in our preliminary analysis with a smaller sample of patients and controls (Sanjuán *et al.*, 2006b). In this study, the short allele of 5-HTTLPR appeared to be more frequent in the psychotic group and it was also found to be associated with the emotional response to AH measured through PSYRATS scale. Although in the present study we have not been able to replicate the association of the short allele with risk for schizophrenia, the association of this allele with the emotional response to AH still remains significant, giving more strength to our finding. These preliminary findings encouraged us to study deeply the impact of *SLC6A4* variation in the emotional aspects of AH.

The literature about the molecular genetics of AH in psychotic patients is brief. In this study, we tackled the analysis of the relationship among the serotonin transporter gene and auditory hallucinations, but especially with its emotional dimension. Until now, there was **only one previous report** (Malhotra *et al.*, 1998) that had analyzed the association between *SLC6A4* and AH in psychosis. In this case, **the long allele of the 5-HTTLPR polymorphism was found to be associated with the intensity of hallucinations** in schizophrenic patients. However, we did not find an association of 5-HTTLPR with any of the items of the PSYRATS scale directly related to the physical dimension of AH (such as Location, Duration or Intensity of AH, among others). In our opinion, these results are not surprising, if we consider that there are different mechanisms (and genes) controlling different aspects of AH. Furthermore, this previous report from Malhotra should be considered carefully. First of all, the small sample size, along with the lack of ethnical homogeneity, could have led to a false positive association. Moreover, the differences in the clinical assessment of AH could also explain the differences between our study and Malhotra's finding.

In summary, our results point out a relationship between higher emotionality in psychotic patients and the presence of low-expression 5-HTTLPR alleles. Ansorge *et al.* (2004) proposed that those genetic polymorphisms that reduce SERT expression may exert their effects during the early development of the CNS by altering the maturation of circuits that modulate the emotional response to novelty and stress. Our present findings are also in agreement with many prior association reports. There is a large body of evidence about the implication of the short, low-expressing allele of 5-HTTLPR in the emotional response to stressful situations. The short allele of the 5-HTTLPR has been described before as a risk allele for the development of

affective disorders (Anguelova *et al.*, 2003; Lasky-Su *et al.*, 2005; Cervilla *et al.*, 2006; Dick *et al.*, 2007), whereas the long allele seems to act as a protective factor.

The significant results involving 5-HTTLPR and emotional response to AH encouraged us to study further the effect of this functional polymorphism. The use of neuroimaging techniques to study those brain regions involved in emotionality represents an important research strategy to understand emotional dysfunction in schizophrenia. For this reason, functional and structural magnetic resonance studies were performed. With regard to fMRI, we found **greater right amygdala activation to emotional words in the *S/S* affected group**, compared to the remaining genotypes. Moreover, we have also demonstrated that both schizophrenic patients with auditory hallucinations and healthy controls carrying at least one *S* allele of the 5-HTTLPR polymorphism have a reduced gray matter density. However, involved areas are different (ACC in patients; hippocampus and parahippocampus in controls).

To the best of our knowledge, no previous neuroimaging study has reported data about the effect of serotonin transporter genetic variation in patients with schizophrenia and auditory hallucinations. Thus, our study would be **the first attempt to discover the underlying genetic mechanisms of auditory hallucinations in psychosis**.

The amygdala is a key structure in the generation of both normal and pathological emotional behaviours. It is densely innervated by serotonergic neurons and 5-HT receptors are particularly abundant. Previous studies have related different activation patterns on amygdala and other related brain regions depending on 5-HTTLPR genotype: it has been shown that the *s* allele of 5-HTTLPR produces an increased amygdala response to fearful stimuli in healthy individuals (Hariri *et al.*, 2002; Hariri *et al.*, 2005; Canli *et al.*, 2005b; Canli *et al.*, 2006; Heinz *et al.*, 2005; Heinz *et al.*, 2007; Rao *et al.*, 2007). This overactivation could be indicative of oversensitivity to threat-related signals in *S* allele carriers (Aleman *et al.*, 2008). Furthermore, higher amygdala activation associated with the *s* allele has been also found in several pathological conditions, such as social phobia (Furmark *et al.*, 2004) and panic disorders (Domschke *et al.*, 2006). All these findings are in agreement with the fMRI results presented in this study. However, all these previous reports have used emotional paradigms based on visual stimuli. By contrast, our fMRI paradigm is of auditory nature, in an attempt to mimic the auditory hallucinations suffered by schizophrenic patients. In the light of our findings, it can be suggested that auditory hallucinations are stressful events, and the final response to them can be modulated by the patient's genetic background. In addition to the neuroimaging approaches, there are also clear evidences relating vulnerability to environmental stress with variations in the *SLC6A4* gene (Caspi *et al.*, 2003; Eley *et al.,* 2004; Kaufman *et al.,* 2004; Grabe *et al.,* 2005; Kendler *et al.,* 2005; Cervilla *et al.,* 2007). Studies with a primate (*Macacca mulata* or Rhesus monkey) model also support the existence of a gene x environment interaction: an interaction between rearing conditions and genotype for the rhesus monkey promoter variation (rh5-HTTLPR) has a significant effect on 5-HIAA concentrations in cerebrospinal fluid (Bennett *et al.*, 2002), as well as in behavioral parameters from nursery-reared monkeys when compared to mother-reared monkeys (Champoux *et al.*, 2002).

Our results in the structural imaging-VBM analysis also support an effect of the serotonin transporter promoter polymorphism in gray matter volumes from limbic areas. The reduction of the GM density in the ventral and dorsal anterior cingulate cortex (ACC) of patients from the

S group can be considered a relevant finding since this structure is abnormally reduced in patients with schizophrenia and plays a key role in the neurobiological bases of this disease (Baiano *et al.*, 2007). Moreover, ACC is involved in the processing of emotional stimuli and mood regulation and these processes are known to be abnormal in patients suffering from schizophrenia or auditory hallucinations. Therefore, we can hypothesize that patients suffering from schizophrenia and AH present a disturbed emotional processing modulated by 5-HTTLPR and expressed by an ACC GM reduction. Supporting this hypothesis, our group has recently reported a coincidence cluster in the right anterior cingulate gyri showing at the same time a reduction in GM density and an increased hemodynamic response to an emotional auditory paradigm in patients with schizophrenia (Martí-Bonmatí *et al.*, 2007). Thus, the genetically-driven effect observed in ACC volume could be directly related to the differences we have observed in right amygdala activation. Pezawas *et al.* (2005) studied the effect of 5-HTTLPR variation in the functional connectivity of ACC and amygdala in healthy subjects. Interestingly, they found a diminished functional coupling between these two regions in *S* allele carriers. As a result, it has been proposed that the decrease in the functional connectivity of amygdala and ACC leads to a dysregulation of amygdala response to emotional (negative) stimuli, which may result in a reduced ability to integrate arousal in cognition and behaviour (Pezawas *et al.*, 2005; Hariri and Holmes, 2006). Moreover, Pezawas and colleagues (2005) also found a reduced GM volume in *S*-allele carriers in ACC and amygdala. Unfortunately, no differences in amygdala GM volume depending on 5-HTTLPR have been detected in our study. Several reasons, such as a sex or sample size effect, could explain these differences. Furthermore, it would be particularly interesting to study the effect of 5-HTTLPR variation on the activation of other limbic areas different from the amygdala, such as the anterior cingulate cortex.

However, the results from our sMRI analysis in control subjects have been different to the schizophrenic group, since hippocampal and parahippocampal volumetric reductions associated to the *s* allele have been found. This finding is in clear contrast to other prior studies, such as the study from Pezawas *et al.* (2005), as well as another report from Canli *et al.* (2005b), who also found an effect of *s* allele on the pregenual region of ACC. Once again, sex effects could also help to explain our discordant results. Both studies also included females while our sample was only comprised of male subjects. Medication effects could also be a confounding factor. Moreover, since our sample was relatively small, a type II error cannot either be ruled out.

The potential role of stress may be a possible explanation for our findings in healthy subjects. The biological effects of stress are mediated by the hypothalamic-pituitary-adrenal (HPA) axis, which regulates the release of cortisol. Accordingly, higher cortisol levels in *s* allele carriers might be contributing to hippocampal GM deficits. Interestingly, the serotonergic system is implicated in the augmentation of hippocampal glucocorticoid receptors during certain developmental periods. Moreover, it has been recently shown that an interaction between the *s* allele and high waking cortisol causes lower hippocampal volumes (O'Hara *et al.*, 2007). These authors suggested that a) *s* allele carrier status could increase vulnerability to negative effects of higher cortisol levels on hippocampal volume, and b) neuronal development in *s* allele carriers might increase vulnerability to dysregulation of HPA function with a subsequent negative effect on hippocampal volume. Finally, severe stress early in life has been associated

with smaller hippocampal volumes (Stein *et al.*, 1997; Vythilingam *et al.*, 2002) and with persistent changes in the hypothalamic-pituitary-adrenal axis (Heim *et al.*, 2001).

Somewhat surprisingly, we did demonstrate a GM deficit in hippocampus in our control group but not in patients. This may indicate the existence of other additional risk factors which are contributing to the determination of volume in ACC and medial temporal structures. In particular, other genes may play a role in determining the volume of these brain structures. Some examples are neurotrophin 3 (*NTF3*), brain-derived neurotrophic factor (*BDNF*) and disrupted in schizophrenia 1 (*DISC1*) (Szeszko *et al.*, 2005; Agartz *et al.*, 2006; Callicott *et al.*, 2005), among others. Moreover, it should be also considered that the environmental conditions influencing a schizophrenic phenotype can greatly differ from those in a healthy subject.

It would be interesting to perform the fMRI study in healthy subjects, since the particular phenomenology of schizophrenia could be masking the real effects of serotonin transporter genetic variation on the activity of limbic areas.

In summary, we have found two SNPs (rs140700 and rs2020936), as well as several protective haplotypes, associated with psychosis and psychosis with auditory hallucinations. The significance is especially high for rs2020936, although its slight deviation from HWE generates reasonable doubts about the validity of this finding. Moreover, the functional promoter polymorphism, 5-HTTLPR appears to be associated with the emotional dimension of auditory hallucinations, with the low expression alleles (L_G, S_A and S_G) associated with a higher emotional response. This initial finding obtained in the linear regression analysis of PSYRATS scale was subsequently confirmed through the use of a fMRI auditory-emotional paradigm as well as a structural neuroimaging approach; in those cases, we found a higher amygdala response to emotional voices in s/s patients, while an effect of 5-HTTLPR s allele on brain regional volumes could also be detected (ACC gray matter reductions in schizophrenia, and both hippocampal and parahippocampal gyri gray matter deficits in healthy controls). In conclusion, our findings suggest the existence of an effect of the serotonin transporter genetic variation on those neural circuits implicated in the regulation of emotionality in both healthy and schizophrenic conditions. It could be also interesting to study in the future the effect of the SNP rs2020936 on brain imaging phenotypes.

2.2. Other serotonergic system genes: the exploratory analysis of *HTR2A* and *TPH2* genes shows an effect of genetic variation on emotionality

Only **two polymorphisms from *HTR2A* gene**, **rs6311** (also known as -1438 A/G) and **rs6313** (known as T102C) were selected and genotyped in the Spanish dataset. Both SNPs are located in the 5' region of the gene (promoter and exon 1, respectively) and they are two of the most studied polymorphisms in the 5-HT_{2A} receptor gene. Our results show that, in agreement with the prior reports, these two SNPs are in almost perfect LD. With regard to ***TPH2* gene,** only one SNP, **rs4570625** (also known as -703G/T), was selected and genotyped in the Spanish sample. However, none of the three SNPs can be considered as tag SNPs, as they do not capture all the variability in those genes. The reason to choose only some representative SNPs instead of a set of tag SNPs is that this was an exploratory analysis whose objective was to evaluate the

involvement of other loci different from the serotonin transporter gene in the vulnerability to schizophrenia and auditory hallucinations.

Unfortunately, **no significant associations were found in the case-control association analysis for none of the two *HTR2A* SNPs** and only some trends can be observed in the haplotype analysis. By contrast, the most suggestive findings are the **associations between both *HTR2A* SNPs and different quantitative variables related to emotionality**: BPRS Anxiety item, KGV Anxiety and Depression items, PANSS Anxiety and Preoccupation items and Intensity of Distress to AH (from PSYRATS subscale for AH). In all cases, G/G (rs6311) and C/C (rs6313) individuals got higher scores. The association is particularly strong for both Anxiety items from PANSS and BPRS. Remarkably, an extraordinary coherence among the different scales can be observed, thus giving more strength to our finding. Our study of the *HTR2A* gene is in agreement with the previous literature. Despite the important role of the serotonin receptor 5-HT_{2A} in the pharmacological treatment of schizophrenia (Worrel *et al.*, 2000) and the existence of several genetic functional variants, there is still uncertainty about the involvement of *HTR2A* variation in the susceptibility to schizophrenia (for meta-analyses, see Williams *et al.*, 1997; Lohmueller *et al.*, 2003; Abdolmaleky *et al.*, 2004; Li *et al.*, 2006). However, there are many studies which had also related this gene to mood disorders, such as depression (Choi *et al.*, 2004), some personality traits of bipolar disorder (Ni *et al.,* 2006), seasonal affective disorder (Levitan *et al.*, 2002), and panic disorder (Unschuld *et al.*, 2007). Another study from Golimbet *et al.* (2002) is particularly interesting, since they also found that, in complete agreement with our present results, the rs6313 polymorphism was associated with some personality traits, including Anxiety and Neuroticism, in psychotic patients. However, a meta-analysis from Anguelova *et al.* (2003) has not detected the existence of association between *HTR2A* polymorphisms and affective disorders. Other complementary approaches will be necessary to better understand how this serotonin receptor is influencing emotional responses. As an example, it should be mentioned the study from Weisstaub and colleagues (Weisstaub *et al.*, 2006), who found that the global disruption of 5-HT_{2A} receptor signalling in mice affects anxiety-like behaviours. Functional neuroimaging studies could also be an interesting approach. It would be useful to study in the near future the effect of *HTR2A* variation in the response to emotional voices through our emotional-auditory fMRI paradigm (Sanjuan *et al.*, 2007).

Regarding the functional ***TPH2* polymorphism rs4570625**, the results have been mainly negative. Only a **slight association between this SNP and several items from the PSYRATS subscale for AH** has been found. These items are: Total Score, Duration, Location, Intensity, Degree of Negative Content and Amount of Distress. However, several criticisms can be made to these results. First, the associations are significant only when the subset of schizophrenic patients is considered, and become only a trend if the whole hallucinatory sample is included in the linear regression analysis. This fact could be indicating that this polymorphism has an effect on the pathogenesis of specific schizophrenia symptoms rather than in psychosis as a general phenomenon; however, *P* values (between 0.02 and 0.043) are not significant enough to ensure this hypothesis. Another explanation could be that the sample size is too small and the differences among all patients and schizophrenic patients are the result of a slight random variation in the genotypic distributions which result in spurious associations. The MAF of rs4570625 is relatively small (0.15 in patients), therefore the number of G/T and T/T

schizophrenic patients with AH is low (43 G/T individuals and 5 T/T individuals). Further studies would be necessary to understand the role of this SNP on the vulnerability to AH in schizophrenic patients.

Anyway, the fact that **G/T genotype is associated with higher distress to AH** (compared with G/G individuals) is a really interesting finding, since it is in coherence with the literature. Gutknecht *et al.* (2007) found that the T allele from rs4570625 was associated with the personality trait Harm Avoidance, which is directly related to anxiety. Moreover, several fMRI studies (Brown *et al.*, 2005; Canli *et al.*, 2005a) support the involvement of this SNP in the responsiveness of amygdala to emotional stimuli. However, our finding is of small relevance and it would need to be confirmed by means of functional neuroimaging approaches or with the use of larger samples of patients.

In conclusion, our results do not strongly support an effect of the genes of the serotonergic pathway on the vulnerability to psychosis and/or auditory hallucinations. By contrast, several small evidences suggest that these three genes, and more especially the serotonin transporter and the 5-HT_{2A} receptor genes, may be partially responsible for the emotional dysregulation suffered by psychotic patients. This emotional dysregulation would also affect how these patients respond to auditory hallucinations.

2.3. Likely effect of *NOS1* genetic variation on several disease traits.

One polymorphism, the VNTR located in exon 1f from the neural nitric oxide synthase gene (*NOS1* Ex1f VNTR), was genotyped in the Spanish case-control dataset. Our main goal was to perform an exploratory analysis with a representative functional polymorphism, to evaluate the likely implication of this gene in the vulnerability to schizophrenia or auditory hallucinations in our sample.

Unfortunately, **no significant results were found in the case-control comparisons** of allelic and genotypic frequencies. With regard the linear regression analysis with quantitative variables, **the polymorphism was found to be associated with several items from PANSS scale**: three General items from the Depressive component (Kay and Sevy, 1990): Preoccupation, Guilt Feelings and Anxiety; a negative item (Lack of Spontaneity and Flow of Conversation); and two positive items: Suspiciousness/Persecution and Hostility. The most significant results were the **associations with Guilt Feelings and Suspiciousness/Persecution**. Moreover, the PANSS General score was also associated to this VNTR. In all cases, **S/S homozygote patients scored higher**, showing a great coherence among items. However, no association was found with the remaining scales.

Although we have not detected any association between the *NOS1* Ex1f VNTR and risk for psychosis or schizophrenia, it is likely that the *NOS1* functional promoter polymorphism has an effect on symptom severity, due to its association with some emotional-depressive, positive and negative measures, with the short alleles associated with higher scores on these measures. These findings are in agreement with those reported by Reif *et al.* (2006). Particularly, they found that the low expression S/S genotype was associated with higher values for the PANSS positive subscale and the Hamilton depression rating scale. However, despite the coherence with previous findings, our results should be considered cautiously, due

to the small sample size (N = 102) and the fact that no association was found with other related items from other psychopathological scales (BPRS, KGV). Therefore, the possibility of a false positive cannot be discarded. Moreover, it would be interesting to analyze additional polymorphisms located on this gene to fully discard the involvement of *NOS1* genetic variation on the liability to schizophrenia. Many previous reports have found an association between other *NOS1* polymorphisms (different from *NOS1* Ex1f-VNTR) and schizophrenia. Thus, it is plausible that certain polymorphisms could have a role on the vulnerability to schizophrenia itself, while other polymorphisms such as the Ex1f-VNTR may modulate the severity of the disease.

In summary, our findings support the implication of the *NOS1* gene in the severity of certain symptoms of schizophrenic patients, such as the positive symptoms, although the significance is weak.

2.4. Involvement of the *STMN1* gene in the severity of negative symptoms.

The *stathmin1* gene is a very small gene located in chromosome 1. According to the linkage disequilibrium pattern around this area, two tag SNPs (rs182455 and rs12037513) were selected and genotyped in the Spanish sample. As far as we know, this is the first attempt to study the effect of this gene on the etiology of psychoses. The results from the association analysis will be commented below.

The most interesting result from the case-control association analysis is the **nominal association between the SNP rs182455 (located in the putative 5' regulatory region of *STMN1*) and risk for schizophrenia**. According to the information presented in table R48, T/T homozygotes were less frequent (OR = 0.67, P = 0.027 uncorrected) in the DSM-IV schizophrenic group compared with the control group. This association was also found when only hallucinatory schizophrenic patients were considered. However, this association became only a trend when all psychotic patients were considered in the comparison. Moreover, neither of the significant *P* values resisted the correction for multiple testing. Although the SNP rs182455 may have an effect on the vulnerability to schizophrenia, other case-control studies with additional independent and larger samples would be necessary to confirm this statement.

The **study of the effect of *STMN1* variation on several quantitative disease traits** may be a complementary approach to case-control analyses, since it could provide us with additional information about how this gene can modulate particular aspects of the symptomatology of schizophrenia. According to this idea, we found that the **SNP rs182455 was nominally associated with several negative items**, namely Flattened Affect and Incoherence of Speech from the KGV scale, as well as Active Social Avoidance, Lack of Spontaneity and Flow of conversation from PANSS, and, finally, the PANSS Negative score. In all cases, the C/C genotype correlated with higher scores. Moreover, this SNP was also **highly associated with the positive item Suspiciousness/Persecution**, which also survived the Bonferroni Sequential correction (P = 0.0026 corrected). However, in this case, those individuals who were C/C homozygotes scored lower than T/C homozygotes. With regard to AH, the polymorphism **rs182455 was found to be nominally associated with two items from PSYRATS subscale for AH: Beliefs about the Origin and Intensity of Distress**. Regarding **SNP rs12037513**, the most reliable findings are the **nominal associations with two items from PANSS scale**: Somatic Concern, a

depression-related item; and Blunted Affect, from the Negative subscale. Interestingly, the association with this last item also survived the correction from multiple testing (P = 0.03).

These findings suggest that *STMN1* variation could be implicated in the severity of Negative symptoms, since an important number of correlations among *STMN1* SNPs and negative items has emerged. However, whether these or other polymorphisms in *STMN1* gene have a functional implication is an unknown issue.

Negative symptoms in schizophrenic patients can be considered as a type of emotional alteration: a decrease in the general response of the individual to different stimuli. According to our findings, this gene may also have a **role in the emotional dysfunction suffered by schizophrenic patients, due to the existence of several associations with emotional items**. The role of stathmin in the emotional dimension of schizophrenia would also involve the emotional response to auditory hallucinations. It has been reported that *STMN1* is highly expressed in the lateral nucleus of the amygdala, as well as in those thalamic and cortical structures which process information related to conditioned and unconditioned stimuli (Shumyatsky *et al.*, 2005). The disruption of *STMN1* expression in a murine model subsequently leads to the alteration of those behavioral patterns associated with fear and threat assessment of the environment (Shumyatsky *et al.*, 2005; Martel *et al.*, 2008). As a result, social behaviors, such as maternal care and social interactions, are also severely affected. Interestingly, schizophrenia is a clear example of a human disease where normal emotional responses and social interactions are clearly compromised. Therefore, alterations on *STMN1* gene expression or regulation could be involved, in conjunction with other genetic and environmental factors, in those pathological manifestations. Our findings would be also in coherence with this hypothesis. However, our results are particularly limited, given the small sample size and the use of a purely clinical approach. Other complementary approaches of functional nature would be of great utility to study the contribution of Stathmin variation to schizophrenia.

In conclusion, according to our results, *STMN1* variation appears to impact on the liability to schizophrenia, as well as the severity of negative symptoms, although these findings need to be checked through other approaches.

2.5. Effect of the gene x gene epistasis on the emotional processing.

The significant associations between certain polymorphisms of the serotonergic pathway and different items related to emotionality led us to investigate the effect of gene-gene epistasis on these emotional factors. Epistasis refers to the non-additive interaction between genes at different loci affecting a phenotype. For our purpose, we selected three polymorphisms (5-HTTLPR from the serotonin transporter gene, rs6313 from the *HTR2A* gene and rs4570625 from the *TPH2* gene) as well as different response variables. As we hypothesized, some gene-gene interactions arose: there was an **effect of *SLC6A4*x*HTR2A* interaction on several items** (All cases versus controls; PSYRATS for AH - Intensity of distress; PSYRATS for AH - Amount of distress; PSYRATS for AH – Disruption; BPRS – Anxiety; and KGV – Anxiety), while **the *TPH2*x*HTR2A* interaction affected the following emotional parameters**: PSYRATS for delusions – Disruption; PSYRATS for delusions – Amount of distress; and PANSS G6 – Depression. These are really interesting findings. Among them, the most significant findings are the effect of

*SLC6A4*x*HTR2A* interaction on the Intensity and Amount of Distress to auditory hallucinations. As it can be seen in figure R10, the effect of the serotonin transporter promoter polymorphism is clearly influenced by the genotype for rs6313. By contrast, no significant results were obtained for *STMN1* or *NOS1* genes

According to our evidences, it is likely that multiple genetic polymorphisms are acting in the same or in opposite directions to model certain emotion-related behavioral phenotype. Moreover, several evidences supporting our findings have emerged in the last years. For example, interactions between 5-HTTLPR, *COMT* and *DRD4* have been found to influence the personality trait of Novelty Seeking (Benjamin *et al.*, 2000; Strobel *et al.*, 2003), and interactions between 5-HTTLPR, *COMT* and *MAOA* appear to influence the regulation of HPA-axis response to acute psychological and endocrine challenges (Jabbi *et al.*, 2007). The effect of gene-gene epistasis on the activity of emotional brain circuits has also been tackled. An effect of a 5-HTTLPR x *TPH2* interaction on the response to emotional visual stimuli has been reported (Herrmann *et al.*, 2007). In an fMRI study with healthy subjects, Smolka and colleagues (2007) also showed an additive effect of *COMT* Val/Met and 5-HTTLPR polymorphisms on the differential activation of limbic regions in response to emotional negative stimuli. Finally, studies in mice also support interacting effects of the serotonin transporter genes with the dopamine transporters (*slc6a3*) the MAOA gene (*maoa*), the 5-HT1B receptor gene (*htr1b*) and the BDNF gene (*bdnf*) (Murphy *et al.*, 2008).

In summary, our results, in coherence with many previous studies, suggest the existence of a genetically driven interaction between different elements of the serotonergic system which have a key role in the processing of emotional stimuli.

3. *ASPM* gene as an example of a positive selection gene with an effect on schizophrenia phenotype.

Whereas much of the sequence of the *ASPM* gene is substantially conserved among primates, specific segments are subject to strong positive selection. This fact can be related to the progressive increasing of the relative brain size in the course of primate evolution. As an important part of this PhD project, we have studied the impact of *ASPM* variation on risk for schizophrenia. Moreover, we also assessed how *ASPM* genotypes affected some endophenotypes related to cognition and brain morphology, which are known to be abnormal in patients with schizophrenia as well as their healthy siblings (Cannon *et al.*, 1998; Kremen *et al.*, 1994; Egan *et al*., 2001b). We tested 8 SNPs in the Spanish sample and 6 SNPs in the American CBDB dataset (five of them were common to both samples: rs3762271, rs12138336, rs41310927, rs10922163, rs6677082) and we found association for schizophrenia for some SNPs and haplotypes in both datasets. Moreover, some polymorphisms were found to be associated with several disease traits available in the Spanish patients. Finally, the SNPs located in exon 18 were found to affect performance on some cognitive phenotypes, which also correlated to variations in regional gray matter volumes and cortical function.

Although we have not obtained a highly significant association with the risk for schizophrenia, the results are enormously coherent:

- When the **Spanish sample** was analyzed, **two SNPs (rs6700180 and rs10922163) were found to be associated with psychosis and psychosis with AH**. The same associations were subsequently found when only schizophrenic patients were compared with controls. In all cases, C/C genotype from rs6700180 and A/A genotype from rs10922163 were significantly more common in the control group. It should be remarked that these associations resisted the correction for multiple testing. By contrast, no significant results were found for the remaining SNPs (see Rivero *et al.*, 2006, for a preliminary analysis with 5 SNPs). Moreover, several common protective haplotypes (which included allele A from rs10922163, in coherence with the single-SNP analysis) were also found. Moreover, several SNPs were found to be nominally associated with the **age at onset of the disease**, as well as with several clinical scores from **BPRS, PANSS and PSYRATS**; however, only some associations remained significant after correction: the associations of both rs4915337 and rs972778 with the PANSS general score and the associations of rs10922163 and rs6700180 with the Negative item from PANSS "Blunted affect". Despite the uncertainty surrounding these correlations, the high number of associations with negative rather than positive items remarks the likely implication of *ASPM* variation on the pathophysiology of the negative dimension of schizophrenia. This issue should be studied deeply through the use of other approaches. In conclusion, according to all these findings, the SNPs rs10922163 and rs6700180 (or other variations in high LD with them) appear to be interesting elements which may modulate the risk for schizophrenia. It is nevertheless true that both polymorphisms were found to be out of HWE in the control sample as well as in the affected group. Although this deviation was slight (not significant after correction), this detail should be taken into consideration. The deviation from HWE could be indicating two situations: on the one hand, it could be an indicator of a genotyping error; on the other hand, it could be the consequence of HWE-modifying factors escaping from our control. Among these factors, we cannot discard the possibility that these polymorphisms are under the effects of a positive selection process. There are evidences that suggest that *ASPM* is still undergoing positive selection (Mekel-Bobrov *et al.*, 2005). However, even if the deviation of HWE of those polymorphisms is related to positive selection processes, it is difficult to establish, with our tools, a relationship among this deviation and variation in the liability to schizophrenia.

- With regard to the association analysis of the **American sample**, allele T from **SNP rs6677082 was associated with schizophrenia** in both case-control and family-based studies. The great coherence between both association approaches would allow us to support with more strength that rs6677082 (or another variation in high linkage disequilibrium with it) is really associated with the disease. We also found that allele G carriers for **SNP rs10922163 were more frequent in the patients' sample compared to the control subset**. Although the odds ratio is not very high (1.44), there was also a trend toward overtransmission of the same allele from parents to affected offspring in the family-based study. Moreover, we observed two common haplotypes which also increased the risk for schizophrenia in both approaches. Interestingly, one of these haplotypes included allele T for SNP rs6677082 and the allele G for SNP rs10922163. Finally, **SNP rs12116571 was found to be associated with schizophrenia**, with an increase of genotype G/G in the patients' sample. Although this polymorphism gave the higher statistical evidence (P =

0.001), **this finding could be a false positive, because this SNP was out of HWE in the patients' sample**. Hence, the reliability of this result is uncertain. However, our understanding is that the possibility of a genotyping error is low. Therefore, we cannot discard the possibility that the lack of HWE in patients is a manifestation of a real association of this polymorphism with the disease.

There are **some encouraging coincidences between both samples**. The polymorphism rs10922163 was found to be associated to schizophrenia in both the Spanish and the American samples. Moreover, in both cases, allele G carriers were more frequent in the affected group. Interestingly, the SNP rs10922163 was also found to be significantly associated with schizophrenia when a case-control analysis with a pooled sample that included American and Spanish cases and controls was performed. However, there are striking differences among both case-control datasets. Particularly, the significant haplotypes differ among the two studies and other polymorphisms different from rs10922163 have been found to be associated with schizophrenia in one of the samples but not in the other. As it was previously commented in the Introduction, **the lack of strict replication is a common issue across genetic studies on schizophrenia**, in contrast with other complex diseases such as type-2 diabetes mellitus (Florez *et al.*, 2003) or age-related macular degeneration (Edwards *et al.*, 2005; Haines *et al.*, 2005; Hageman *et al.*, 2005). This fact suggests several explanations for our results, including the possibility of false positives. However, another reasonable explanation is that the variability in the genetic structure among different populations can be influencing which are the risk genotypes and haplotypes in each population. Although our results show that there is a great similarity in the genetic structure of *ASPM* among both the Spanish and the American sample, the existence of differences in other interacting genes (related to both the *ASPM* gene and/or the risk for schizophrenia) can be the underlying cause of the heterogeneous results between both datasets.

Our findings would support the relationship between the *ASPM* gene and vulnerability to schizophrenia, although the statistical evidence is weak. This fact is not surprising if we consider that some of the SNPs have a low minor allele frequency and therefore, in these cases, the power to detect association is reduced. Furthermore, it is worth mentioning that **the association with clinical phenotypes has a limited power to identify causative genes for complex diseases such as schizophrenia**, since each gene is expected to have a low effect on the observed clinical phenotype (Risch, 1990; Riley and Mc Guffin, 2000; Weiss and Terwilliger, 2000). In our opinion, this is especially clear for *ASPM*, because, in contrast with other genes possibly related to schizophrenia, this gene is expressed in the developing brain, but not in the adult one (Kouprina *et al.*, 2005). As a result, *ASPM* exerts its effects only during neurodevelopment and especially during cortex formation. Therefore, we would expect that its effect would be more influenced by other genetic or environmental factors. We think that it is necessary **to test the effects of variation in *ASPM* on other cognitive and morphological phenotypes**. Many previous reports (Hariri *et al.*, 2002; Egan *et al.*, 2004; Fusar-Poli *et al.*, 2007) have shown that genes associated with neuropsychiatric disorders show greater effects at these intermediate phenotypes. This approach is a good complement to the family-based and case-control association analyses because such underlying traits have more power to detect the influence of candidate genes on cognition. Thus, we first explored how *ASPM*

genotypes affected cognition and we found that three **polymorphisms** (rs3762271, rs12138336 and rs41310927) **influenced the scores of several cognitive measures studied on the American sample**: WCST and N-Back in controls and Digit Span in patients. Interestingly, those three polymorphisms are located in exon 18, which codes for the most important part of the calmodulin-binding domain, consisting of long and short isoleucine-glutamine (IQ) repeats (Kouprina *et al.*, 2005). The IQ domain has experienced the highest evolution rate in the *ASPM* protein (Kouprina *et al.*, 2004) and it has been speculated that this domain could have an important role in microtubule polymerization during cell division in the central nervous system (Kouprina *et al.*, 2005). Even more, Mekel-Bobrov *et al.* (2005) showed that rs3762271 and rs41310927 (which are in almost complete linkage disequilibrium with each other) are subject to recent positive selection in humans. Therefore, those nonsynonymous changes tested in this study may have functional implications which could explain our results in the association analysis with the cognitive dimensions. Since the discovery by Mekel-Bobrov and colleagues, several authors have explored how these SNPs affect general intelligence measures (Mekel-Bobrov *et al.*, 2007; Rushton *et al.*, 2007). Unfortunately, no clear association between these nonsynonymous changes and intelligence has been found in all these studies. Our understanding is that, despite its high heritability (Bouchard and McGue, 2003; Toga and Thompson, 2005), intelligence is not a good target phenotype, as it is composed of different cognitive dimensions, each of them likely modulated by genetic and environmental factors, which can be partly common to the different dimensions but probably acting in a different manner or degree. Following this idea, we analysed the impact of *ASPM* on seven cognitive factors which represented different domains of cognition. As a result of this approach, we have found association with three cognitive dimensions (WCST, N Back and Digit Span) which are directly related to working memory. In conclusion, this approach appears to be more powerful to detect the contribution of genetic variation on cognitive phenotypes. Our results also suggest that subtle variations in the *ASPM* sequence may have an impact on the formation of those CNS structures with an important role in cognition.

Surprisingly, these 3 SNPs associated with changes in cognitive performance were not associated with the risk for schizophrenia (with the exception of SNP rs41310927, which was part of a risk haplotype). It is difficult to explain this finding, but it can be related to differences in the characteristics of each sample which could affect the statistical power to detect association. Moreover, we cannot discard the possibility that each polymorphism or haplotype can have a different effect. We then decided to study the effect of three *ASPM* SNPs (rs3762271, rs41310927 and rs10922163) on VBM and fMRI phenotypes. As we expected, **we did not detect an effect of *ASPM* variation in global intracranial volume.** This finding is coherent with previous reports (Woods *et al.*, 2006; Rushton *et al.*, 2007; Timpson *et al.*, 2007; Dobson-Stone *et al.*, 2007; Wang *et al.*, 2008). As general intelligence, global brain size is too complex because an important number of genetic and environmental factors, also interacting with each other, are influencing and modelling the final phenotype. However, in our study, we have been able to find an **association of three *ASPM* SNPs with changes in regional volumes**. Basal ganglia, prefrontal cortex, namely Brodmann area (BA) 9, and subgenual cingulate cortex (BA25) are affected in controls, while, in patients, there are differences in PFC (BA9) and cerebellum volumes. Moreover, in the BOLD fMRI assay during the N Back task, allele G from SNP rs10922163 was also associated with a **poorer hemodynamic response of the anterior**

cingulate cortex (BA32) in control subjects. All these findings show a great coherence. Prefrontal cortex, especially dorsolateral prefrontal cortex (DLPFC), which includes Brodmann areas 9 and 46, plays a key role in a wide variety of functions. It is also connected with different cortical and subcortical circuits, which include the striatum (caudate and putamen) (Alexander *et al.*, 1986). This prefrontal-midbrain functional network is crucial for cognitive function and specially working memory (Alexander *et al.*, 1986; Dunnett *et al.*, 2005). Moreover, it has been also shown that the anterior cingulate cortex, also well connected with the DLPFC, plays an outstanding role in a several brain functions, including motor control as well as cognitive and working memory related tasks, such as N Back task (Paus, 2001; Callicott *et al.*, 1999). Furthermore, the DLPFC dysfunction has been widely accepted as a key feature of schizophrenia (Weinberger *et al.*, 2001). Our results support the correlation between *ASPM* variation and morphological and functional alterations in those areas directly related to working memory, both in patients and healthy subjects. Moreover, an effect of *ASPM* genotype on cerebellar volume in schizophrenic patients has been also detected. This finding is in complete agreement with the preliminary report presented by Andreasen's group in the International Congress on Schizophrenia Research held in Colorado in 2007. In this report, an effect of SNP rs3762271 on cerebellar volumes (AA > AC > CC) was found. Previous hypotheses have pointed out the possible role of cerebellum in the "cognitive dysmetria" observed in schizophrenic patients (Andreasen *et al.*, 1998). This model proposed by Andreasen more than ten years ago is supported by evidences from different fields. First, the cerebellum is well connected with neurocortical regions which include the prefrontal cortex (Middleton and Strick, 1994; Andreasen *et al.*, 1998). Second, neuropathological abnormalities in the cerebellum of schizophrenic patients have also been reported (Picard *et al.*, 2008). Finally, functional brain imaging studies have suggested the implication of cortico-cerebellar networks in several domains of cognition (Picard *et al.*, 2008). Thus, our data could be indicating a **role of *ASPM* in the development of cortical, subcortical and cerebellar structures** which would account for the differences in cognitive performance and brain structure detected in this study. Moreover, the differences observed between patients and controls suggest the existence of other genetic and/or environmental susceptibility factors which are modulating the manifestation of the phenotype observed in patients.

In summary, although there are authors who have suggested that the *ASPM* gene could have been positively selected for its function outside the brain (Woods *et al.*, 2006; Dobson-Stone *et al.*, 2007), we provide evidences of the role of the *ASPM* gene in cognition and brain size as well as in the risk for schizophrenia. All our data suggest that the *ASPM* gene, through its key role during neurodevelopment, has an effect in the size and function of certain brain areas which are critical in the pathogenesis of schizophrenia. These findings would also correlate with our association analysis results. Our results support our initial working hypothesis; therefore, *ASPM* variation could be related to the increase of the cognitive abilities throughout human evolution. As a result, *ASPM* could have also had a role in the origin of schizophrenia and the cognitive dysfunction associated to this disorder.

4. Effects of other novel candidate genes on schizophrenia and other related phenotypes

4.1. *PDE4D* gene

PDE4D can be considered to be an interesting candidate gene in psychiatric genetics research for several reasons. First, *PDE4D* has been found to be a partner of *DISC1*, which is one of the most promising schizophrenia candidate genes (Murdoch *et al.*, 2007). Moreover, all PDE4 isoforms can be inhibited by rolipram, a drug with important effects as an antidepressant. Finally, a CNV study performed by Professor Lesch at the Department of Psychiatry and Psychotherapy from the University of Würzburg showed that the smallest, brain-expressed *PDE4D* isoform (PDE4D6, according to Wang *et al.*, 2003b) was associated to another psychiatric condition, namely the attention-deficit hyperactivity disorder or ADHD (personal communication). All these premises encouraged us to study the effect of several SNPs, located in the PDE4D6 isoform, on the risk for schizophrenia. Two samples were used for this purpose, the Spanish sample and the German sample, both of Caucasian origin. Nine SNPs were analyzed in the Spanish case-control sample, while ten polymorphic variants (the nine SNPs genotyped in the Spanish sample plus an additional SNP, rs10056492) were studied in the German sample.

The **results from our case-control association analyses are mainly negative.** Regarding the Spanish sample, only the SNP rs12656462 was found to be nominally associated with DSM-IV schizophrenia, although the significant value became a trend when all psychotic patients were considered. Moreover, a 2-marker protective haplotype as well as a 2-marker risk haplotype, both differing only in the SNP rs12656462, were also found. However, these findings should not be considered, since the SNP rs12656462 has a low MAF (0.047 in controls) and the number of A/A individuals is extremely low. Thus, the statistical power to detect association with this SNP is very limited and our positive findings could be in fact false positives. With regard to the case-control association analyses performed on the German case-control dataset, only three SNPs (rs10056492, rs4700316 and rs7714708) were found to be associated with bipolar disorder. Although the significance was relatively high and resisted the sequential Bonferroni correction, these findings need to be considered cautiously, however. The reason is that only 88 German patients with bipolar disorder were available and it is very risky to draw any conclusion from that finding. However, it would be interesting to continue the study of the impact of *PDE4D* variation on the vulnerability to bipolar disorder. In this respect, it is worth mentioning the study from Sun and colleagues (Sun *et al.*, 2004), who found that lithium, a drug commonly used for the treatment of bipolar disorder, decreased the expression of *PDE4D* in lymphoblasts from bipolar patients. Moreover, bipolar disorder symptoms also include several mood alterations which may be partly mediated by alterations in cAMP signaling.

Since case-control association studies did not provide us with any clear conclusion, we tackled the study of how *PDE4D* variation could influence several clinical measures and disease traits. As a result, **two *PDE4D* polymorphisms** (rs17291089 and rs7713345) **were nominally associated with the age at onset**, although the association did not survive the correction for

multiple testing. Particularly, patients who were A/A homozygotes for the SNP rs17291089 were more frequent in the Early-Onset group (the first psychotic outbreak took place before 21 years old). Regarding rs7713345, G-carriers were significantly more frequent in the Early-Onset group. These findings could be interpreted as evidences of different risk factors for psychosis depending on the age of onset. However, the number of subjects with early-onset psychosis included in the comparison is small (around 90) and the minor allele frequency of the SNP rs17291089 is also low. Due to these limitations, it is difficult to draw any clear conclusion from these data.

Moreover, **several *PDE4D* SNPs were also associated with a wide variety of clinical measures**. The polymorphism rs17719378 is undoubtedly of special interest, since it has been related to a high number of measures, including the following:

- All items from the PSYRATS subscale for delusions.
- The “Anxiety” item from BPRS.
- “Incoherence of Speech” from the KGV scale.
- Several scores from the PANSS: Total score, “Poor Attention” (G11), Positive score, “Delusions” (P1), “Hallucinatory behavior” (P3) and “Grandiosity” (P5).

In all cases, A/A genotype was associated with lower scores than A/G genotype. Moreover, it is remarkable the fact that the SNP rs17719378 was especially associated with positive symptoms, rather than other type of clinical manifestations, suggesting the possibility that this polymorphism (or another variation in high LD with this SNP) may have a role, together with other interacting elements, in the **severity of positive symptoms, particularly delusions**. Furthermore, the association with the Anxiety item from BPRS is also particularly encouraging, due to the previous evidences that have related this gene with depression and neuroticism (Henkel-Tigges and Davis, 1990; Shifman *et al.*, 2008; Heck *et al.*, 2008). Nevertheless, it should be reminded that the **number of patients who underwent a clinical evaluation through PANSS is low**. Another problem was the important number of fails during the genotyping process of *PDE4D* SNPs. As a result, sample size was reduced considerably.

In summary, although our exploratory analysis suggests that genetic variation affecting the PDE4D6 isoform may have a role in bipolar disorder and psychotic symptoms, additional studies are necessary. Particularly, an association study covering the complete *PDE4D* genomic sequence could also provide us with useful information. Finally, the use of larger samples would be also useful. In this regard, although the association analysis performed with a pooled sample of schizophrenic patients and controls from Spain and Germany did not give any significant result, this finding could be related to differences in the genetic structure of both populations (Seldin *et al.*, 2006; Heath *et al.*, 2008). In fact, the distribution of three SNPs was found to be significantly different between the German and the Spanish sample. The use of larger, homogeneous and non-stratified samples would be helpful to achieve a better understanding of the role of *PDE4D* variation in the vulnerability to a wide spectrum of psychiatric disorders.

4.2. *PLEKHB1* and *RAB6A* genes

As a result of a search for copy number variations (CNVs) in a sample of ADHD patients and controls of German origin, the research group from professor Lesch (University of Würzburg, Germany) detected differences among patients and controls in a region located at chromosome 11 which included two small adjacent genes, *PLEKHB1* and *RAB6A* (unpublished results). For this reason, these two genes were also considered to be interesting candidate genes for the present study. As a result, an exploratory analysis was made to investigate the existence of a relationship among variation in those genes and vulnerability to psychosis. Several polymorphisms were selected to be studied in both the Spanish and the German case-control datasets. However, some polymorphisms were monomorphic in those samples, and an additional SNP was completely out of HWE, probably due to genotyping problems. These variations were therefore discarded for the statistical analysis. Finally, 6 SNPs were studied in the Spanish cohort, while 11 SNPs were studied in the German sample.

With regard to the **Spanish dataset, one polymorphism was found to be associated with psychosis**: rs663303 (located in the putative 5' regulatory region from *PLEKHB1*) and rs3741147 (located in intron 5 from *PLEKHB1* gene). This SNP presented significant differences in the distribution of both genotypic and allelic frequencies. The same results were obtained when the subset of hallucinatory patients was considered, suggesting that the effect of this SNP would be related to the global psychotic process rather than the hallucinatory process in particular. A risk haplotype (T-G-T) as well as a protective haplotype (C-G-G), both including SNPs rs663303, rs940828 and rs3741147, respectively, were also found. These haplotypes were in coherence with the single SNP analysis. Unfortunately, despite all the suggestive findings regarding the *PLEKHB1* gene, neither of the significant results resisted the correction for multiple testing, excepting the protective haplotype (P = 0.047 corrected). Another issue which should be taken into account is that the low MAF of rs663303 (0.09 in controls) drastically reduces the statistical power of the association analysis.

Regarding the **German sample, some nominally significant findings** (not significant after correction) arose, although the significant SNPs were different from the Spanish dataset. Particularly, one polymorphism from the *PLEKHB1* gene (rs940828, located in intron 5) was found to be associated with schizophrenia and bipolar disorder. Interestingly, this SNP is also located in the same area as SNP rs663303, the polymorphism which was significantly associated with psychosis in the Spanish sample, although the LD among both markers is moderate (D' = 1; r^2 = 0.436). Moreover, two other SNPs from the *PLEKHB1* gene (rs4944850 and rs6592527) were found to be associated with bipolar disorder in the German sample. Furthermore, one SNP located in the first intron from the *RAB6A* gene (rs7127066) was nominally associated with schizophrenia and bipolar disorder. However, no risk or protective haplotypes were found, and all results became not significant when Bonferroni sequential correction was applied.

It is important to remark that several nominal associations were found in both the Spanish and the German cohort, particularly involving the *PLEKHB1* gene. However, we can only refer to these findings as a partial replication, since the significant polymorphisms differ between both samples. The only coincident polymorphism is rs940828, which appeared to be associated with

schizophrenia in both samples (as a single SNP in the German cohort, as a part of two haplotypes in the Spanish dataset). However, this SNP is particularly problematic, since the same allele was found to be part of a risk as well as a protective haplotype, so it can be hypothesized that the contribution of this SNP to both haplotypes is nonexistent. Finally, the use of a pooled sample with German and Spanish subjects did not give significant results.

Although the **lack of strict replication** is not an isolated finding (it has been documented by many researchers in the genetic psychiatrics field, see the Introduction, section 2.3.3., as a reminder), it is difficult to explain why two relatively close populations present such differences. One of the most plausible explanations would be the existence of a false positive, probably related to the insufficient sample size or the extremely low minor allele frequencies. Moreover, the existence of false negatives cannot be discarded. Furthermore, it should be also considered that the differences in the genetic structure of Spanish and German populations can involve other genetic interacting factors, which, in turn, may modulate the effect of the polymorphism of interest on the vulnerability to a complex disease. Although European populations share an important part of their genetic structure, they also present clear differences which should be taken into account when a case-control association analysis is designed (Marchini *et al.*, 2004; Seldin *et al.*, 2006; Heath *et al.*, 2008). In agreement with this idea, we found enormous differences in the distribution of certain *PLEKHB1* and *RAB6A* SNPs (rs3742247, rs12274970 and rs11235876) between the German and the Spanish datasets. These differences, together with other differences in the genetic patterns among European populations, can explain our divergences between samples.

In conclusion, several *PLEKHB1* SNPs and one *RAB6A* SNP were nominally associated with psychosis and bipolar disorder. The main evidences are focused on the *PLEKHB1* gene, although the significance is relatively low due to the small sample size and the low MAFs of certain polymorphisms, such as rs663303. Moreover, it is risky to establish a causal relationship between this gene and mental disorders, since a likely role of this gene on behavior and mental disorders has not been explored yet. As far as we know, this is the first study which has tried to find a link between this gene and psychoses. Moreover, little is known about the function of this membrane protein with a pleckstrin homology domain. It has been suggested that this protein, expressed in different cells with a sensory function (Xu *et al.*, 1999; Xu *et al.*, 2004), may have a role in modifying signal transduction processes in photoreceptors (Xu *et al.*, 2004). According to this probable role, we can hypothesize that alterations on this gene may have an active role in the development of hallucinations. Furthermore, high *PLEKHB1* expression has been documented in fetal and adult brain. Interestingly, according to the microarray expression data available at Genomics Institute of the Novartis Research Foundation website (http://symatlas.gnf.org/), this expression is particularly high in those regions which appear to have a role in the physiopathology of schizophrenia, such as temporal lobe, prefrontal cortex, cingulate cortex, thalamus and hypothalamus, among others. These evidences, together with our results from the case-control analysis, encouraged us to go further into the study of this region located at chromosome 11.

The impact of *PLEKHB1-RAB6A* genetic variation on several disease traits was also assessed through linear regression analysis. As a result, an important number of nominal associations, involving the six polymorphisms genotyped in the Spanish patients, were found. However,

many significant findings should not be considered as relevant, especially when the significant model is the recessive one and the number of individuals who were homozygotes for the minor allele is extremely low. Despite this fact, some significant findings resisted the correction for multiple testing. These include:

- The association of the SNP rs12274970 (located in the intergenic region between *PLEKHB1* and *RAB6A*) and the PANSS item G13 - disturbing of volition (P = 0.032 corrected).
- The association of the SNP rs11235976 (located in the third intron of the *RAB6A* gene) and the following PANSS items: G13 - disturbing of volition (P = 0.032 corrected); N4 – apathetic-social withdrawal (P = 0.046 corrected); P7 – Hostility (P = 0.047 corrected)

The disease traits which appear to be modulated by these SNPs are of varied nature. Thus, it is difficult to elaborate a hypothesis about the real meaning of these associations. Anyway, these SNPs are different from those associated with the liability to schizophrenia. Moreover, neither of these polymorphisms seems to be functional, according to the results obtained from PupaSuite (Conde *et al.*, 2006) (data not shown). It could be also possible that these SNPs are in high LD with other functional variants. Another disadvantage is that the number of patients with genotypic information who were assessed for PANSS is very low. Thus, another possibility which should be also considered is that these are false positive findings.

Unfortunately, although we hypothesized that the *PLEKHB1* gene may be implicated in the vulnerability to auditory hallucinations, no clear association between any *PLEKHB1* SNP and hallucinations has been found.

In conclusion, the evidences in favor of a relationship between *PLEKHB1* and *RAB6A* genes and psychosis are weak, mainly due to the small sample sizes and low minor allele frequencies. However, some nominal associations involving *PLEKHB1* have been found in both cohorts. A more profound study would be necessary to confirm these findings.

5. Towards a general model of vulnerability

When studying a common and complex disease, such as schizophrenia, which is characterized by a non-Mendelian inheritance and a high prevalence in human populations, one of the most feasible etiopathogenic explanations is the Common Disease - Common Variant (CDCV) hypothesis. The CDCV hypothesis predicts that the spectrum of allelic variants which confer susceptibility to a complex disease is made up of common variants, each of them with a moderate effect on the disease liability and shared by different subpopulations. This hypothesis is indeed the framework of the present study and for this reason we have focused on the study of different genetic polymorphisms located in several candidate genes involved in different neural processes which could be related to the pathophysiology of schizophrenia and other psychoses. We expected that the contribution of these candidate genes would be modest, and our results are in coherence with our initial assumption.

Despite all the considerations we have taken into account, some interesting and coherent findings have been found in the present study. As a result, our overall findings support a possible integrative model of vulnerability (figure D2). First, there is a **general vulnerability to psychoses** which would be influenced by an important number of several genetic and non-genetic factors. We have evidences that the serotonin transporter gene (*SLC6A4*) and *ASPM* gene may have an important role on this general vulnerability through single-gene as well as epistatic effects, since several highly significant SNPs have been detected (rs140700 and rs2020936 from the *SLC6A4* gene; the replicated SNP rs10922163 from the *ASPM* gene). Thus, according to this scenario, the serotonin transporter and the ASPM protein may have an effect on those neural systems which are known to be involved in the appearance of the schizophrenic manifestations. Anyway, other genes, such as *STMN1* or *PLEKHB1,* could also have an impact on the liability to schizophrenia, however, there are less evidences on this respect, since the polymorphisms located on these genes did not reach the significance threshold for multiple testing. The existence of such different types of genes may also help to explain the great variability in the clinical manifestation of schizophrenia among patients.

Second, genetic variation on several polymorphic variants appeared to impact **symptom severity** (see also figure D2). A clear example is the effect of *STMN1* variation on the severity of negative symptoms. Moreover, slight evidences suggest a possible impact of *NOS1* and *PDE4D* genes on positive symptoms. These findings are not surprising, particularly if we consider the existence of different types of risk alleles and genes with a different impact on the disease (Fanous and Kendler, 2005). Therefore, according to the model presented by Fanous and Kendler, some genes would increase the liability to suffer schizophrenia without altering or impacting its associated clinical features (susceptibility genes), while other genes would affect these clinical features without modifying the susceptibility to illness by themselves (modifier genes).

Furthermore, it is worth mentioning that this study also contributes to the vulnerability to psychosis with a new approach. This approach, which we named **"deconstructing the symptom"**, allows the study of the neurobiological systems implicated in the most characteristic symptoms of psychosis, such as **auditory hallucinations** (one of the most specific symptoms suffered by schizophrenic patients), **cognitive impairment** (one of the most disabling features of schizophrenia) and the **emotional disturbances** and affective deterioration associated to this pathology. We believe it is necessary to study psychoses from a categorical but also from a dimensional point of view. Current classification of mental disorders (based on the classical Kraepelinian assumptions) appears to be partially valid in neurobiological and genetic research. The unstability of psychoses over time, as well as the existence of intermediate conditions (such as schizoaffective disorder), can be justified by a continuum of psychotic manifestations, ranging from nonaffective psychoses to psychotic mood disorders (Peralta and Cuesta, 2008). Therefore, the deconstruction of symptoms into subsymptoms is a necessary step to unravel the neurobiological processes underlying schizophrenia (Cuesta and Peralta, 2008). Following this methodology, some interesting findings have been found in the present study.

With regard to **cognition**, an effect of *ASPM* variation on several cognitive abilities and neuroanatomical variables has been found (figure D2), suggesting an important role of this

gene during the development of those brain regions implicated in the pathogenesis of schizophrenia. Thus, it can be hypothesized that abnormal expression of the *ASPM* gene during neurodevelopment would affect the normal formation of different brain areas clearly associated with the cognitive deficits associated to schizophrenia, such as the prefrontal cortex, the anterior cingulate cortex or the basal ganglia. Furthermore, apart from pathological conditions, we can argue that *ASPM* has a necessary role in normal cognition and can be considered as a strong candidate for understanding human brain evolution. In conclusion, the active role of *ASPM* in the evolution of human brain function and cognition may have triggered, somehow as a collateral effect, the emergence of cognitive deficits and mental disturbances associated to schizophrenia.

Moreover, of particular interest is the relationship between variation in the serotonergic system and **emotional alterations**. Our overall results suggest that the alteration of the expression of several key elements of this system, namely the serotonin transporter, the serotonin receptor 2A and, to less extent, the tryptophan hydroxylase 2, leads to an altered emotional state in those patients. Moreover, stathmin protein may also be involved in the pathogenesis of affective disturbances observed in schizophrenic patients, although in this case the evidences are weaker. All these findings support the emotional nature of schizophrenia and other psychotic disorders. The understanding of schizophrenia as an emotional disorder should be integrated together with cognitive hypotheses for a better understanding of the psychotic phenotype.

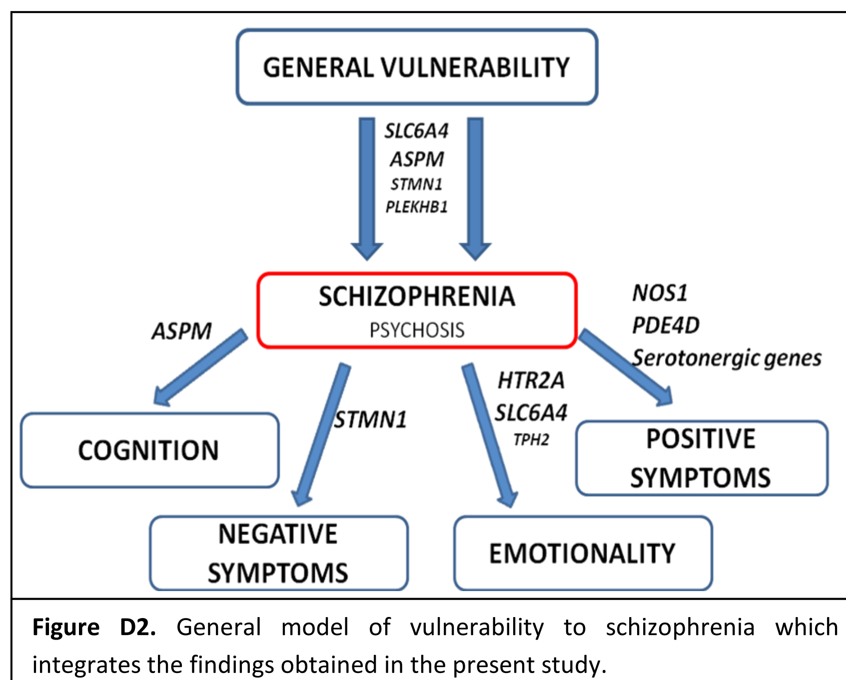


Figure D2. General model of vulnerability to schizophrenia which integrates the findings obtained in the present study.

Finally, this study supports the involvement of several polymorphic variants in the **pathogenesis of auditory hallucinations**. Particularly, this study is part of a more ambitious project whose main objective is to disentangle, from a dimensional point of view, the molecular mechanisms responsible for the vulnerability to auditory hallucinations. As a

consequence of our results in this field, we have developed a **possible model that integrates all our findings** (figure D3), allowing us to provide an explanation for the aetiopathogenesis of AH. According to this model, two different pathways can be considered to be involved in the genetic vulnerability to AH in psychosis. The first of them is a **vulnerability to language disorders**, which could increase the probability of hearing voices. This vulnerability could be due to changes in the *FOXP2* gene, among others (Sanjuán *et al.*, 2006a). *FOXP2* is the first gene that has been related to a language disorder and is associated with functional and structural abnormalities in the temporal lobe (Liegeois *et al.*, 2003; Vargha-Khadem *et al.*, 2005). Second, there is another important dimension in the neurobiology of AH, which is one of the central aspects of our present work: the **vulnerability to abnormal emotional responses**. We have reasons to support the role of emotional processing in the pathophysiology of AH. In a previous work, our group designed an auditory emotional functional magnetic resonance imaging (fMRI) paradigm to elicit the emotional states experienced by schizophrenic patients when suffering from AH (Sanjuán *et al.*, 2007). In that work, an enhanced activation of limbic and frontal brain areas was detected in a group of persistent hallucinatory patients in response to emotional words. Thus, we hypothesized that a) this activation is directly related to the emotional response that occurs when suffering from auditory hallucinations and b) this emotional response is partially controlled by genetic variation in certain candidate genes. Interestingly, the present study correlates our previous and presents results in the genetics of the emotional response to AH: analogously to the effect of genetic variation on the general emotional state of schizophrenic patients, we have also noticed an important effect of several serotonergic genes on the emotional response to auditory hallucinations (Sanjuán *et al.*, 2006b). Finally, environmental factors cannot be discarded from this model. Particularly, cultural aspects can influence the content and social adjustment of voices (Sanjuán *et al.*, 2005; Sanjuán, 2006; Aleman and Larøi, 2008).

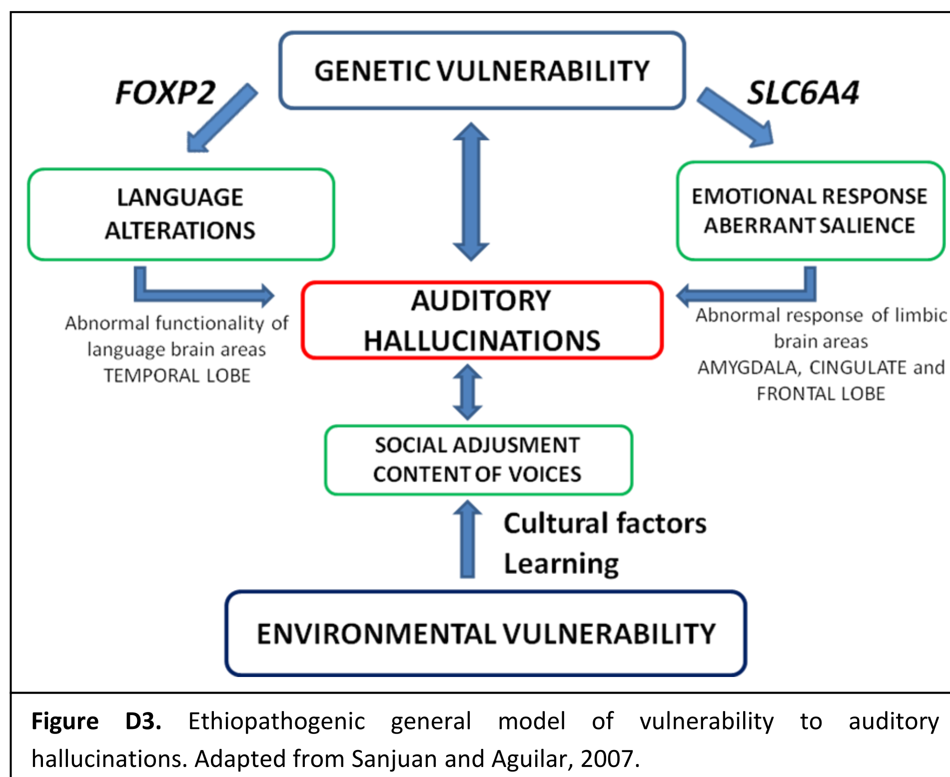


Figure D3. Ethiopathogenic general model of vulnerability to auditory hallucinations. Adapted from Sanjuan and Aguilar, 2007.

However, despite the present study as well as other prior studies may have unmasked some key elements in the pathogenesis of schizophrenia, many other elements remain unclear: there is still a lot of uncertainty and several unknown factors of genetic, epigenetic and environmental nature are waiting to be discovered and understood. However, in the light of recent findings, association studies (classical or genome-wide) appear to be a limited tool, at least with the current sample sizes and statistical methodologies. Moreover, it is also very plausible that severe mental disorders are due to a mutation-selection balance: old harmful and rare mutations that have yet to go extinct, which are transmitted from parents to their offspring (Keller and Miller, 2006). This hypothesis appears to be consistent with the data on mental disorder prevalence rates, fitness costs, the likely rarity of susceptibility alleles, and the increased risks of mental disorders with brain trauma, inbreeding, and paternal age. Moreover this scenario would help to understand schizophrenia from an evolutionary point of view. New approaches, such as the search for copy number variations, the use of animal models, epigenetic studies, whole expression analyses, neuroimaging studies, or gene x environment interaction approaches, are required to unravel the neurophysiological mechanisms which are responsible for one of the most severe mental diseases.

CONCLUSIONS

In summary, taken together our results support the following conclusions:

1. Case-control association analysis indicates the existence of association between SNP rs2020936 from the serotonin transporter gene and psychosis. Several protective haplotypes, which are more frequent in the control group than in the affected group, have also been found.

2. Results from the present study suggest a strong relationship between variation at 5-HTTLPR locus and abnormal emotional response in schizophrenic patients. Particularly, low expression alleles have been associated with a higher score in emotional items from PSYRATS subscale for auditory hallucinations. The S allele has been also associated with higher amygdala activation in response to emotional words as well as a lower gray matter density in limbic areas (anterior cingulate cortex in schizophrenic patients; hippocampus and parahippocampus in healthy subjects).

3. *HTR2A* gene appears to have an effect on emotionality in psychotic patients, assessed through several psychopathological scales: PANSS, BPRS and PSYRATS. *TPH2* gene has been also associated with some items from PSYRATS subscale for auditory hallucinations, although in this latter case evidences are weaker.

4. Interaction analyses point out the existence of epistatic effects involving three genes from the serotonergic system: *SLC6A4*, *HTR2A* and *TPH2*. These interactions appear to modulate different variables related to the emotional processing, especially the emotional response to auditory hallucinations.

5. An effect of *NOS1* ex1f-VNTR on the scores of several PANSS items (three items from the Depressive component and two positive items) has been detected.

6. *STMN1* gene is weakly associated with the liability for schizophrenia, as well as with several clinical items mainly related to the negative/affective symptomatology of schizophrenia.

7. Through both case-control and family-based association analyses in two different datasets, *ASPM* gene has been found to be associated with the risk for schizophrenia. Particularly, SNP rs10922163 has been associated with schizophrenia in both samples. *ASPM* gene has been also associated with several cognitive measures mainly related to working memory, as well as with the physiological response of the anterior cingulate cortex during a working memory task. Finally, *ASPM* variation has an effect on gray matter volumes from certain areas related to cognition and pathogenesis of schizophrenia, such as cerebellum, prefrontal cortex, subgenual anterior cingulate cortex and basal ganglia.

8. One SNP located at the *PDE4D* locus has been weakly associated with schizophrenia in the Spanish sample, while different SNPs have been associated with bipolar disorder in

the German case-control dataset. This gene has been also associated with the severity of different symptoms, mainly of positive nature.

9. Several SNPs located at the *PLEKHB1* gene have been found to be associated with the risk for schizophrenia and bipolar disorder.

10. Overall, our findings support a general model of vulnerability. According to this model, certain genes would have a major effect on the vulnerability to suffer from schizophrenia, while other genes would have an effect on symptom severity. Regarding auditory hallucinations, there is a genetic vulnerability to aberrant emotional response, which is modulated by variation in different genes, namely the serotonin transporter gene, among others.

RESUMEN EN CASTELLANO

Introducción

El término esquizofrenia fue acuñado por Eugen Bleuler en 1908 para referirse a un grupo de desórdenes caracterizados por graves síntomas de naturaleza cognitiva, emocional y comportamental (Bleuler, 1911). Los síntomas de la esquizofrenia afectan a diferentes dominios y pueden agruparse en tres categorías: a) síntomas positivos o psicóticos: manifestaciones clínicas que siguen un patrón episódico y que no están presentes en la población normal: alucinaciones, delirios y trastorno del pensamiento; b) síntomas negativos: se refieren a la ausencia o afectación de ciertos comportamientos como la interacción social, motivación o la habilidad para experimentar placer (Wong y Van Tol, 2003); c) deterioro cognitivo, que afecta a la atención, la función ejecutiva y la memoria. Esta variada sintomatología lleva a una gran heterogeneidad clínica, la cual es el origen de importantes problemas a la hora de abordad esta enfermedad desde un punto de vista biológico.

Debido a su naturaleza crónica, severidad, incidencia (entre el 1.6% y el 4.3% en todas las poblaciones según Jablenski *et al.,* 1992) e inicio temprano, la esquizofrenia, así como las psicosis en general, implican un alto coste humano y económico (UK National Health Service Executive, 1996). Esto ha generado también un gran interés por estudiar los mecanismos neurofisiopatológicos subyacentes a este trastorno. Hasta el momento, se conocen numerosos factores que pueden aumentar el riesgo de padecer esquizofrenia, (Jablensky, 2003). Estas variaciones apoyan la existencia de modelos de interacción vulnerabilidad x estrés.

Asimismo, existen hasta el momento un importante número de hallazgos neuropatológicos relacionados con la esquizofrenia. Lamentablemente, estos hallazgos no pueden ser usados como criterios diagnósticos, puesto que también pueden aparecer en sujetos sanos, y además no se dan en todos los pacientes. Entre los hallazgos más importantes destacan los siguientes:

- Anormalidades de tipo morfológico. Destacan la reducción global del tamaño cerebral (Harrison *et al.*, 2003), engrosamiento de los ventrículos tercero y lateral (Elkis *et al.*, 1995; Harrison *et al.*, 2003), ausencia de gliosis (Iritani, 2007) y, por último, reducciones en el neurópilo (Selemon y Goldman-Rakic, 1999). También se han hallado reducciones regionales de materia gris, que afectan principalmente los lóbulos temporal medial y superior (Honea *et al.*, 2005), el córtex prefrontal (PFC) y el tálamo (Tandon *et al.*, 2008).

- Los estudios sobre los mecanismos de acción de los medicamentos antipsicóticos llevaron también al desarrollo de diferentes hipótesis sobre el origen de la esquizofrenia. Una de las más importantes es la hipótesis dopaminérgica (Weinberger, 1987), que postula la existencia de un desequilibrio de los niveles de dopamina en diferentes áreas del cerebro, lo cual explicaría la existencia de los síntomas positivos y negativos. Una reformulación llevó a la hipótesis glutamatérgica, que considera que el causante de los desequilibrios en los niveles de dopamina es la existencia de un desajuste en los niveles de glutamato. Por último, también se han descrito alteraciones en el sistema serotoninérgico.

- Los estudios de neuroimagen funcional, mediante el empleo de diferentes paradigmas y tareas cognitivas, han supuesto una aproximación muy interesante. Mediante estas técnicas, se ha encontrado una hipoactivación a nivel del córtex prefrontal en pacientes

esquizofrénicos (Weinberger *et al.,* 1986; Glahn *et al.,* 2005). Además, estudios de espectroscopía de resonancia magnética nuclear (Wong y Van Tol, 2003) han detectado alteraciones en los niveles de algunos metabolitos como el N-Acetil aspartato (marcador de integridad glial y pérdida neuronal).

Todos los hallazgos comentados anteriormente sugieren que la esquizofrenia es el resultado de la compleja interacción entre la predisposición biológica de cada individuo (determinada por factores genéticos) y ciertos factores ambientales. Por este motivo, en las últimas décadas se han venido realizando importantes esfuerzos para comprender mejor los factores genéticos subyacentes y para entender cómo éstos interaccionan con el ambiente para producir el fenotipo esquizofrénico. Por ello, se han realizado diferentes aproximaciones al campo de la genética de la esquizofrenia. Los estudios de epidemiología genética (estudios familiares, de gemelos y de adopción) fueron la primera aproximación a la genética de los trastornos mentales. Mediante estos estudios, se ha podido constatar que la esquizofrenia es una enfermedad heredable (heredabilidad en torno al 80%) y que los parientes de pacientes esquizofrénicos, especialmente los de primer grado, tienen un riesgo mayor de padecer la enfermedad que la población general (Kendler y Diehl, 1993; Shih *et al.,* 2004; Burmeister *et al.,* 2008). Sin embargo, el patrón de herencia es complejo y no se ajusta al patrón mendeliano de las enfermedades monogénicas.

La aparición de los marcadores moleculares en las décadas de 1980 y 1990 permitió el desarrollo de los análisis de ligamiento, que permitían localizar de los loci causantes de enfermedades monogénicas mediante el empleo de grandes genealogías (Knowlton *et al.,* 1985; Guilford *et al.,* 1994). Desafortunadamente, los resultados de los análisis de ligamiento no han sido tan satisfactorios en el caso de enfermedades complejas, debido a la herencia poligénica de estas enfermedades. En el caso de la esquizofrenia, aunque se han encontrado diferentes picos de ligamiento a lo largo de todo el genoma, los resultados son muy controvertidos, debido a la existencia de resultados contradictorios o no replicados (Burmeister *et al.,* 2008). Además la realización de meta-análisis tampoco ha ayudado a clarificar la situación. Son por tanto necesarios otros enfoques para dilucidar cuáles son realmente los genes implicados en la susceptibilidad a padecer esquizofrenia.

Hace 13 años, Risch y Merinkangas demostraron que, para poder estudiar variantes genéticas con un efecto pequeño sobre el fenotipo (como es el caso de la esquizofrenia), existe una aproximación más potente que los estudios de ligamiento: los estudios de asociación (Risch y Merikangas, 1996). Estos estudios se basan en el uso de polimorfismos genéticos localizados en los genes de interés, seleccionados bien por su localización cercana a un pico de ligamiento (genes candidatos posicionales) o bien porque se sospecha que su función podría estar relacionada con la patogénesis de la enfermedad (genes candidatos biológicos) (Burmeister *et al.,* 2008). Una vez seleccionados estos polimorfismos, se evalúa si cierto alelo, genotipo o haplotipo aparece en mayor frecuencia de lo esperado en la población de afectados. En este caso, se considera que ese alelo, genotipo o haplotipo está asociado con la enfermedad. Los polimorfismos empleados en los estudios de asociación pueden ser de diferente naturaleza: variaciones en el número de repeticiones en tándem (VNTRs), inserciones-deleciones (indels) y, más comúnmente, polimorfismos de un único nucleótido (SNPs), estos últimos ampliamente

distribuidos por todo el genoma. En un primer momento, la mayoría de los estudios de asociación se centraron en el estudio de variantes funcionales, las cuales eran consideradas como probables variantes causales (o "polimorfismos candidatos"). Sin embargo, esta "aproximación directa" (Risch, 2000) presenta dificultades, puesto que no siempre se conocen cuáles son la variantes funcionales de un gen, y además la búsqueda de estas variantes es especialmente costosa. Por ello, existe otra alternativa ("aproximación indirecta"), que consiste en el análisis de polimorfismos genéticos sin tener en cuenta su funcionalidad. Esta técnica aprovecha la estructura de desequilibrio de ligamiento (LD) del genoma humano para seleccionar aquellos polimorfismos que permiten capturar toda la variabilidad genética existente en una determinada zona. Estos SNP reciben el nombre de tagSNPs (de Bakker *et al.,* 2005). Es también importante remarcar que esta técnica sería casi imposible sin la existencia del proyecto Hapmap (Internacional Hapmap Consortium, 2003).

Hay diferentes tipos de asociación, dependiendo del tipo de muestra con el que se trabaje. Uno de los estudios más comunes es el estudio de asociación caso-control, que compara dos grupos de individuos no relacionados (uno afectado y uno control) que presentan características similares (sexo, edad, etnia…) para evitar problemas de estratificación (Langefeld y Fingerlin, 2007). El análisis se basa en testar la hipótesis nula de ausencia de diferencias en las frecuencias alélicas, genotípicas o haplotípicas entre ambos grupos. Hay diferentes maneras de testar la hipótesis nula, como el test de Pearson, el test exacto de Fisher, el test Cochran-Armitage o el análisis de regresión logística (Balding, 2006). Asimismo, también es interesante calcular un parámetro como la *"Odds Ratio"* (OR), que permite evaluar el impacto de cada factor genético sobre el riesgo de padecer la enfermedad. Sin embargo, también es posible realizar estudios de asociación basados en familias. En este caso, los familiares sanos pueden ser usados como controles internos, por lo que se reduce el riesgo de estratificación de la muestra (Langefeld y Fingerlin, 2007). Uno de los tests basados en familias más usados es el test de desequilibrio de transmisión o TDT (Spielman *et al.,* 1993), que permite evaluar si alguno de los alelos se transmite con mayor frecuencia de la esperada a los hijos enfermos. Otro test bastante popular es el test de asociación basado en familias o FBAT (Laird *et al.,* 2000), cuya peculiaridad reside en el hecho de que puede funcionar con datos parentales ausentes. Esto es especialmente importante para enfermedades de inicio más bien tardío como la esquizofrenia.

Desde que comenzaron a realizarse los primeros estudios de asociación en esquizofrenia y hasta la actualidad, el número anual de trabajos y genes analizados ha crecido enormemente. Pero al mismo tiempo también ha ido creciendo la incertidumbre por la falta de replicación en algunos casos. Sin embargo, hay ciertos genes candidatos que presentan una mayor evidencia de asociación con esquizofrenia. Entre ellos, cabe destacar la Neurorregulina1 (*NRG1*), la disbindina (*DTNBP1*), los genes *DAOA* y *DAAO*, el gen "*Regulator of G protein signaling*" (*RGS4*), la catecol-O-metil transferasa (*COMT*) y el gen *Disrupted in Schizophrenia* 1 (*DISC1*) (Harrison and Weinberger, 2005; Norton *et al.,* 2006; Lang *et al.*, 2007; Burmeister *et al.,* 2008). Estos genes cumplen importantes funciones en el sistema nervioso central y están muy relacionados con las hipótesis dopaminérgica y glutamatérgica. Sin embargo, a pesar de los datos procedentes de los estudios de asociación que apoyan la implicación de estos y otros genes en la patogénesis de la esquizofrenia, existen todavía un importante número de

resultados inconsistentes, debido a la existencia de resultados negativos incluso para los genes anteriormente citados. Asimismo, además de los resultados negativos, hay también inconsistencias entre los trabajos positivos, puesto que dependiendo del estudio, el polimorfismo significativo también es diferente. Por ello, en un intento de dar respuesta a estos problemas, en los últimos años se han realizado estudios de asociación de genoma amplio (GWAS) que analizan polimorfismos localizados a lo largo de todo el genoma en muestras de gran tamaño. Sin embargo, esta nueva aproximación tampoco ha conseguido clarificar la situación, al menos en el campo de las enfermedades psiquiátricas (Burmeister *et al.,* 2008), puesto que los genes asociados con la enfermedades varian de unos estudios a otros.

Las inconsistencias en los estudios de asociación plantean una serie de interrogantes: ¿son los estudios de asociación una aproximación adecuada? ¿Esta heterogeneidad en los resultados se debe a diferencias entre poblaciones? ¿Es la esquizofrenia una enfermedad más heterogénea que otras enfermedades complejas como la diabetes? Hay diversas razones para explicar esta existencia de inconsistencias en los estudios de asociación. En primer lugar, la heterogeneidad en los resultados puede ser una señal de la heterogeneidad genética de la esquizofrenia, aunque tampoco se debe descartar la existencia de falsos positivos (error de tipo I) y falsos negativos (error de tipo II), como resultado del uso de muestras muy pequeñas o muestras estratificadas de diferente origen genético, lo que podría haber llevado a una distorsión de los resultados. Asimismo, tampoco se puede olvidar que la esquizofrenia es una enfermedad muy heterogénea también a nivel clínico, lo que todavía dificulta más su estudio. Por último, otros factores importantes a tener en cuenta serían la existencia de mecanismos epigenéticos o ambientales, que podrían estar actuando en solitario o a través de interacciones con los factores genéticos (Caspi y Moffitt, 2006).

Como se ha visto, la complejidad fenotípica y genética de la esquizofrenia, unido a la probable falta de validez de la actual clasificación clínica (Cuesta y Peralta, 2008), hace necesario el uso de otras aproximaciones complementarias a los estudios clásicos de asociación. Una de estas aproximaciones es centrarse en el síntoma. A este respecto, no puede olvidarse que, en neuropsiquiatría, el síntoma está pobremente estudiado. Además, la deconstrucción de la esquizofrenia en elementos más simples podría permitir disminuir la complejidad. Asimismo, es necesario buscar fenotipos más cercanos a los procesos biológicos, para facilitar el descubrimiento de aquellos genes implicados en la fisiopatología de la esquizofrenia. De esta necesidad de nuevos fenotipos alternativos surgió el concepto de endofenotipo hace más de 30 años (Gottesman y Shields, 1973). El endofenotipo se define como un rasgo interno que está presumiblemente más cercano al gen que la patología global y puede ser detectado a través de tests bioquímicos o microscópicos. Asimismo, otro concepto relacionado es el de “fenotipo alternativo”, que se refiere al uso de síntomas clínicos en vez de los criterios diagnósticos tradicionales.
Para el presente estudio se han seleccionado dos fenotipos alternativos: las alucinaciones auditivas y el deterioro cognitivo.

Las alucinaciones auditivas son el tipo más común de alucinación en los pacientes psicóticos y están presentes en un importante número de pacientes (entre un 50 y un 70%, según Slade y

Bentall, 1988; y Andreasen y Flaum, 1991). Aunque este fenómeno se da en un variado número de pacientes psiquiátricos y neurológicos, e incluso en sujetos sanos, las alucinaciones auditivas presentan unas características particulares en los pacientes psicóticos: suelen ser voces humanas que le hablan al paciente, con una intensa carga emocional e interpretadas por el sujeto como ajenas a él (alto grado de "*insight*"). Se caracterizan además por su naturaleza dimensional (Aleman y Larøi, 2008). El componente emocional es una de las dimensiones más interesantes, puesto que se sabe de su importancia en el propio fenómeno alucinatorio, así como en la respuesta del individuo a esas voces. Asimismo, se han hecho numerosos estudios de neuroimagen para dilucidar cuáles son las regiones cerebrales implicadas en este fenómeno. Los hallazgos más replicados son una reducción de la circunvolución temporal superior, así como una actividad anormal en aquellas áreas relacionadas con diferentes aspectos del lenguaje (Silbersweig *et al.,* 1995; Woodruff, 2004; Aleman y Larøi, 2008). Asimismo, nuestro grupo ha desarrollado un paradigma de fMRI de tipo auditivo-emocional, mediante el cual se ha detectado la activación anormal de ciertas áreas como respuesta a palabras de contenido emocional (Sanjuán *et al.,* 2007). A la luz de todos estos hallazgos, consideramos que las alucinaciones auditivas pueden ser un fenotipo alternativo útil en el campo de la genética psiquiátrica. La integridad de los circuitos responsables de la aparición de las alucinaciones auditivas podría pues estar modulada y controlada por factores genéticos. Sin embargo, hasta el momento, los estudios de genética molecular de las alucinaciones auditivas son más bien escasos y se han centrado principalmente en pacientes neurológicos (Aleman y Larøi, 2008). Es por tanto necesario un conocimiento más profundo de las bases genéticas y biológicas de las alucinaciones auditivas, con una especial atención a su componente emocional.

El deterioro cognitivo es también un síntoma de gran importancia en la esquizofrenia, por ser uno de los más severos y con un peor tratamiento, si se compara con los síntomas psicóticos. Además, es el causante de gran parte de la discapacidad psicosocial de estos pacientes (Breier *et al.,* 1991; Lewis, 2004). En un meta-análisis (Heinrichs y Zakzanis, 1998), se estimó que el 61%-78% de los pacientes esquizofrénicos presentaban problemas de tipo cognitivo. Además, se ha visto que la memoria de trabajo es uno de los elementos clave para entender el deterioro cognitivo en pacientes esquizofrénicos. La memoria de trabajo se define como un sistema de capacidad limitada que mantiene, almacena y procesa información relevante, a través de procesos complejos que implican al pensamiento y el lenguaje, entre otros (Baddeley, 2003). Muchos estudios han hallado un funcionamiento anormal de la memoria de trabajo en pacientes esquizofrénicos (Goldberg *et al.,* 1987; Goldman-Rakic, 1994; Silver *et al.,* 2003). Esto suscitó el interés de muchos investigadores por conocer las bases biológicas de la memoria de trabajo y otros aspectos cognitivos de vital importancia en la patogénesis de la esquizofrenia. Las habilidades cognitivas pueden ser consideradas un endofenotipo válido por varias razones. En primer lugar, son altamente heredables en humanos. Además, las técnicas de neuroimagen funcional permiten correlacionar el rendimiento en un test cognitivo con alteraciones en ciertos sistemas cerebrales. De hecho, hay claras evidencias de la implicación del cortex prefrontal dorsolateral en la memoria de trabajo (revisado en Tan *et al.,* 2007). Asimismo, la neuroimagen estructural permite detectar cambios en el tamaño de ciertas áreas cerebrales. Se sabe de la existencia de déficits volumétricos que están asociados con el riesgo genético de padecer esquizofrenia (Cannon *et al.,* 1998; Goldman *et al.,* 2008; Honea *et al.,*

2008). Como se puede ver, hay importantes motivos para estudiar la genética de los procesos cognitivos en pacientes esquizofrénicos. Uno de los genes más estudiados ha sido el de la *COMT*, lo que ha permitido constatar la implicación del polimorfismo funcional Val158Met en la memoria de trabajo (revisado en Meyer-Linderberg *et al.,* 2006; Roffman *et al.,* 2006; y Tan *et al.,* 2007).

En este estudio, diferentes cuestiones se han tenido en cuenta para seleccionar diferentes genes candidatos. Principalmente, la selección se ha basado en tres aspectos de la esquizofrenia fuertemente interrelacionados: las alucinaciones auditivas, la disfunción cognitiva y la respuesta emocional. De esta manera se han seleccionado 9 genes candidatos.

Genes del sistema serotoninérgico. La serotonina es un neurotransmisor de tipo monoamina con un importante papel en los sistemas nerviosos central y periférico. Como resultado de su amplia localización y la expresión ubicua de sus numerosos receptores, juega un papel clave en la regulación de diferentes comportamientos cognitivos, fisiológicos y emocionales (Jacobs y Azmitia, 1992; Baumgarten y Grozdanovic, 1997). El sistema serotoninérgico es la diana de muchos medicamentos para el tratamiento de enfermedades psiquiátricas y por esta y otras razones este sistema se ha relacionado con la patofisiología de numerosos trastornos psiquiátricos. Por estos motivos, en el presente estudio se han seleccionado 3 genes que codifican para tres elementos clave del sistema serotoninérgico: el transportador de serotonina, el receptor 5-HT_{2A} y la triptófano hidroxilasa 2.

El transportador de serotonina es un elemento de vital importancia para el sistema serotoninérgico, ya que esta proteína localizada en la membrana presináptica se encarga de la recaptación de la serotonina presente en el espacio sináptico, controlando así la duración de la señalización serotoninérgica. El gen humano del transportador de serotonina (*SLC6A4*, *SERT* o *5-HTT*) está localizado en el cromosoma 17 (17q11.2). Su actividad transcripcional está principalmente regulada por un elemento repetitivo localizado en una región reguladora aguas arriba del inicio de transcripción. Este polimorfismo recibe el nombre de 5-HTTLPR (*5-HTT gene linked polimorphic region*) y mayoritariamente da lugar a la aparición de dos alelos: el alelo l (largo) y el s (corto)(Heils *et al.*, 1996). Se ha visto que el alelo s presenta unos niveles más bajos de expresión y función que el alelo l (revisado en Murphy *et al.*, 2008). Además, otro polimorfismo (rs25531), un cambio A/G en la región del 5-HTTLPR, también afecta a los niveles de expresión del gen, dando lugar al alelo L_A , de alta expresión, y a los alelos L_G, S_A y S_G, de baja expresión. Se han llevado a cabo un número importante de estudios para evaluar la posible contribución del 5-HTTLPR en la susceptibilidad a diferentes trastornos mentales, como el trastorno bipolar (Cho *et al.*, 2005), trastorno obsesivo-compulsivo (Bengel *et al.*, 1999) o la depresión mayor (Kraft *et al.*, 2005). El alelo s también se ha asociado con altos niveles de neuroticismo (Lesch *et al.*, 1996), mayor depresión como respuesta a sucesos vitales estresantes (Caspi *et al.*, 2003) y una mayor activación de la amígdala como respuesta a estímulos amenazantes (Hariri *et al.*, 2002). También se ha estudiado la relación del 5-HTTLPR y otros polimorfismos del gen con la esquizofrenia, aunque los resultados son más contradictorios (Fan y Sklar, 2005; Allen *et al.*, 2008; Shi *et al.*, 2008). Con respecto a las alucinaciones auditivas, hay un trabajo previo de Malhotra y colaboradores (1998) que asoció el alelo largo con una mayor intensidad de las alucinaciones auditivas.

El receptor de serotonina 5-HT_{2A} está codificado por el gen *HTR2A*, localizado en 13q14-q21. Dos polimorfismos de este gen (T102C o rs6313, localizado en el primer intrón, y -1438A/G o rs6311, localizado en la región promotora) han sido ampliamente estudiados en relación con la susceptibilidad a padecer esquizofrenia, debido a la conocida afinidad de los antipsicóticos atípicos por los receptores 5-HT_{2A} (Worrel *et al.*, 2000). Además, también se sabe que algunos agonistas de este receptor, como el LSD y la mescalina, tienen potentes efectos alucinógenos (Glennon *et al.*, 1984). Sin embargo, los resultados existentes hasta el momento son contradictorios y no permiten llegar a ninguna conclusión clara. Lo mismo ha ocurrido también al estudiar cómo influye la variación de este gen en los trastornos afectivos (Golimbet *et al.*, 2002; Anguelova *et al.*, 2003) y en la respuesta a la medicación en pacientes esquizofrénicos (Arranz *et al.*, 1995; Anttila *et al.*, 2007).
La **enzima triptófano hidroxilasa (TPH)** es el principal elemento regulador en la síntesis de serotonina. La isoforma específica de cerebro fue denominada TPH2 (Walther y Bader, 2003) y está codificada por el gen del mismo nombre. La búsqueda de variantes genéticas con un efecto sobre la actividad de la enzima se ha convertido en una aproximación interesante para estudiar la neurobiología de los desórdenes psiquiátricos, especialmente de aquellos que implican alteraciones afectivas y de tipo emocional. Siguiendo esta hipótesis, se han estudiado diferentes polimorfismos, aunque uno de los más estudiados es el SNP rs4570625, que tiene un impacto sobre la funcionalidad de la enzima. Hasta el momento, se han asociado diferentes variaciones en el gen *TPH2* con a) diferentes desórdenes afectivos, como depresión y ansiedad (Zhang *et al.*, 2005; Zhou *et al.*, 2005) y trastorno bipolar (Harvey *et al.*, 2004; Cichon *et al.*, 2008); b) enfermedades con alteraciones cognitivas o que afectan al control ejecutivo, como el trastorno por déficit de atención e hiperactividad y trastorno obsesivo-compulsivo (Baehne *et al.*, 2008); y c) rasgos de la personalidad relacionados con la emocionalidad (Gutknecht *et al.*, 2007; Herrmann *et al.*, 2007). Sin embargo, los resultados relacionados con esquizofrenia son hasta el momento negativos (De Luca *et al.*, 2005a; Higashi *et al.*, 2007). A pesar de ello, consideramos que este gen podría tener un papel importante en el procesado emocional de las alucinaciones auditivas en pacientes esquizofrénicos.

Gen de la óxido nítrico sintasa 1. El óxido nítrico (NO) es una molécula gaseosa que puede actuar como neurotransmisor en el sistema nervioso central, en estrecha relación con la transmisión glutamatérgica (Garthwaite, 2008). El NO es sintetizado en el sistema nervioso por la NO sintasa 1 (NOS-I). Esta enzima está codificado por un gen extremadamente complejo localizado en el cromosoma 12 (Hall *et al.*, 1994; Zhang *et al.*, 2004), que presenta diferentes exones no codificantes. Entre estos exones, destaca el exón 1f, puesto que contiene una región de elementos repetitivos, concretamente nucleótidos CA (Reif *et al.*, 2006). Este VNTR es de especial importancia, puesto que se ha visto que los alelos cortos reducen la eficiencia transcripcional del promotor. Éste y otros polimorfismos del gen *NOS1* se han asociado con diferentes patologías psiquiátricas, entre ellas la esquizofrenia. Así, diferentes estudios de asociación apoyan el papel del gen *NOS1* en la patogénesis de la esquizofrenia y otras enfermedades psiquiátricas (Detera-Wadleigh *et al.*, 1999; Shinkai *et al.*, 2002; Fallin *et al.*, 2005; Reif *et al.*, 2006; Tang *et al.*, 2008; O'Donovan *et al.*, 2008), aunque también existen resultados negativos (Liou *et al.*, 2003).

Stathmin. Stathmin pertenece a una familia de proteínas encargada de regular la dinámica de polimerización-despolimerización de los microtúbulos (Belmont y Mitchinson, 1996; Gavet *et al.*, 1998). En humanos, esta proteína está codificada por un pequeño gen (*STMN1*) localizado en 1p36-1-p35. Entre las diferentes tareas en las que participa, destacan la regulación de la proliferación celular; transporte, motilidad celular y mantenimiento de la forma celular; migración celular; y por último, desarrollo, maduración y regulación funcional del sistema nervioso central (Curmi *et al.*, 1999). Esta última función es de gran interés para el presente estudio. Se ha comprobado que stathmin se expresa enormemente durante el crecimiento de neuritas y la formación de sinapsis, lo que apoyaría su papel en la diferenciación neuronal (Di Paolo *et al.*, 1997). Sin embargo, es intrigante el hecho de que la inactivación de este gen en ratón no parecía producir un fenotipo aparente (Schubart *et al.*, 1996). Sin embargo, recientemente se ha comprobado que esto no es realmente así, puesto que el gen *STMN1* es clave en el ratón para la inducción de la potenciación a largo plazo en aquellos tractos que proyectan hacia la amígdala, así como para regular el miedo innato y aprendido. Esto tiene consecuencias en comportamientos relacionados con estos sistemas, como el cuidado materno innato y las interacciones sociales (Shumyatsky *et al.*, 2005; Martel *et al.*, 2008). Stathmin podría por tanto tener un papel clave en aquellos trastornos psiquiátricos donde el procesado del miedo y las interacciones sociales están comprometidos. Los desórdenes afectivos y la esquizofrenia son un claro ejemplo de ello.

Genes de selección positiva: *ASPM*. Desde un punto de vista evolutivo, se puede hipotetizar que el incremento de habilidades cognitivas sufrido por la especie humana pudo haber favorecido la aparición de ciertas patologías que cursan con alteraciones cognitivas, tales como la esquizofrenia (Crow, 1997; Horrobin, 1998). Esta idea genera una paradoja que podría explicar por qué la esquizofrenia mantiene una frecuencia más o menos invariable a lo largo del tiempo a pesar de su gravedad. Por ello, aquellos genes que hayan sufrido una evolución acelerada en el linaje humano y que tengan un papel clave en el funcionamiento del sistema nervioso central son excelentes genes candidatos a estar implicados en la vulnerabilidad a padecer esquizofrenia. En este estudio, hemos seleccionado para su estudio un gen de selección positiva: el gen *ASPM* (*abnormal spindle-like microcephaly associated*). Este gen tiene un papel muy importante en el desarrollo del cortex cerebral. Está localizado en 1q31 (Bond *et al.*, 2002) y codifica una proteína con varios dominios, siendo el más grande e importante un dominio de repeticiones Isoleucina-Glutamina (IQ), que da lugar a una estructura de orden superior que podría tener un papel importante en las interacciones entre la proteína ASPM y proteínas poliméricas como la actina y la calmodulina (Kouprina *et al.*, 2005). El gen *ASPM* se expresa durante el desarrollo embrionario en un gran número de tejidos, incluido el cerebro. Sin embargo, no se expresa en el cerebro adulto (Bond *et al.*, 2002; Kouprina *et al.*, 2005). Diferentes estudios han permitido constatar que la proteína se localiza en los polos durante la mitosis celular y parece ser necesaria para asegurar la simetría proliferativa de los progenitores que darán lugar al cortex cerebral. (Fish *et al.*, 2006). Sin embargo, el rasgo más llamativo del gen *ASPM* son las evidencias que apoyan una evolución acelerada de este gen, presumiblemente por selección positiva, en el linaje humano (Zhang, 2003; Evans *et al.*, 2004; Kouprina *et al.*, 2004). Asimismo, esta selección positiva parece que sigue actuando en la actualidad (Mekel-Bobrov *et al.*, 2005). Este hecho sugiere que el gen *ASPM* podría haber tenido un papel clave en la expansión del cerebro observada a lo largo de la evolución de los

primates. Sin embargo, a pesar de los indicios de selección positiva, ninguna variación polimórfica de este gen ha podido ser asociada todavía con el tamaño cerebral global (Woods *et al.*, 2006; Rushton *et al.*, 2007; Timpson *et al.*, 2007; Wang *et al.*, 2008) o con parámetros relacionados con la inteligencia (Mekel-Bobrov *et al.*, 2007; Rushton *et al.*, 2007) en población sana. Son necesarios más estudios para conocer el verdadero papel de este gen en la cognición y la susceptibilidad a la esquizofrenia.

Otros genes candidatos. Los tres últimos genes que se han incluido en este estudio se seleccionaron a partir de un trabajo dirigido por el Dr. Lesch cuyo principal objetivo era la búsqueda de variaciones en el número de copias en una muestra de pacientes con trastorno por déficit de atención e hiperactividad (TDAH). A partir de este estudio, se seleccionaron tres genes, *PDE4D*, *PLEKHB1* y *RAB6A*.

***PDE4D*.** Este gen codifica una fosfodiesterasa (PDE), proteína encargada de degradar el AMP cíclico (cAMP), molécula de gran importancia como segundo mensajero en un importante número de procesos neurales (Dudai, 1986). Concretamente la familia de fosfodiesterasas PDE4 se caracteriza por su inhibición mediante rolipram, sustancia neuroactiva con efecto antidepresivo. El gen *PDE4D* es un gen de gran tamaño localizado en el cromosoma 5 que presenta procesado alternativo, lo que da lugar a un elevado número de isoformas diferentes (Wang *et al.*, 2003b). De especial interés es la isoforma PDE4D6, isoforma de pequeño tamaño específica de cerebro, cuya localización celular es hasta el momento desconocida. El hecho de que la proteína PDE4D se vea inhibida por rolipram (Henkel-Tigges y Davis, 1990) sugiere que podría tener un papel clave en el comportamiento humano. Asimismo, se ha comprobado que PDE4D interacciona con *DISC1* (Murdoch *et al.*, 2007). Hay por tanto suficientes razones para sugerir que variaciones en el gen *PDE4D* podrían impactar sobre el riesgo de padecer esquizofrenia o desórdenes afectivos. De acuerdo con esta hipótesis, varios autores (Shifman *et al.*, 2008b; Heck *et al.*, 2008) han encontrado muy recientemente asociaciones entre polimorfismos de este gen y ciertos rasgos de la personalidad como el Neuroticismo o la Evitación de Daño, tanto en sujetos sanos como en pacientes depresivos.

PLEKHB1* y *RAB6A. Son dos genes adyacentes pero en orientaciones opuestas localizados en el cromosoma 11. PLEKHB1 (anteriormente llamado PHR1) es una proteína integral de membrana que contiene un dominio de homología con la pleckstrina. Se expresa en las células fotorreceptoras, en las células pilosas cocleares y vestibulares, así como en las neuronas receptoras olfatorias (Xu *et al.*, 1999; Xu *et al.*, 2004). Su función parece estar relacionada con la transducción de señales. La existencia de dos promotores diferentes y el procesado alternativo del exón 7 da lugar a 4 isoformas diferentes. Por otra parte, *RAB6A* es un gen que da lugar a dos isoformas diferentes, Rab6A y Rab6A' (Echard *et al.*, 2000), pertenecientes a la familia de pequeñas GTPasas, proteínas de vital importancia en el tráfico intracelular de membranas (Wanschers *et al.*, 2008). Aunque ambos genes (*PLEKHB1* y *RAB6A*) se expresan en el cerebro, su relación con la esquizofrenia no es tan clara a priori como en los casos anteriores. Sin embargo, el hecho de estar asociados con una enfermedad psiquiátrica como el TDAH los convierte en interesantes genes candidatos.

Objetivos

Este estudio es parte de un proyecto de investigación multidisciplinario más amplio que buscar profundizar en las bases genéticas de la esquizofrenia y sus síntomas más característicos. De acuerdo con este marco general, la hipótesis general y los objetivos de este estudio son los siguientes:

Hipótesis general: variaciones en los genes previamente descritos modulan la vulnerabilidad a padecer esquizofrenia o alguno de sus rasgos más importantes, particularmente las alucinaciones auditivas, el deterioro cognitivo, alteraciones neuroanatómicas y, finalmente, la disfunción emocional asociada a las condiciones patológicas descritas previamente.

Objetivos generales:

1. Análisis de varios polimorfismos localizados en los genes candidatos mediante estudios de asociación caso-control y basados en familias. Para este propósito, diferentes muestras de pacientes y sujetos sanos serán incluidas.
2. Búsqueda de asociaciones entre alelos, genotipos o haplotipos y diferentes variables cuantitativas y rasgos patológicos, incluyendo a) puntuaciones en escalas generales y de alucinaciones, y b) rendimiento en diferentes tareas de naturaleza cognitiva.
3. Evaluación del impacto de algunos polimorfismos de interés sobre ciertos fenotipos de neuroimagen que están afectados en pacientes esquizofrénicos.

Resultados y conclusiones

En este estudio se ha trabajado con tres muestras de diferente origen:

- Un grupo de 328 pacientes psicóticos (76% de ellos diagnosticados con esquizofrenia; además 246 sufrían alucinaciones auditivas) y 348 controles sanos no emparentados, todos ellos de Valencia (España). Los pacientes fueron evaluados mediante diferentes escalas clínicas psiquiátricas generales (BPRS, PANSS, KGV), así como con escalas específicas (subescalas PSYRATS de alucinaciones auditivas y delirios).

- Una muestra de 336 pacientes (247 sufrían de esquizofrenia, 89 presentaban desorden bipolar) y 540 controles sanos, procedentes de la Baja Franconia (Alemania).

- Una muestra procedente de los Estados Unidos de América, consistente en 466 familias con individuos afectados de esquizofrenia o trastorno esquizoafectivo, así como 483 individuos sanos no emparentados. Las familias incluidas se componían, en total, de 498 pacientes, 612 padres y 427 hermanos no afectados. Tanto los pacientes como los controles sanos fueron sometidos a una batería de tests cognitivos. A partir de sus resultados, se elaboraron una serie de medidas numéricas derivadas de los tests cognitivos (Genderson *et al.*, 2007): Memoria Verbal; Memoria de Trabajo-*N Back*; Memoria Visual; Cociente Intelectual (CI)-Velocidad de Procesamiento-Fluidez; Función Ejecutiva-*Wisconsin Card Sorting Test* (WCST); Atención; y Repetición de Números (*Digit Span*).

A partir del ADN obtenido, bien de sangre o de saliva, se genotiparon una serie de polimorfismos en cada uno de los 9 genes candidatos elegidos en este estudio. A continuación se llevaron a cabo diferentes estudios de asociación dependiendo de la muestra de individuos (caso-control, basado en familias, asociación con variables cuantitativas). Asimismo, se analizó el impacto de ciertos polimorfismos sobre el volumen cerebral total o regional y sobre la activación de ciertas áreas mediante el uso de determinados paradigmas de resonancia magnética funcional (fMRI).

Los resultados para cada gen y las conclusiones obtenidas se muestran a continuación.

Gen *SLC6A4* (Transportador de serotonina)

Se seleccionaron 12 polimorfismos a lo largo del gen (incluyendo tagSNPs y otros polimorfismos adicionales seleccionados por su posible funcionalidad), que fueron genotipados en la muestra caso-control española.

Los resultados procedentes del análisis de asociación caso-control revelaron la existencia de dos SNPs (rs140700 y rs2020936) asociados con el riesgo de psicosis (tabla C1) y psicosis con alucinaciones (datos no mostrados). Estos resultados se obtuvieron tanto en el análisis de asociación de las frecuencias alélicas como en el de las genotípicas (tabla C2). Además, los resultados significativos obtenidos con el SNP rs2020936, localizado en el intrón 1A del gen, resistieron la corrección para comparaciones múltiples. Sin embargo, el polimorfismo funcional

5-HTTLPR no pareció impactar sobre el riesgo de psicosis; de hecho, este dato es coherente con la literatura existente (Fan y Sklar, 2005; Levinson, 2005). Asimismo, se encontraron varios haplotipos protectores de 2, 3 y 4 marcadores, los cuales eran variaciones de los mismos alelos de los SNPs rs3813034, rs140700, rs2020942, rs2020939 y rs2020936 (datos no mostrados). Desafortunadamente, estos resultados dejaron de ser significativos tras 1000 permutaciones.

Tabla C1. Comparación de las frecuencias alélicas de los polimorfismos del gen *SLC6A4* en pacientes psicóticos y controles sanos de la muestra española.

Polimorfismo	Alelo	Frecuencia pacientes	Frecuencia controles	OR (95% IC)	χ^2	P[a]
rs3813034	G	0.4715	0.4693	1	0.006	0.938 (1)
	T	0.5285	0.5307	0.991 (0.796-1.235)		
rs1042173	G	0.4815	0.4721	1	0.113	0.737 (1)
	T	0.5185	0.5279	0.963 (0.773-1.2)		
rs140700	A	0.04833	0.08254	1	5.45	**0.0196** (0.2152)
	G	0.9517	0.9175	1.772(1.09-2.879)		
rs2020942	A	0.3658	0.3284	1	1.959	0.162 (1)
	G	0.6342	0.6716	0.848 (0.673-1.068)		
STin2	12 rep	0.6429	0.6776	1	1.668	0.197 (1)
	10 rep	0.3571	0.3224	1.168 (0.923-1.478)		
rs2020939	C	0.5406	0.558	1	0.359	0.549 (1)
	T	0.4594	0.442	1.073 (0.852-1.35)		
rs2020936	C	0.1644	0.2141	1	5.075	**0.0243** (0.2427)
	T	0.8356	0.7859	1.384 (1.042-1.838)		
rs2066713	C	0.646	0.6711	1	0.892	0.345 (1)
	T	0.354	0.3289	1.118 (0.887-1.409)		
rs4251417	A	0.06506	0.07166	1	0.198	0.657 (1)
	G	0.9349	0.9283	1.109 (0.702-1.753)		
5-HTTLPR (incluyendo rs25531)	L_A	0.4505	0.4797	1	1.104	0.576 (1)
	L_G	0.04785	0.05072	1.005 (0.599 - 1.686)		
	S_A	0.4901	0.4638	1.125 (0.899 - 1.409)		
	S_G	0.01155	0.005797	2.122 (0.615 - 7.324)		
rs12945042	G	0.7286	0.719	1	0.133	0.715 (1)
	A	0.2714	0.281	0.9532 (0.737-1.233)		

Los valores de *P* significativos (P < 0.05) se indican en negrita.
a. El valor de *P* corregido se indica entre paréntesis.
Abreviaturas: OR, *Odds Ratio*; IC, Intervalos de confianza; rep, repeticiones.

Tabla C2. Comparación de las distribuciones genotípicas de los polimorfismos del gen *SLC6A4* en pacientes psicóticos y controles sanos de la muestra española.

Polimorfismo	Genotipo	Frecuencia controles	Frecuencia pacientes	OR (95% IC)	*P* [b]	AIC	Modelo
rs3813034	T/T-G/T	270 (79%)	230 (77.2%)	1.00	0.59 (1)	887.9	Recesivo
	G/G	72 (21.1%)	68 (22.8%)	1.11 (0.76-1.61)			
rs1042173	T/T-T/G	268 (78.6%)	228 (76.5%)	1.00	0.53 (1)	886.5	Recesivo
	G/G	73 (21.4%)	70 (23.5%)	1.13 (0.78-1.64)			
rs140700	G/G	265 (84.1%)	243 (90.3%)	0.56(0.34-0.91)[a]	**0.017** (0.17)	804.3	Aditivo
	A/G	48 (15.2%)	26 (9.7%)				
	A/A	2 (0.6%)	0 (0%)				
rs2020942	G/G	156 (45.8%)	119 (39.9%)	1.00	0.14 (1)	884.7	Dominante
	A/G-A/A	185 (54.2%)	179 (60.1%)	1.27 (0.93-1.74)			
STin2	12/12	156 (46.6%)	118 (41.1%)	1.00	0.17 (1)	860.7	Dominante
	10/12-10/10	179 (53.4%)	169 (58.9%)	1.25 (0.91-1.72)			
rs2020939	C/C-C/T	259 (81.2%)	214 (79%)	1.00	0.5 (1)	817.5	Recesivo
	T/T	60 (18.8%)	57 (21%)	1.15(0.77-1.72)			
rs2020936	T/T	204 (59.8%)	211 (70.8%)	1.00	**0.0045 (0.0495)**	878.1	Codominante
	T/C	128 (37.5%)	76 (25.5%)	0.57 (0.41-0.81)			
	C/C	9 (2.6%)	11 (3.7%)	1.18 (0.48-2.91)			
rs2066713	C/C	156 (45.6%)	121 (40.6%)	1.00	0.2 (1)	886.6	Dominante
	C/T-T/T	186 (54.4%)	177 (59.4%)	1.23 (0.90-1.68)			
rs4251417	G/G	269 (85.7%)	234 (87%)	1.00	0.64 (1)	808.5	N/A
	A/G	45 (14.3%)	35 (13%)	0.89 (0.56-1.44)			
5-HTTLPR (including rs25531)	low/low	257 (75.4%)	239 (80.2%)	1.00	0.14 (1)	884.8	Recesivo
	high/low-high/high	84 (24.6%)	59 (19.8%)	0.76 (0.52-1.10)			
rs12945042	G/G-G/A	285 (90.5%)	253 (94%)	1.00	0.11 (0.99)	807.4	Recesivo
	A/A	30 (9.5%)	16 (6%)	0.60 (0.32-1.13)			

Los valores de *P* significativos (P < 0.05) se indican en negrita.
a. El valor indicado se corresponde con la diferencia que se le atribuye a cada alelo en el modelo aditivo.
b. El valor de *P* corregido se indica entre paréntesis.
Abreviaturas: OR, *Odds Ratio*; IC, Intervalos de confianza; N/A, no aplicable.

El polimorfismo rs2020936 no había sido asociado previamente con la susceptibilidad a padecer esquizofrenia aunque Kim *et al.* (2002) encontraron una transmisión preferencial del alelo T en una muestra de pacientes autistas. Sin embargo, nuestros resultados deberían ser interpretados con cuidado, puesto que este polimorfismo estaba ligeramente fuera del Equilibrio de Hardy-Weinberg (HWE por sus siglas en inglés) en la muestra de controles, lo que dificulta la interpretación y valoración de los resultados. Aunque tampoco podemos descartar que este SNP, u otro SNP en alto desequilibrio de ligamiento (LD) con él, pueda tener algún efecto sobre la funcionalidad del transportador de serotonina.

Con respecto al análisis de asociación con variables cuantitativas, se encontraron un gran número de resultados significativos, aunque los resultados más interesantes son las asociaciones del 5-HTTLPR con parámetros relacionados con aspectos emocionales (tabla C3),

como los ítems Excitación y Hostilidad de la escala PANSS, que forman parte del Componente Excitativo de esta escala (Kay y Sevy, 1990). Asimismo, este polimorfismo localizado en el promotor del gen se asoció con tres ítems emocionales de la escala PSYRATS de alucinaciones auditivas: Intensidad de la Ansiedad, Frecuencia de la Ansiedad y Repercusión.

Tabla C3. Asociaciones significativas del polimorfismo 5-HTTLPR del transportador de serotonina con diferentes ítems clínicos relacionados con emocionalidad.

Polimorfismo	Distribución genotípica[a]	Puntuación media (SE)	Diferencia (95% IC)	Modelo	P^b	BIC
KGV - Ansiedad						
5-HTTLPR	baja/baja : 78	1.72 (0.119)	0.00	Dominante	**0.04** (0.44)	789
	alta/baja-alta/alta: 135	1.44 (0.08)	-0.29 (-0.57 - -0.01)			
PANSS P4 - Excitación						
5-HTTLPR	baja/baja : 37	2.43 (0.18)	0.00	Dominante	**0.0014** **(0.015)**	394.3
	alta/baja-alta/alta:92	1.77 (0.11)	-0.67 (-1.07 - -0.27)			
PANSS P7 - Hostilidad						
5-HTTLPR	baja/baja : 37	2.19 (0.2)	0.00	Dominante	**0.0004** (**0.0044**)	364.9
	alta/baja - alta/alta : 92	1.53 (0.09)	-0.67 (-1.03 - -0.32)			
PSYRATS AA – Frecuencia de la ansiedad						
5-HTTLPR	baja/baja : 68	1.78 (0.21)	0.00	Dominante	**0.011** (0.121)	799.5
	alta/baja-alta/alta:140	1.21 (0.13)	-0.61 (-1.08 - -0.14)			
PSYRATS AA – Intensidad de la ansiedad						
5-HTTLPR	baja/baja : 68	1.79 (0.2)	0.00	Dominante	**0.0061** (0.0671)	790.1
	alta/baja-alta/alta:140	1.18 (0.13)	-0.65 (-1.11 - -0.19)			
PSYRATS AA - Repercusión						
5-HTTLPR	baja/baja : 68	1.84 (0.18)	0.00	Dominante	**0.012** (0.132)	765.3
	alta/baja-alta/alta:140	1.28 (0.12)	-0.56 (-0.99 - -0.13)			

Los valores de *P* significativos (P < 0.05) se indican en negrita.
a. La designación "baja" o "alta" hace referencia a los alelos de alta y baja expresión del polimorfismo 5-HTTLPR.
b. El valor de *P* corregido se indica entre paréntesis.
Abreviaturas: SE, error estándar; IC, intervalo de confianza; AA, alucinaciones auditivas; N/A, no aplicable.

Estos interesantes resultados nos animaron a profundizar en el efecto de este polimorfismo sobre aquellas regiones cerebrales asociadas con el procesado de las emociones. Por ello, se realizaron estudios de resonancia magnética funcional (fMRI) y estructural (sMRI) y los datos obtenidos se correlacionaron con los datos genéticos. Con respecto al estudio de neuroimagen funcional, se empleó un paradigma de tipo auditivo-emocional mediante el cual se detectó una mayor activación de la amígdala derecha frente a palabras emocionales en los pacientes S/S en comparación con los L/L (figura C1). Además, los estudios de neuroimagen estructural han permitido detectar que tanto los pacientes esquizofrénicos alucinadores como los controles sanos portadores de al menos un alelo S presentan una reducción en la densidad de materia gris en ciertas zonas del sistema límbico. Sin embargo, las áreas implicadas son diferentes (hipocampo y parahipocampo en controles, ver figura C2; córtex anterior cingulado en pacientes, ver figura C3).

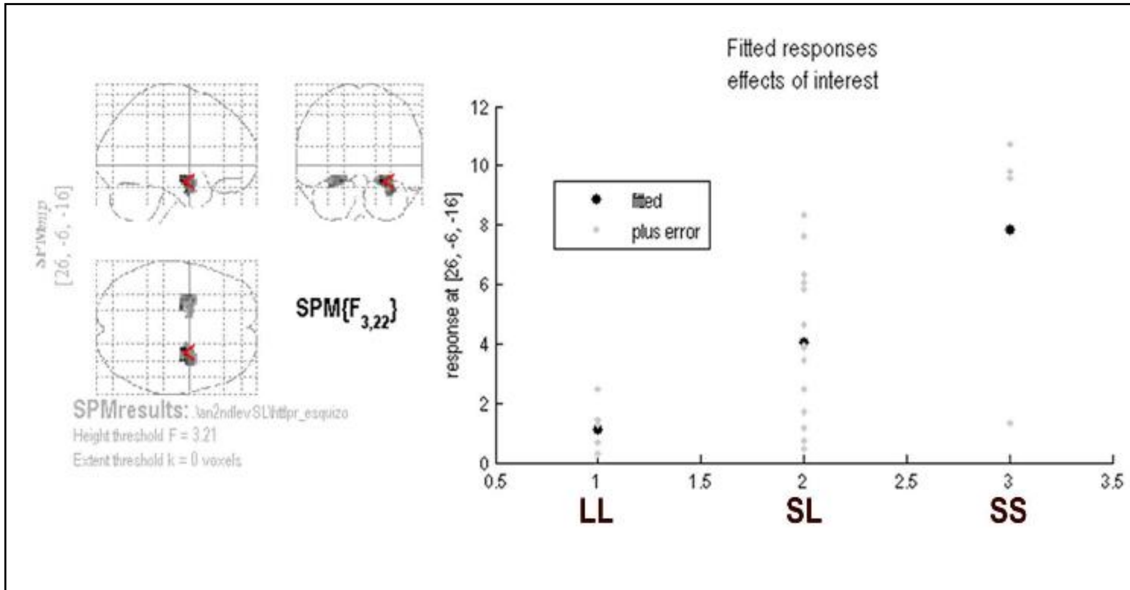


Figura C1. Diferencias en la activación de la amígdala derecha ante palabras emocionales en pacientes esquizofrénicos dependiendo del genotipo para el polimorfismo 5-HTTLPR.

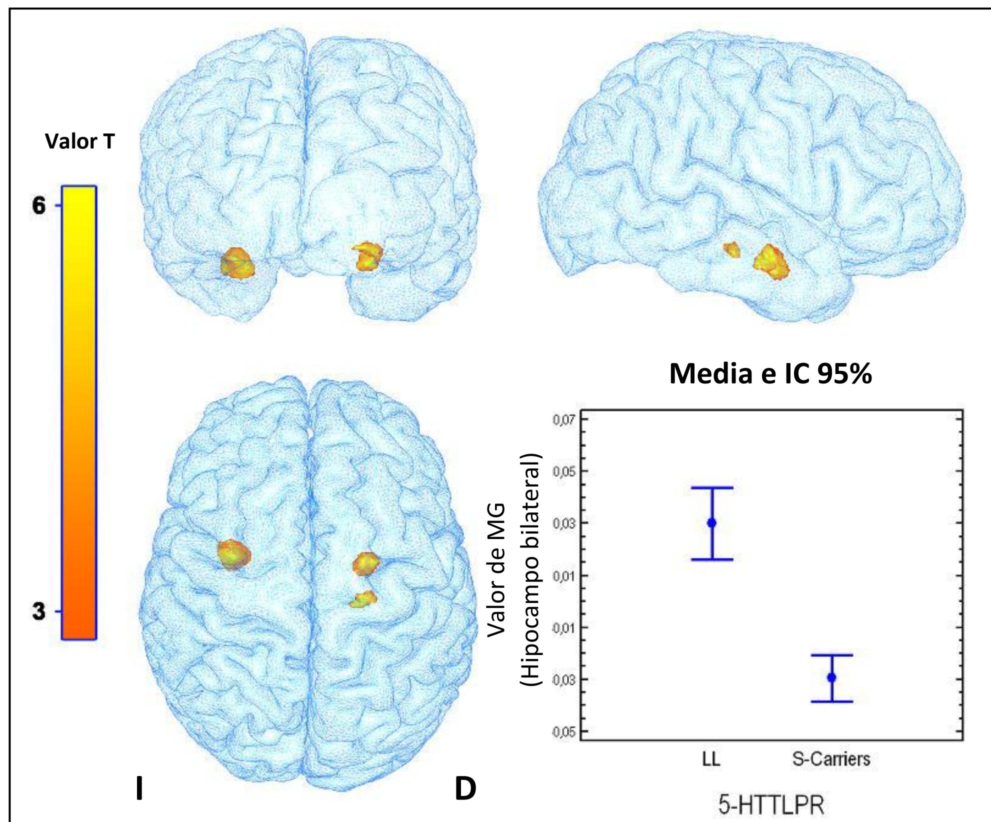


Figura C2. Áreas con reducción en la densidad de material gris en sujetos control con al menos una copia del alelo corto del polimorfismo 5-HTTLPR y gráfico con los valores normalizados de materia gris en el hipocampo (bilateral) y la circunvolución parahipocámpica (izquierda). $p < 0.05$ FDR corregido k=34.
Abreviaturas: MG, material gris; LSD, *least significant difference*; FDR, *false discovery rate*; I, izquierda; D, derecha.

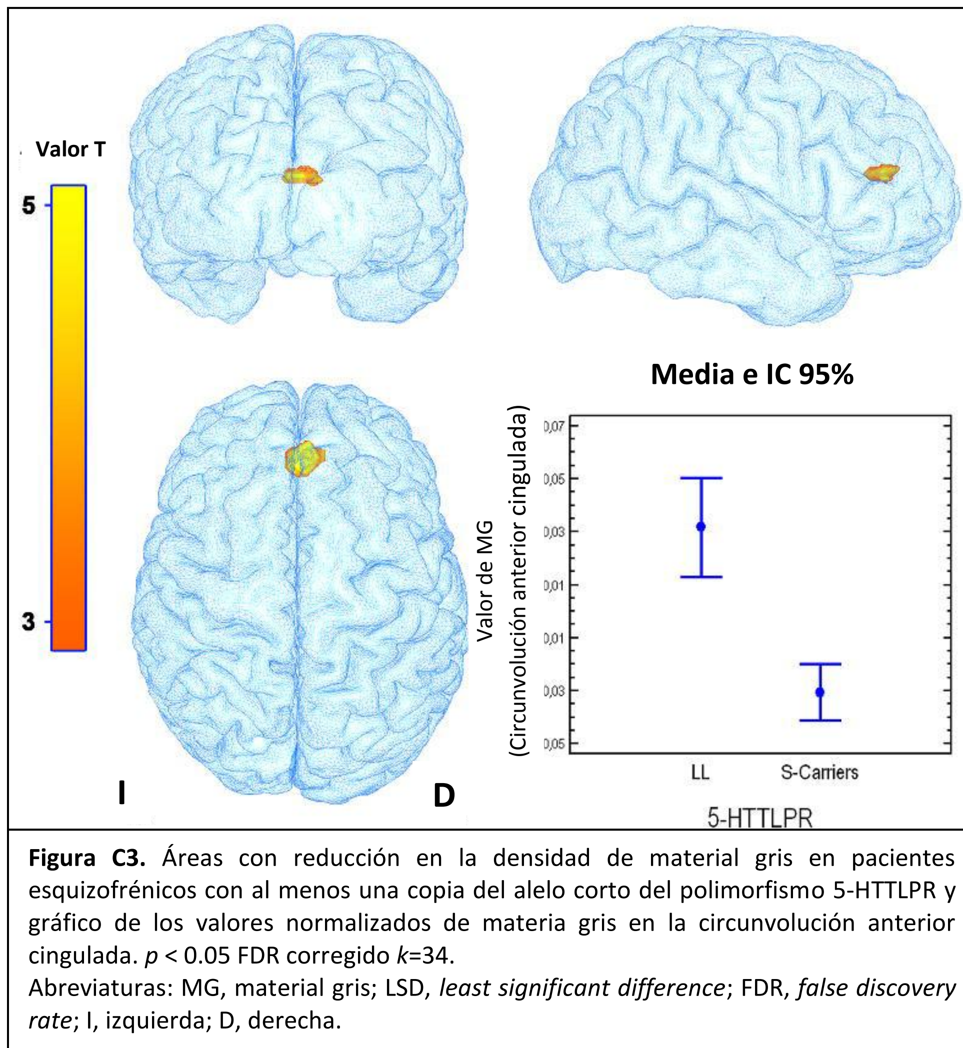


Figura C3. Áreas con reducción en la densidad de material gris en pacientes esquizofrénicos con al menos una copia del alelo corto del polimorfismo 5-HTTLPR y gráfico de los valores normalizados de materia gris en la circunvolución anterior cingulada. $p < 0.05$ FDR corregido k=34.
Abreviaturas: MG, material gris; LSD, *least significant difference*; FDR, *false discovery rate*; I, izquierda; D, derecha.

A nuestro entender, éste es el primer estudio que ha intentado profundizar en las bases genéticas de las alucinaciones auditivas en la psicosis, teniendo en cuenta además un punto de vista dimensional. Nuestros resultados claramente sugieren la existencia de un efecto de la variación genética del transportador de serotonina en aquellos circuitos neurales implicados en el procesado emocional, tanto en sujetos sanos como psicóticos. La amígdala, así como otros elementos del sistema límbico (hipocampo, córtex anterior cingulado) juegan un papel clave en estos procesos tanto en situaciones normales como patológicas (Hariri *et al.*, 2002; Furmark *et al.*, 2004; Hariri *et al.*, 2005; Canli *et al.*, 2005b; Heinz *et al.*, 2005; Canli *et al.*, 2006; Domschke *et al.*, 2006; Heinz *et al.*, 2007; Rao *et al.*, 2007). Nuestros resultados también sugieren que las alucinaciones auditivas son sucesos estresantes, y que la respuesta del individuo a ellas puede ser modulada por el acervo genético del individuo. En este sentido, también hay estudios que relacionan claramente este mismo polimorfismo con la vulnerabilidad a eventos estresantes y la tendencia a la depresión (Caspi *et al.*, 2003; Eley *et al.*, 2004; Kaufman *et al.*, 2004; Grabe *et al.*, 2005; Kendler *et al.*, 2005; Cervilla *et al.*, 2007). Los resultados del análisis estructural de neuroimagen también apoyan un efecto del polimorfismo del promotor del transportador de serotonina en áreas límbicas. La reducción de

la densidad de materia gris en el córtex anterior cingulado (ACC) ventral y dorsal observada en pacientes del grupo S es especialmente relevante puesto que esta estructura desempeña un papel importante en la neurobiología de la esquizofrenia (Baiano et el al., 2007). Por otra parte, el ACC está implicado en la regulación del estado de ánimo y estos procesos están afectados en pacientes esquizofrénicos. Por lo tanto, podemos presumir que los pacientes que sufren de esquizofrenia y alucinaciones auditivas presentan una alteración del procesado emocional, que está modulada por el 5-HTTLPR y se traduce a su vez en una reducción de materia gris en el ACC. Esta reducción podría a su vez relacionarse con las diferencias observadas en la activación de la amígdala derecha. En coherencia con esta hipótesis, Pezawas y colaboradores (2005) encontraron un volumen reducido en el ACC y la amígdala de sujetos sanos portadores de al menos un alelo corto del 5-HTTLPR. Desafortunadamente, en nuestro estudio no se han detectado diferencias en el volumen de materia gris de la amígdala dependiendo del 5-HTTLPR. Varias razones, tales como un efecto del sexo o del tamaño muestral, podían explicar estas diferencias. Sin embargo, los resultados de nuestro análisis de sMRI en sujetos control han sido diferentes a los del grupo esquizofrénico, puesto que para este grupo hemos encontrado reducciones volumétricas en el hipocampo y el parahipocampo asociadas al alelo S. Una vez más, ciertos factores, como el sexo, los efectos de la medicación, el reducido tamaño muestral o el papel potencial del estrés sobre el eje hipotalámico-hipofisario-suprarrenal podrían explicar las divergencias. Con relación al efecto del estrés sobre el eje hipotalámico-hipofisario-suprarrenal, que controla la liberación de cortisol, se ha encontrado una interacción entre el alelo S del 5-HTTLPR y niveles altos de cortisol, que se asocia a una reducción en el volumen del hipocampo (O'Hara *et al.*, 2007). También hay estudios previos que relacionan situaciones de estrés severo en la infancia con un menor tamaño del hipocampo (Stein *et al.*, 1997; Vythilingam *et al.*, 2002).

Desafortunadamente, no encontramos ninguna diferencia en el volumen hipocámpico dependiente del genotipo en nuestro grupo de pacientes. Esto sugiere la existencia de otros factores de riesgo adicionales (genéticos o ambientales) que contribuyen a la determinación del volumen de las estructuras temporales. Por otra parte, tampoco se puede olvidar que las condiciones ambientales que influencian un fenotipo esquizofrénico pueden diferir enormemente de las de un sujeto sano.

En resumen, en este estudio se han encontrado dos SNPs (rs140700 and rs2020936), así como varios haplotipos protectores, asociados con la psicosis y la psicosis con alucinaciones auditivas. Asimismo, el polimorfismo funcional del promotor (5-HTTLPR) parece estar asociado con la dimensión emocional de las alucinaciones auditivas, lo que ha sido corroborado mediante diferentes aproximaciones.

Otros genes del sistema serotoninérgico: *HTR2A* (receptor 5-HT$_{2A}$) y *TPH2* (triptófano hidroxilasa 2)

Los interesantes resultados obtenidos con el gen del transportador de serotonina nos llevaron a hacer un análisis preliminar de otros dos genes del sistema serotoninérgico: *HTR2A* y *TPH2*. Para ello, seleccionamos una serie de polimorfismos de interés: del gen *HTR2A*, los SNPs rs6311 (también conocido como -1438A/G y localizado en la región promotora) y rs6313 (conocido como T102C y situado en el exón 1); del gen *TPH2*, el polimorfismo rs4570625

(también llamado -703G/T y situado en el promotor del gen). Los 3 SNPs se genotiparon en la muestra caso-control española.

Desafortunadamente, no se detectó ninguna asociación significativa en las comparaciones caso control (datos no mostrados). Por el contrario, los resultados más interesantes son las asociaciones entre los dos polimorfismos del gen *HTR2A* y diferentes variables cuantitativas relacionadas con la emocionalidad (tabla C4): ítem "Ansiedad" de la escala BPRS, ítems "Ansiedad" y "Depresión" de la escala KGV, ítems "Ansiedad" y "Preocupación" de la escala PANSS; y, finalmente, "Intensidad de la Ansiedad", de la subescala PSYRATS de alucinaciones auditivas. En todos los casos, los individuos G/G (rs6311) y C/C (rs6313) puntuaron más alto. Esta asociación fue particularmente fuerte para los niveles de ansiedad medidos con las escalas PANSS y BPRS. Además, los resultados presentan una gran coherencia entre diferentes escalas.

Tabla C4. Asociaciones significativas de los polimorfismos del gen *HTR2A* con diferentes ítems clínicos relacionados con emocionalidad.

SNP	Distribución genotípica	Puntuación media (SE)	Diferencia (95% IC)	Modelo	P [b]	BIC
			KGV - Ansiedad			
rs6313	C/C : 87 T/C - T/T : 180	1.75 (0.11) 1.42 (0.08)	0.00 -0.33 (-0.59 - -0.07)	Dominante	**0.014 (0.014)**	781.6
rs6311	G/G : 72 A/G - A/A : 195	1.82 (0.12) 1.42 (0.07)	0.00 -0.40 (-0.68 - -0.13)	Dominante	**0.0041 (0.0082)**	779.4
			KGV - Depresión			
rs6313	C/C : 87 T/C : 130 T/T : 50	1.09 (0.11) 0.93 (0.09) 0.64 (0.11)	-0.22 (-0.38 - -0.05)[a]	Aditivo	**0.011 (0.022)**	756.3
rs6311	G/G - A/G : 216 A/A : 51	1 (0.07) 0.65 (0.11)	0.00 -0.35 (-0.65 - -0.05)	Recesivo	**0.022 (0.022)**	757.6
			BPRS - Ansiedad			
rs6313	C/C : 52 T/C : 93 T/T : 34	3.38 (0.21) 2.96 (0.14) 2.76 (0.27)	-0.32 (-0.63 - -0.02)[a]	Aditivo	**0.041** (0.06)	652
rs6311	G/G : 53 A/G : 91 A/A : 35	3.4 (0.2) 2.96 (0.14) 2.74 (0.26)	-0.34 (-0.64 - -0.04)[a]	Aditivo	**0.03** (0.06)	651.5
			PANSS G2 – Ansiedad			
rs6313	C/C : 39 T/C : 67 T/T : 23	3.18 (0.22) 2.6 (0.14) 2.22 (0.25)	-0.50 (-0.80 - -0.19)[a]	Aditivo	**0.0021 (0.0038)**	430.7
rs6311	G/G : 39 A/G : 66 A/A : 24	3.18 (0.22) 2.61 (0.14) 2.21 (0.24)	-0.50 (-0.80 - -0.19)[a]	Aditivo	**0.0019 (0.0038)**	430.4

Tabla C4 (continuación). Asociaciones significativas de los polimorfismos del gen *HTR2A* con diferentes ítems clínicos relacionados con emocionalidad.

SNP	Distribución genotípica	Puntuación media (SE)	Diferencia (95% IC)	Modelo	*P* [b]	BIC
			PANSS G15 - Preocupación			
rs6313	C/C : 39	3.08 (0.23)	0.00	Dominante	**0.012 (0.024)**	457.8
	T/C - T/T : 90	2.41 (0.14)	-0.67 (-1.18 - -0.16)			
rs6311	G/G : 39	3.08 (0.23)	0.00	Dominante	**0.012 (0.024)**	457.8
	A/G - A/A : 90	2.41 (0.14)	-0.67 (-1.18 - -0.16)			
			PSYRATS AA – Intensidad de la ansiedad			
rs6311	G/G : 53	1.74 (0.24)	-0.33 (-0.64 - -0.01) [a]	Additive	**0.042** (0.084)	788.2
	A/G : 112	1.32 (0.14)				
	A/A : 43	1.09 (0.23)				

Los valores de *P* significativos (P < 0.05) se indican en negrita.
a. El valor indicado se corresponde con la diferencia que se le atribuye a cada alelo en el modelo aditivo.
b. El valor de *P* corregido se indica entre paréntesis.
Abreviaturas: SE, error estándar; IC, intervalo de confianza; AA, alucinaciones auditivas.

Diferentes estudios previos han relacionado el gen *HTR2A* con enfermedades afectivas, como depresión (Choiy *et al.*, 2004), algunos rasgos de personalidad relacionados con el desorden bipolar (Ni *et al.*, 2006), trastorno afectivo estacional (Levitan *et al.*, 2002) y desorden de pánico (Unschuld *et al.*, 2007). Un estudio de Golimbet y colaboradores (2002) es particularmente interesante, puesto que asocia el polimorfismo rs6313 con algunos rasgos de personalidad, como Ansiedad y Neuroticismo, en pacientes psicóticos. Sin embargo, serías necesarias otras aproximaciones para entender mejor el papel de este gen en la emocionalidad y los trastornos afectivos.

Con respecto al polimorfismo funcional del gen *TPH2* (rs4570625), los resultados han sido principalmente negativos. Únicamente al considerar la submuestra de pacientes esquizofrénicos alucinadores, se ha encontrado una ligera asociación entre este SNP y varios parámetros de la escala PSYRATS de alucinaciones auditivas (tabla C5): Puntuación Total, Duración, Localización, Intensidad, Frecuencia de Contenido de Negativo y Frecuencia de la Ansiedad.

Tabla C5. Asociaciones significativas del polimorfismo del gen *TPH2* con diferentes ítems de la subescala PSYRATS de alucinaciones auditivas.

SNP	Distribución genotípica	Puntuación media (SE)	Diferencia (95% IC)	Modelo	*P*	BIC
PSYRATS AA - Total						
	G/G : 124	16.94 (1.38)	1.00			
rs4570625	G/T : 43	22.98 (2.41)	6.03 (0.69 - 11.38)	Codominante	**0.037**	1446.5
	T/T : 5	9 (5.51)	-7.94 (-21.72 - 5.83)			
PSYRATS AA - Duración						
	G/G : 124	1.62 (0.15)	1.00			
rs4570625	G/T : 43	2.35 (0.26)	0.73 (0.15 - 1.31)	Codominante	**0.031**	684.1
	T/T : 5	1 (0.63)	-0.62 (-2.12 - 0.88)			
PSYRATS AA - Localización						
	G/G : 124	1.47 (0.14)	1.00			
rs4570625	G/T : 43	2.07 (0.26)	0.60 (0.05 - 1.16)	Codominante	**0.042**	668.2
	T/T : 5	0.6 (0.4)	-0.87 (-2.30 - 0.57)			
PSYRATS AA - Intensidad						
	G/G : 124	1.24 (0.11)	1.00			
rs4570625	G/T : 43	1.67 (0.21)	0.43 (-0.01 - 0.87)	Codominante	**0.043**	588.4
	T/T : 5	0.4 (0.24)	-0.84 (-1.98 - 0.30)			
PSYRATS AA – Cantidad de contenido negativo						
	G/G : 124	1.49 (0.15)	1.00			
rs4570625	G/T : 43	2.16 (0.26)	0.67 (0.10 - 1.24)	Codominante	**0.037**	675.5
	T/T : 5	0.8 (0.49)	-0.69 (-2.16 - 0.77)			
PSYRATS AA – Intensidad de la ansiedad						
	G/G : 124	1.33 (0.14)	1.00			
rs4570625	G/T : 43	2 (0.25)	0.67 (0.12 - 1.22)	Codominante	**0.02**	665.8
	T/T : 5	0.4 (0.4)	-0.93 (-2.35 - 0.49)			

Los resultados corresponden a la submuestra de pacientes esquizofrénicos con alucinaciones auditivas.
Los valores de *P* significativos ($P < 0.05$) se indican en negrita.
Abreviaturas: SE, error estándar; IC, intervalo de confianza; AA, alucinaciones auditivas.

El hecho de que el genotipo G/T se asocie con una mayor ansiedad frente a las alucinaciones auditivas es particularmente interesante, puesto que es coherente con trabajos previos (Gutknecht *et al.*, 2007; Brown *et al.*, 2005; Canli *et al.*, 2005a). Sin embargo, los resultados obtenidos en este estudio habría que considerarlos con cautela, puesto que la significación es baja y no resistió la corrección para comparaciones múltiples. Además, hay que considerar que la frecuencia del alelo menor es relativamente baja (0.15 en pacientes), por lo que el número de individuos homozigotos para el alelo menor también es bajo. Sería por tanto necesaria una muestra de mayor tamaño, para poder afirmar si estos resultados están indicando una verdadera asociación o por el contrario son el resultado de una variación aleatoria de las distribuciones genotípicas que han dado como resultado un falso positivo.

Gen *NOS1* (Óxido Nítrico Sintasa 1)

Se realizó un análisis preliminar y exploratorio para conocer si este gen podría tener alguna implicación en la vulnerabilidad a padecer esquizofrenia o en el desarrollo de alguno de sus síntomas. Por ello, un polimorfismo situado en el gen de la Óxido Nítrico Sintasa 1 (*NOS1*), concretamente la VNTR funcional localizada en el exón 1f, fue genotipada en la muestra de origen español.

Desafortunadamente, no se obtuvo ningún resultado significativo en las comparaciones caso-control. Con respecto al análisis de regresión con variables cuantitativas, se detectó una asociación entre este polimorfismo y diferentes ítems de la escala PANSS (tabla C6): tres ítems Generales del componente Depresivo (Kay y Sevy, 1990): Preocupación, Sentimientos de Culpa y Ansiedad; un ítem negativo (Fluidez de la Conversación); y dos ítems positivos (Suspicacia/Perjuicio y Hostilidad). En todos los casos, los pacientes homozigotos para alelos cortos (S/S) puntuaban más alto. A la luz de estos resultados, es muy probable que este polimorfismo funcional tenga un efecto en la severidad de los síntomas de la esquizofrenia. Nuestros resultados son además coherentes con otros trabajos previos, como el trabajo de Reif y colaboradores (2006), en el que encontraron que el genotipo S/S se asociaba con mayores puntuaciones en la subescala positiva de la PANSS y en la escala de depresión de Hamilton. Sin embargo, a pesar de que nuestros resultados son bastante sugerentes, deben ser considerados con cautela, debido al reducido tamaño muestral (N = 102) y al hecho de que no se encontró asociación con aquellas escalas donde el número de pacientes evaluados fue mayor, como la BPRS o la KGV. Sería además de gran interés estudiar otros polimorfismos adicionales localizados en este gen.

Tabla C6. Asociaciones significativas del polimorfismo del gen *NOS1* con diferentes ítems de la PANSS.

Polimorfismo	Distribución genotípica	Puntuación media (SE)	Diferencia (95% IC)	Modelo	*P*	BIC
			PANSS General			
***NOS1* Ex1f-VNTR**	L/L - S/L : 78	30.14 (1.04)	0.00	Recesivo	**0.048**	750.7
	S/S : 24	34.38 (1.74)	4.23 (0.09 - 8.38)			
			PANSS G2 - Ansiedad			
***NOS1* Ex1f-VNTR**	L/L - S/L : 78	2.55 (0.15)	0.00	Recesivo	**0.044**	353.4
	S/S : 24	3.17 (0.26)	0.62 (0.02 - 1.21)			
			PANSS G3 – Sentimientos de culpa			
***NOS1* Ex1f-VNTR**	L/L - S/L : 78	1.54 (0.11)	0.00	Recesivo	**0.0012**	325.9
	S/S : 24	2.42 (0.32)	0.88 (0.36 - 1.39)			
			PANSS G15 - Preocupación			
***NOS1* Ex1f-VNTR**	L/L : 35	2.2 (0.2)	0.46 (0.11 - 0.82)[a]	Aditivo	**0.012**	365.5
	S/L : 43	2.67 (0.23)				
	S/S : 24	3.12 (0.28)				
			PANSS N6 – Falta de espontaneidad y fluidez de la conversación			
***NOS1* Ex1f-VNTR**	L/L : 35	2.09 (0.23)	0.00	Dominante	**0.032**	309.8
	S/L - S/S : 67	1.61 (0.1)	-0.47 (-0.90 - -0.05)			

Tabla C6 (continuación). Asociaciones significativas del polimorfismo del gen *NOS1* con diferentes ítems de la PANSS.

Polimorfismo	Distribución genotípica	Puntuación media (SE)	Diferencia (95% IC)	Modelo	*P*	BIC
PANSS P6 – Suspicacia / Persecución						
***NOS1* Ex1f-VNTR**	L/L - S/L : 78	2.08 (0.15)	0.00	Recesivo	**0.0018**	378.4
	S/S : 24	3.17 (0.37)	1.09 (0.42 - 1.76)			
PANSS P7 - Hostilidad						
***NOS1* Ex1f-VNTR**	L/L : 35	1.51 (0.16)	0.30 (0.03 - 0.56)[a]	Aditivo	**0.029**	304.1
	S/L : 43	1.7 (0.15)				
	S/S : 24	2.12 (0.23)				

Los valores de *P* significativos ($P < 0.05$) se indican en negrita.
a. El valor indicado se corresponde con la diferencia que se le atribuye a cada alelo según un modelo aditivo.
Abreviaturas: SE, error estándar; IC, intervalo de confianza.

Gen *STMN1* (Stathmin)

Teniendo en cuenta los valores de desequilibrio de ligamiento de la región genómica donde se sitúa el gen *STMN1*, se seleccionaron dos *tag* SNPs (rs182455 y rs12037513), que fueron genotipados en la muestra española.

El resultado más interesante del estudio de asociación caso-control es la asociación entre el SPN rs182455 (localizado en la supuesta región 5' reguladora del gen) y el riesgo de esquizofrenia (tabla C7) y esquizofrenia con alucinaciones (datos no mostrados). De acuerdo a la información presentada, los individuos homozigotos T/T eran menos frecuentes en el grupo de pacientes diagnosticados con esquizofrenia (según los criterios del DSM-IV) que en el grupo control. Sin embargo, estas asociaciones no resistieron la corrección de Bonferroni secuencial (Rice, 1989). Estudios en otras muestras caso-control serían necesarios para confirmar nuestros resultados.

Tabla C7. Comparación de las distribuciones genotípicas de los polimorfismos del gen *STMN1* en pacientes esquizofrénicos y controles de la muestra española.

SNP	Genotipo	Frecuencia controles	Frecuencia pacientes SCZ	OR (95% IC)	P^{b}	AIC	Modelo
rs12037513	T/T	149 (47.5%)	107 (52.2%)	0.86 (0.65-1.13)[a]	0.28 (0.28)	699.3	Aditivo
	T/C	136 (43.3%)	82 (40%)				
	C/C	29 (9.2%)	16 (7.8%)				
rs182455	C/C - T/C	285 (77.5%)	185 (84.9%)	1.00	**0.027** (0.54)	772.7	Recesivo
	T/T	83 (22.6%)	33 (15.1%)	0.61 (0.39-0.95)			

Los valores de *P* significativos ($P < 0.05$) se indican en negrita.
a. El valor indicado se corresponde con la diferencia que se le atribuye a cada alelo según un modelo aditivo.
b. El valor de *P* corregido se indica entre paréntesis.
Abbreviations: SCZ, esquizofrenia; OR, *Odds Ratio*; IC, intervalo de confianza.

Asimismo, el SNP rs182455 se ha asociado con diferentes parámetros que evalúan los síntomas negativos de la esquizofrenia (tabla C8): Afecto Aplanado e Incoherencia del Lenguaje (escala KGV); Evitación Social Activa y Fluidez de la Conversación (PANSS); y finalmente, la puntuación negativa total de la PANSS. En todos los casos, el genotipo C/C se correlacionaba con mayores puntuaciones en los ítems enumerados. Asimismo, este mismo SNP se asoció con el ítem positivo de la PANSS Suspicacia/Persecución, resistiendo la corrección de Bonferroni Secuencial. Con respecto a la escala PSYRATS de alucinaciones auditivas, el SNP rs182455 se asoció ligeramente (sin resistir la corrección para comparaciones múltiples) con dos parámetros: Creencias sobre el Origen de las Voces e Intensidad de la Ansiedad.

Con respecto al otro SNP, rs12037513, también se ha asociado con ciertos parámetros de la escala PANSS (tabla C8): Preocupación Somática (un ítem relacionado con la depresión) y Afecto Aplanado, de la subescala negativa.

Todos nuestros hallazgos sugieren que pequeñas variaciones en el gen *STMN1* pueden estar implicadas en la severidad de los síntomas negativos. Sin embargo, por el momento se desconoce si los dos polimorfismos analizados (u otros en alto desequilibrio de ligamiento con éstos) tienen alguna implicación funcional. Además, las asociaciones con parámetros emocionales también indican que este gen podría tener también un papel importante en la disfunción emocional que sufren los pacientes esquizofrénicos, así como en la respuesta emocional a las alucinaciones auditivas.

Tabla C8. Resultados significativos del análisis de asociación entre los polimorfismos del gen *STMN1* y diferentes escalas clínicas.

SNP	Distribución genotípica	Puntuación media (SE)	Diferencia (95% CI)	Modelo	P^a	BIC
KGV - Afecto aplanado						
rs182455	C/C - T/C : 222	1.29 (0.07)	0.00	Recesivo	**0.038** (0.076)	813.1
	T/T : 45	0.93 (0.15)	-0.37 (-0.71 - -0.02)			
KGV – Incoherencia del lenguaje						
rs182455	C/C : 85	0.87 (0.13)	0.00	Dominante	**0.027** (0.054)	799.6
	T/C - T/T : 182	0.57 (0.07)	-0.31 (-0.58 - -0.04)			
PANSS G1 – Preocupación somática						
rs12037513	T/T : 63	1.49 (0.12)	0.00	Dominante	**0.029** (0.058)	345.8
	T/C - C/C : 45	1.98 (0.19)	0.48 (0.06 - 0.91)			
PANSS G16 – Evitación social activa						
rs182455	C/C - T/C : 106	1.96 (0.15)	0.00	Recesivo	**0.027** (0.054)	471.4
	T/T : 23	1.26 (0.16)	-0.73 (-1.36 - -0.09)			
PANSS Negativa						
rs182455	C/C - T/C : 106	15.57 (0.61)	0.00	Recesivo	**0.05** (0.1)	840.7
	T/T : 23	12.96 (0.94)	-2.70 (-5.36 - -0.03)			
PANSS N1 – Afecto aplanado						
rs12037513	T/T : 63	2.43 (0.16)	0.00	Dominante	**0.015 (0.03)**	352
	T/C - C/C : 45	1.91 (0.15)	-0.55 (-0.99 - -0.11)			

Tabla C8 (continuación). Resultados significativos del análisis de asociación entre los polimorfismos del gen *STMN1* y diferentes escalas clínicas.

SNP	Distribución genotípica	Puntuación media (SE)	Diferencia (95% CI)	Modelo	*P* [a]	BIC
PANSS N6 – Falta de espontaneidad y fluidez en la conversación						
rs182455	C/C - T/C : 106	1.83 (0.11)	0.00	Recesivo	**0.029** (0.058)	395.7
	T/T : 23	1.3 (0.12)	-0.54 (-1.01 - -0.06)			
PANSS P3 – Comportamiento alucinatorio						
rs12037513	T/T - T/C : 106	2.32 (0.2)	0.00	Recesivo	**0.023** **(0.046)**	480.7
	C/C : 2	6 (0)	3.45 (0.52 - 6.38)			
PANSS P6 - Suspicacia / Persecución						
rs182455	C/C : 38	1.76 (0.17)	0.00	Codominante	**0.0013** **(0.0026)**	465.6
	T/C : 68	2.68 (0.19)	0.91 (0.37 - 1.45)			
	T/T : 23	1.83 (0.32)	0.03 (-0.68 - 0.73)			
PSYRATS AA – Creencias sobre el origen						
rs182455	C/C : 70	1.43 (0.2)	0.00	Dominante	**0.041** (0.082)	832
	T/C - T/T : 138	1.94 (0.15)	0.52 (0.02 - 1.01)			
PSYRATS AH – Intensidad de la ansiedad						
rs182455	C/C : 70	1.07 (0.17)	0.00	Dominante	**0.044** (0.088)	793.6
	T/C - T/T : 138	1.54 (0.14)	0.46 (0.02 - 0.91)			

Los valores de *P* significativos (P < 0.05) se indican en negrita.
a. El valor de *P* corregido se indica entre paréntesis.
Abreviaturas: SE, error stándar; IC, Intervalo de confianza; AA, alucinaciones auditivas.

Se sabe que el gen *STMN1* se expresa en la amígdala basolateral, así como en otras estructuras talámicas y corticales también asociadas con el procesado de estímulos condicionales e incondicionales (Shumyatsky *et al.*, 2005). Además, la inactivación de la expresión de este gen en el ratón altera patrones de comportamiento relacionados con el miedo y la evaluación de situaciones amenazadoras (Shumyatsky *et al.*, 2005), lo que a su vez repercute en determinados comportamientos sociales, como el cuidado de las crías y las interacciones sociales (Martel *et al.*, 2008). Puesto que la esquizofrenia es una enfermedad que afecta la emocionalidad y las interacciones sociales del individuo, podemos hipotetizar que este gen tienen un papel importante en la etiopatogénesis de la enfermedad. Sin embargo, serían necesarios estudios complementarios para corroborar esta hipótesis.

Efecto de las interacciones gen x gen en el procesado emocional

El término epistasia hace referencia a la interacción no aditiva entre diferentes loci que afecta a un determinado fenotipo. Como resultado de nuestros propios hallazgos, así como por estudios previos que relacionaban ciertos genes con desórdenes emocionales, se investigaron en este trabajo el efecto de las epistasias gen x gen en diferentes ítems emocionales. Para este fin, seleccionamos una serie de polimorfismos localizados en genes implicados en el procesado de emociones: 5-HTTLPR (gen *SLC6A4*), rs6313 (gen *HTR2A*), rs4570625 (gen *TPH2*), ex1f VNTR (gen *NOS1*) y rs182455 (gen *STMN1*). Posteriormente, se estudió el posible efecto de

interacciones entre estos polimorfismos sobre diferentes variables de tipo emocional. Como se esperaba, se encontraron algunas interacciones significativas (tabla C9):

- Se encontró un efecto de la interacción *SLC6A4*x*HTR2* sobre las variables Intensidad de la Ansiedad, Frecuencia de la Ansiedad y Repercusión (escala PSYRATS de alucinaciones auditivas), así como sobre los ítems "Ansiedad" de las escalas BPRS y KGV.
- La interacción *TPH2*x*HTR2A* afectó a su vez a los siguientes parámetros: Repercusión y Frecuencia de la Ansiedad (subescala PSYRATS de delirios) y el ítem Depresión de la PANSS.

Entre estos hallazgos, los más significativos son el efecto de la interacción *SLC6A4* x *HTR2A* sobre la intensidad y la frecuencia de la ansiedad frente a las voces, ya que resistieron la corrección de Bonferroni secuencial. Sin embargo, no se encontró ninguna interacción significativa que implicara a los genes *STMN1* y *NOS1*.

De acuerdo con nuestros resultados, es probable que haya múltiples polimorfismos genéticos que estén actuando en la misma dirección o en direcciones opuestas, llevando a cabo el modelado de ciertos fenotipos asociados a comportamientos afectivos. Además, hay un importante número de evidencias previas que sugieren que esto es realmente así. Por ejemplo, se han descrito interacciones entre los genes *SLC6A4*, *COMT* y *DRD4* que influyen sobre el rasgo de personalidad Búsqueda de Novedad (Benjamin *et al.*, 2000; Strobel *et al.*, 2003), así como interacciones entre los genes *SLC6A4* y *TPH2* que modulan la respuesta a estímulos visuales emocionales (Herrmann *et al.*, 2007). Asimismo, se ha descrito un efecto aditivo de los genes *COMT* y *SLC6A4* sobre la activación de áreas límbicas en respuesta a estímulos negativos emocionales (Smolka *et al.*, 2007). También hay resultados en modelos de ratón que apoyan la existencia de relaciones epistáticas entre diferentes genes del sistema serotoninérgico (Murphy *et al.*, 2008).

Tabla C9. Resultados significativos del análisis de interacción entre diferentes polimorfismos relacionados con el procesado emocional.

Polimorfismo 1	Polimorfismo 2	Variable de estudio	OR o β[a]	Stadístico	P[b]
rs6313 *(HTR2A)*	5-HTTLPR (*SLC6A4*)	PSYRATS AA – Intensidad Ansiedad	0.6877	9.921	**0.001634 (0.0098)**
rs6313 *(HTR2A)*	5-HTTLPR (*SLC6A4*)	PSYRATS AA –Frecuencia Ansiedad	0.6726	9.058	**0.002616 (0.0156)**
rs6313 *(HTR2A)*	5-HTTLPR (*SLC6A4*)	PSYRATS AA - Repercusión	0.4607	4.94	**0.02625** (0.1575)
rs6313 *(HTR2A)*	5-HTTLPR (*SLC6A4*)	BPRS - Ansiedad	0.4756	4.184	**0.0408** (0.2448)
rs6313 *(HTR2A)*	5-HTTLPR (*SLC6A4*)	KGV - Ansiedad	0.2709	4.127	**0.04221** (0.2533)
rs4570625 *(TPH2)*	rs6313 *(HTR2A)*	PSYRATS delirios - Repercusión	0.6679	5.62	**0.01775** (0.1065)
rs4570625 *(TPH2)*	rs6313 *(HTR2A)*	PSYRATS delirios – Frecuencia ansiedad	0.6692	5.462	**0.01944** (0.1166)
rs4570625 *(TPH2)*	rs6313 *(HTR2A)*	PANSS G6 - Depresión	0.6451	3.86	**0.04945** (0.2967)

Los valores de *P* significativos ($P < 0.05$) se indican en negrita.
a. Este valor se refiere a los valores β que dan una idea sobre la magnitud de cada epistasia gen x gen sobre una determinada variable cuantitativa.
b. El valor de *P* corregido se indica entre paréntesis.
Abbreviatura: OR, *Odds ratio*.

El efecto de un gen de selección positiva, el *ASPM*, sobre la esquizofrenia y el deterioro cognitivo.

Como una parte importante del presente estudio, se ha llevado a cabo el análisis del impacto de la variación en el gen *ASPM* sobre el riesgo de padecer esquizofrenia. Asimismo, también se abordó el estudio de la influencia de este gen sobre ciertos fenotipos relacionados con la cognición y la estructura cerebral, los cuales se sabe que están alterados en pacientes esquizofrénicos y sus hermanos sanos (Cannon *et al.*, 1998; Kremen *et al.*, 1994; Egan et al 2001). Con este propósito, se estudiaron 8 SNPs en la muestra española y 6 SNPs en la muestra americana (5 de ellos comunes a ambas muestras: rs3762271, rs12138336, rs41310927, rs10922163 y rs6677082). Los resultados obtenidos son bastante coherentes.

Con respecto a la muestra española, dos SNPs en alto desequilibrio de ligamiento entre ellos (rs6700180 y rs10922163) se asociaron con el riesgo de psicosis (tabla C10) y psicosis con alucinaciones auditivas (datos no mostrados). Los resultados permanecieron significativos tras aplicar la corrección de Bonferroni secuencial. Además, se detectaron varios haplotipos protectores que también incluían el SNP rs10922163 (datos no mostrados).

Además, varios SNPs se asociaron con diferentes variables, como la edad de inicio de la enfermedad, y diferentes parámetros de las escalas BPRS, PANSS y PSYRATS. Además, como se observa en la tabla C11, el gran número de asociaciones con síntomas negativos sugiere que el gen *ASPM* podría estar implicado en la patofisiología de la dimensión negativa de la esquizofrenia. Sin embargo, tampoco se puede olvidar el hecho de que los polimorfismos rs6700189 y 10922163 están fuera del equilibrio de Hardy-Weinberg en la muestra de sujetos control. Aunque esta desviación es sutil, podría indicar un error de genotipado (poco probable en este caso) o bien podría ser consecuencia de factores que escapan de nuestro control, como la existencia de procesos de selección. En este sentido, hay evidencias que sugieren que el gen *ASPM* está en la actualidad bajo un proceso de selección positiva (Mekel-Bobrov *et al.*, 2005).

Tabla C10. Comparación de las frecuencias genotípicas de los polimorfismos del gen *ASPM* entre pacientes psicóticos y controles sanos de la muestra española.

Marcador	Genotipo	Frecuencia controles	Frecuencia pacientes	OR (95% IC)	*P* [b]	AIC	Modelo
rs6700180	C/C	102 (32%)	62 (22.9%)	1.00	**0.0091** (0.0637)	810.6	Codominante
	T/C	139 (43.6%)	151 (55.7%)	1.79 (1.21-2.64)			
	T/T	78 (24.4%)	58 (21.4%)	1.22 (0.77-1.95)			
rs3762271	C/C - A/C	275 (80.7%)	253 (84.9%)	1.00	0.16 (0.84)	884.9	Recesivo
	A/A	66 (19.4%)	45 (15.1%)	0.74 (0.49-1.12)			
rs12138336	G/G	298 (87.4%)	248 (83.2%)	1.00	0.14 (0.84)	884.7	Dominante
	C/G - C/C	43 (12.6%)	50 (16.8%)	1.40 (0.90-2.17)			
rs41310927	A/A - A/G	302 (81.8%)	253 (84.9%)	1.00	0.29 (1)	920	Recesivo
	G/G	67 (18.2%)	45 (15.1%)	0.80 (0.53-1.21)			
rs10922163	A/A	104 (33%)	61 (22.7%)	1.00	**0.005 (0.04)**	801.4	Codominante
	G/A	137 (43.5%)	151 (56.1%)	1.88 (1.27-2.78)			
	G/G	74 (23.5%)	57 (21.2%)	1.31 (0.82-2.10)			

Tabla C10 (continuación). Comparación de las frecuencias genotípicas de los polimorfismos del gen *ASPM* entre pacientes psicóticos y controles sanos de la muestra española.

Marcador	Genotipo	Frecuencia controles	Frecuencia pacientes	OR (95% IC)	P^b	AIC	Modelo
rs4915337	A/A	277 (81.2%)	235 (78.9%)				
	A/T	62 (18.2%)	61 (20.5%)	1.15 (0.79-1.67)[a]	0.46 (1)	886.4	Aditivo
	T/T	2 (0.6%)	2 (0.7%)				
	A/A	277 (81.2%)	235 (78.9%)	1.00	0.45 (1)	886.4	Dominante
	A/T - T/T	64 (18.8%)	63 (21.1%)	1.16 (0.79-1.71)			
rs6677082	T/T	248 (78.7%)	214 (79.5%)				
	T/C	66 (20.9%)	54 (20.1%)	0.96 (0.65-1.41)[a]	0.82 (1)	809.9	Aditivo
	C/C	1 (0.3%)	1 (0.4%)				
	T/T	248 (78.7%)	214 (79.5%)	1.00	0.81 (1)	809.9	Dominante
	T/C - C/C	67 (21.3%)	55 (20.5%)	0.95 (0.64-1.42)			
rs9726778	G/G	269 (78.9%)	229 (76.8%)				
	C/G	70 (20.5%)	66 (22.1%)	1.14 (0.80-1.62)[a]	0.48 (1)	886.4	Aditivo
	C/C	2 (0.6%)	3 (1%)				

Los valores de *P* significativos (P < 0.05) se indican en negrita.
a. El valor indicado se corresponde con la diferencia que se le atribuye a cada alelo según un modelo aditivo.
b. El valor de *P* corregido se indica entre paréntesis.
Abreviaturas: OR, *Odds Ratio*; IC, intervalo de confianza.

Tabla C11. Resultados significativos del estudio de asociación entre los SNPs del gen *ASPM* y diferentes parámetros clínicos que evalúan los síntomas negativos.

Polimorfismo	Distribución genotípica	Puntuación media (SE)	Diferencia (95% IC)	Modelo	P^a	BIC
KGV – Afecto aplanado						
rs6700180	C/C - T/C : 189	1.38 (0.08)	0.00	Recessive	**0.031** (0.248)	739.2
	T/T : 51	1.04 (0.14)	-0.38 (-0.71 - -0.04)			
rs10922163	A/A - G/A : 189	1.36 (0.08)	0.00	Recessive	**0.049** (0.343)	721.3
	G/G : 50	1.04 (0.14)	-0.34 (-0.68 - -0.00)			
PANSS Negativa						
rs3762271	C/C : 49	13.84 (0.73)				
	A/C : 60	15.58 (0.81)	1.63 (0.16 - 3.09)[a]	Additive	**0.031** (0.217)	839.9
	A/A : 20	16.75 (1.59)				
rs41310927	A/A : 49	13.69 (0.74)				
	A/G : 60	15.58 (0.81)	1.77 (0.31 - 3.22)[a]	Additive	**0.019** (0.152)	839
	G/G : 20	17.1 (1.54)				
PANSS N1 – Afecto aplanado						
rs6700180	C/C - T/C: 87	2.36 (0.13)	0.00	Recessive	**0.0053** **(0.0424)**	354.6
	T/T : 23	1.7 (0.16)	-0.76 (-1.28 - -0.24)			
rs41310927	A/A : 49	1.88 (0.14)				
	A/G : 60	2.28 (0.15)	0.32 (0.04 - 0.59)[a]	Additive	**0.026** (0.156)	410.1
	G/G : 20	2.45 (0.31)				
rs10922163	A/A - G/A:84	2.37 (0.14)	0.00	Recessive	**0.007** **(0.049)**	350.6
	G/G : 24	1.67 (0.16)	-0.73 (-1.24 - -0.21)			

Los valores de *P* significativos (P < 0.05) se indican en negrita.
a. El valor indicado se corresponde con la diferencia que se le atribuye a cada alelo según un modelo aditivo.
b. El valor de *P* corregido se indica entre paréntesis.
Abreviaturas: OR, *Odds Ratio*; IC, intervalo de confianza.

Con respecto a la muestra Americana, el alelo T del SNP rs677082 se asoció con la esquizofrenia tanto en el estudio caso-control (tabla C12) como en el basado en familias (tabla C13). Además, los portadores del alelo G del SNP rs10922163 eran más frecuentes en la muestra de afectados que en la de controles (tabla C12), y también se observó una tendencia a un exceso de transmisión de ese mismo alelo de los padres a los hijos afectados (tabla C13). También se encontraron dos haplotipos de riesgo que incluían los polimorfismos rs6677082 y rs10922163 (datos no mostrados). Por último, el SNP rs12116571 se asoció también con la esquizofrenia mediante la aproximación caso-control (tabla C12), aunque este dato hay que considerarlo con cautela, puesto que este SNP no estaba en equilibrio de Hardy-Weinberg en la muestra de afectados. Este dato podría estar sugiriendo que la falta de equilibrio es una manifestación de la asociación de este SNP con la enfermedad.

Tabla C12. Análisis de asociación caso-control de los SNPs del gen *ASPM* en la muestra Americana.

SNP	Genotipo	Frecuencia controles	Frecuencia pacientes	*P* test exacto de Fisher[a,b]	OR (95% IC)	*P* Regresión logística[b]
rs3762271	C/C	149 (34.57%)	151 (37.94%)	0.580 (1)	0.927 (0.672-1.279)[c]	0.646 (1)[c]
	C/A	209 (48.49%)	185 (46.48%)		0.831 (0.541 - 1.279)[d]	0.401 (0.813)[d]
	A/A	73 (16.94%)	62 (15.58%)			
rs12138336	G/G	371 (86.08%)	324 (83.08%)	0.417 (1)	1.263 (0.838 - 1.901)[c]	0.264 (1)[c]
	C/G	58 (13.46%)	65 (16.67%)		0.659 (0.0386-11.254)[d]	0.774 (0.813)[d]
	C/C	2 (0.46%)	1 (0.26%)			
rs41310927	A/A	143 (33.18%)	148 (37.47%)	0.397 (1)	0.910 (0.659 - 1.257)[c]	0.568 (1)[c]
	A/G	211 (48.96%)	185 (46.84%)		0.786 (0.512 - 1.206)[d]	0.271 (0.813)[d]
	G/G	77 (17.87%)	62 (15.70%)			
rs10922163	A/A	119 (27.74%)	89 (21.92%)	0.135 (0.54)	1.441 (1.012 - 2.049)[c]	**0.042** (0.21)[c]
	A/G	218 (50.82%)	217 (53.45%)		1.481 (0.975 - 2.248)[d]	0.065 (0.325)[d]
	G/G	92 (21.45%)	100 (24.63)			
rs12116571	G/G	321 (75.65%)	320 (81.42%)	**0.001 (0.006)**	0.604 (0.416 - 0.877)[c]	**0.008 (0.048)**[c]
	G/A	105 (24.42%)	61 (15.52%)		2.508 (0.769 - 8.179)[d]	0.127 (0.508)[d]
	A/A	4 (0.93%)	12 (3.05%)			
rs6677082	T/T	353 (80.78%)	329 (83.29%)	**0.03** (0.15)	0.829 (0.536 - 1.22)[c]	0.344 (1)[c]
	T/C	77 (17.62%)	66 (16.71%)			
	C/C	7 (1.60%)	0 (0.00%)			

Los valores de *P* significativos (P < 0.05) se indican en negrita.
a. Este test compara las frecuencias genotípicas de pacientes y controles asumiendo un modelo codominante.
b. El valor de *P* corregido se indica entre paréntesis.
c. Resultado obtenido cuando los homozigotos para el alelo más frecuente se comparan con los heterozigotos.
d. Resultado obtenido cuando los homozigotos para el alelo más frecuente se comparan con los homozigotos para el alelo menor.
Abreviaturas: OR, *Odds Ratio*.

Tabla C13. Resultados del gen *ASPM* para el test de desequilibrio de transmisión basado en familias.

Polimorfismo	Alelo transmitido en exceso	Nº de familias[a]	Estadístico[b]	Estadístico esperado[c]	Z	P^d
rs3762271	C	144	179.000	170.281	1.206	0.21 (0.8)
rs12138336	C	64	37.000	34.000	0.688	0.53 (1)
rs41310927	A	150	187.000	176.348	1.444	0.20 (0.8)
rs10922163	C	157	173.000	157.898	2.017	0.071 (0.355)
rs12116571	G	76	108.000	106.550	0.280	0.75 (1)
rs6677082	A	71	118.000	108.667	2.132	**0.038** (0.228)

Los valores de *P* significativos ($P < 0.05$) se indican en negrita.
a. Número de familias informativas.
b. Estadístico del test proporcionado por el programa FBAT para el número observado de alelos transmitidos.
c. Valor esperado del estadístico si se cumple la hipótesis nula de no asociación.
d. El valor de *P* corregido se indica entre paréntesis.

Hay algunas coincidencias especialmente alentadoras entre la muestra española y la americana, ya que en ambos casos el SNP rs10922163 se ha asociado con la enfermedad; además, en ambos casos es el alelo G el más frecuente en el grupo afectado. Esta asociación también se detectó al realizar un análisis de asociación caso-control usando una muestra en la que se combinaron los sujetos españoles y americanos (datos no mostrados). Sin embargo también hay datos no coincidentes. Como se ha comentado anteriormente, la falta de replicación estricta es algo habitual en los estudios de asociación, en contraposición a otras enfermedades como la diabetes tipo 2 (Florez *et al.*, 2005) o la degeneración macular asociada a la edad (Edwards *et al.*, 2005; Haines *et al.*, 2005; Hageman *et al.*, 2005). Este hecho podría tener diversas explicaciones, como la posibilidad de falsos positivos, o diferencias en la estructura genética de diferentes poblaciones o subpoblaciones que podrían a su vez influenciar cuáles son los alelos y haplotipos de riesgo en cada una de estas poblaciones.

Sin embargo, ya ha sido anteriormente comentado que la asociación con fenotipos de tipo clínico puede ser poco potente para identificar los genes causantes de enfermedades complejas como la esquizofrenia, debido al pequeño efecto que se le presupone a cada gen (Risch, 1990; Riley y Mc Guffin, 2000; Weiss y Terwilliger, 2000). Por este motivo, consideramos interesante estudiar el efecto de la variación en el gen *ASPM* sobre otros fenotipos intermediarios relacionados con la cognición, usando para ello la información disponible en la muestra americana de la *Clinical Brain Disorders Branch*. Con este enfoque, encontramos que 3 SNPs (rs3762271, rs12138336 y rs41310927) influenciaban las puntuaciones de diferentes medidas cognitivas directamente relacionadas con la memoria de trabajo (tabla C14): *Wisconsin Card Sorting Test* (WCST) y *N Back* en controles, y Repetición de Números (*Digit Span*) en pacientes. Los tres SNPs asociados con parámetros cognitivos están situados en el exón 18, que codifica para gran parte del dominio IQ, una de las regiones más importantes de la proteína y que además ha sufrido la mayor tasa de evolución (Kouprina *et al.*, 2004), por lo que se ha especulado sobre su posible papel durante la división celular en el desarrollo del Sistema Nervioso Central (Kouprina *et al.*, 2005). Además, rs3762271 y rs41310927 (ambos en casi completo desequilibrio de ligamiento) han estado sujetos a un

reciente proceso de selección positiva (Mekel-Bobrov *et al.*, 2005), lo que sugiere que estos dos cambios podrían tener implicaciones funcionales que explicarían nuestros resultados.

Tabla C14. Efecto de tres SNPs del gen *ASPM* en diferentes medidas cognitivas.

Polimorfismo	Contraste	Coef. regresión	*P*[a]	Submuestra
		N Back		
rs12138336	C/C vs C/A	-1.748	**0.011** (0.066)	Controles
		WCST		
rs3762271	C/C vs C/A	-1.609	**0.017** (0.085)	Controles
rs41310927	A/A vs A/G	-1.834	**0.008 (0.048)**	Controles
		Repetición de números (*Digit Span*)		
rs12138336	G/G vs G/A	-1.616	**0.023** (0.138)	Pacientes

Los valores de *P* significativos ($P < 0.05$) se indican en negrita.
a. El valor de *P* corregido se indica entre paréntesis.
Abreviaturas: WCST, *Wisconsin Card Sorting Test*; Coef, coeficiente del análisis de regresión lineal; vs, versus.

Asimismo, se evaluó el efecto de diferentes polimorfismos del gen *ASPM* sobre el volumen cerebral y sobre la activación de ciertas áreas cerebrales durante la realización de la tarea *N Back*. En este caso, pudimos asociar 3 SNPs con cambios en el volumen de los ganglios basales, el córtex prefrontal y el córtex cingulado subgenual en controles, mientras que los pacientes presentaban diferencias según el genotipo en el córtex prefrontal y el cerebelo (en las figuras C4 y C5 se muestran los resultados para el SNP rs3762261). Además el estudio de resonancia magnética funcional permitió detectar que el SNP rs10922163 tenía un efecto sobre la activación del córtex anterior cingulado en controles, durante la realización de la tarea *N Back* (figura C6).

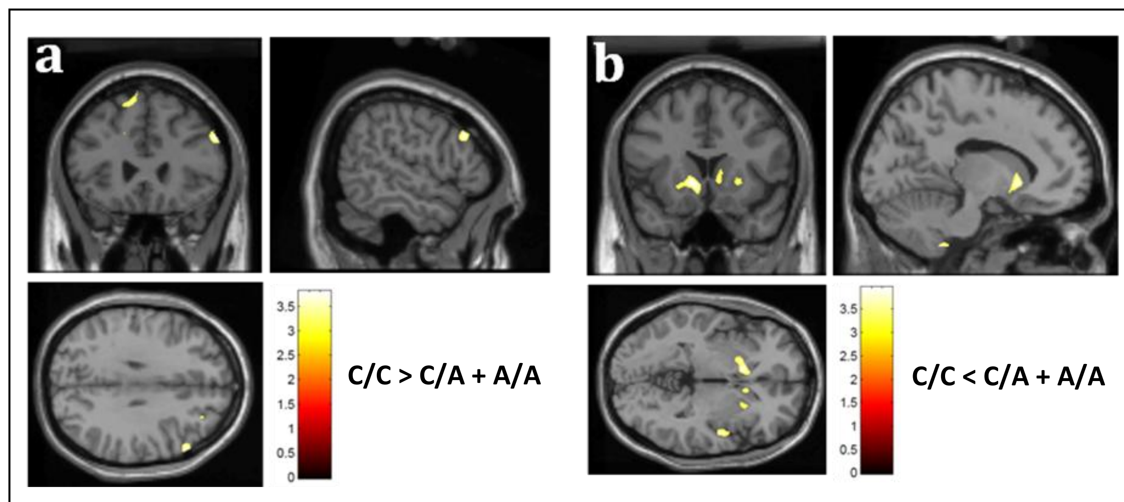


Figura C4. Correlación entre los datos estructurales de morfometría basada en vóxels (VBM) y el SNP rs3762271 en sujetos sanos. Los portadores del alelo A mostraban (**a**) un volumen reducido en el córtex prefrontal (área de Brodmann 9) comparados con los homozigotos C/C, así como (**b**) un incremento de volumen en el córtex cingulado subgenual (área de Brodmann 25).
Umbral del mapa: $P < 0.001$ no corregido.

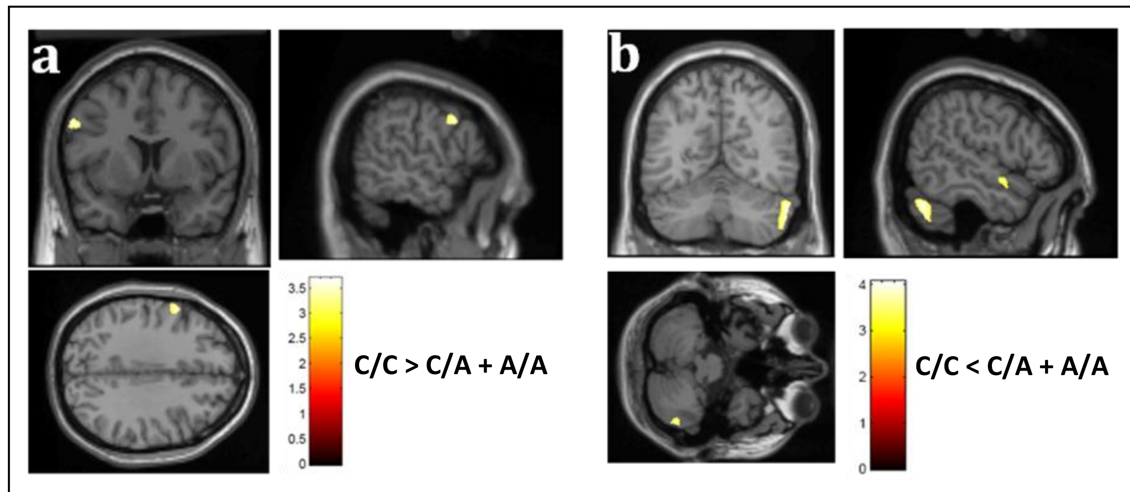


Figura C5. Correlación entre los datos estructurales de morfometría basada en vóxels (VBM) y el SNP rs3762271 en pacientes esquizofrénicos. Los portadores del alelo A mostraban (**a**) un volumen reducido en el córtex prefrontal (área de Brodmann 9) comparados con los homozigotos C/C, así como (**b**) un incremento de volumen en el cerebelo.
Umbral del mapa: $P < 0.001$ no corregido.

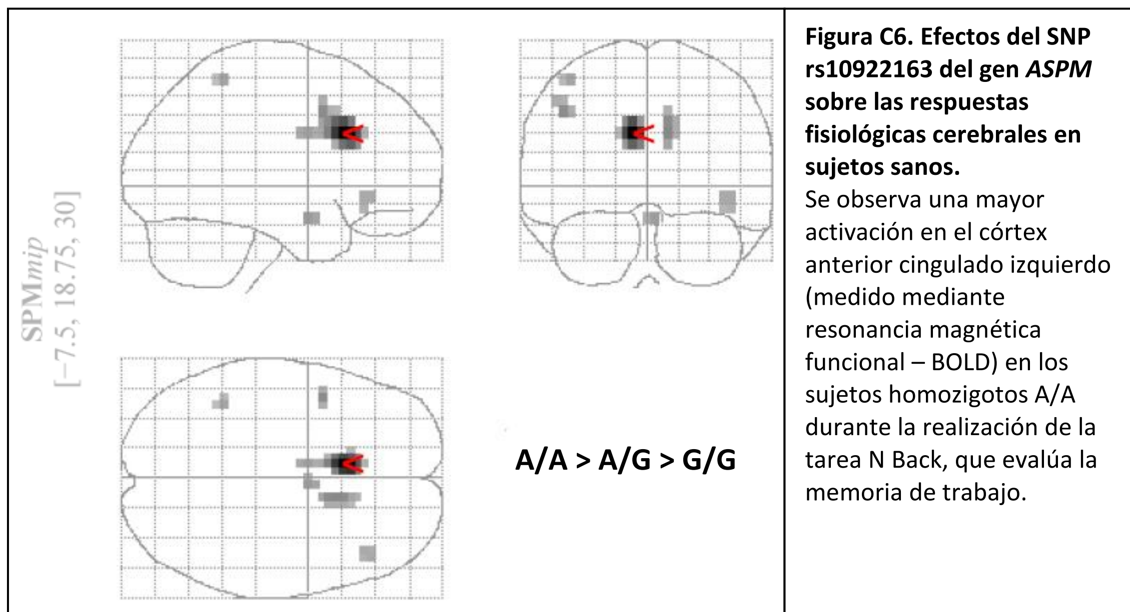


Figura C6. Efectos del SNP rs10922163 del gen *ASPM* sobre las respuestas fisiológicas cerebrales en sujetos sanos.
Se observa una mayor activación en el córtex anterior cingulado izquierdo (medido mediante resonancia magnética funcional – BOLD) en los sujetos homozigotos A/A durante la realización de la tarea N Back, que evalúa la memoria de trabajo.

Estos resultados son muy interesantes. Se sabe que el córtex prefrontal, y especialmente el área dorsolateral, está conectado con diferentes áreas corticales y subcorticales, incluyendo el estriado (caudado y putamen) (Alexander *et al.*, 1986). Esta red es crucial para el buen funcionamiento de la función cognitiva, especialmente de la memoria de trabajo (Alexander *et al.*, 1986; Dunnett *et al.*, 2005). Además se ha visto también que el córtex cingulado, que está profusamente conectado con el córtex prefrontal, también juega un papel clave en numerosas funciones cerebrales, entre ellas la memoria de trabajo (Callicott *et al.*, 1999; Paus, 2001). Por último, no se debe olvidar que se ha postulado que un elemento clave en la fisiopatogenia de la esquizofrenia es la disfunción del córtex prefrontal dorsolateral (Weinberger *et al.*, 2001).

Nuestros resultados apoyan la correlación entre variaciones sutiles del gen *ASPM* y alteraciones morfológicas y funcionales en aquellas áreas directamente relacionadas con la memoria de trabajo, tanto en sujetos sanos como en pacientes esquizofrénicos. Además, el efecto de este gen sobre el volumen del cerebelo de los pacientes esquizofrénicos sería coherente con las hipótesis de algunos autores, los cuales han postulado el papel importante del cerebelo en el fenotipo observado en los pacientes esquizofrénicos (Andreasen *et al.*, 1998). En resumen, nuestros resultados sugieren un efecto del gen *ASPM* sobre la vulnerabilidad a padecer esquizofrenia. Además los datos de neuroimagen podrían indicar que este gen tiene un papel en el desarrollo de estructuras corticales, subcorticales y cerebelares. Asimismo, las diferencias observadas entre pacientes y controles sugieren la existencia de otros factores de susceptibilidad genéticos o ambientales que estarían modulando la manifestación del fenotipo patológico en los pacientes.

Gen *PDE4D*

El gen *PDE4D* se presenta como un interesante candidato en genética psiquiátrica. En primer lugar, se sabe que interacciona con DISC1, otra proteína fuertemente relacionada con la fisiopatología de la esquizofrenia (Murdoch *et al.*, 2007). Además, todas las proteínas de la familia PDE4 pueden ser inhibidas por rolipram, una sustancia con propiedades antidepresivas. Finalmente, la isoforma más pequeña codificada por el gen *PDE4D* (PDE4D6, de acuerdo a la nomenclatura de Wang *et al.*, 2003) ha sido asociada con una condición psiquiátrica, el Trastorno por Déficit de Atención e Hiperactividad (TDAH) en un estudio de búsqueda de variaciones en el número de copias dirigido por el profesor K.P. Lesch en la Universidad de Würzburg (Alemania). Todos estos hallazgos nos llevaron a estudiar el efecto de la variación del gen *PDE4D* sobre el riesgo de esquizofrenia. Para ello, nos centramos en la región del gen que codifica para la isoforma PDE4D6. Se usaron dos muestras caso-control, la muestra de Valencia (España) y la muestra de Alemania. Asimismo, se analizaron 9 SNPs en la muestra española y diez marcadores en los individuos de origen alemán (los 10 SNPs de la muestra española más un décimo SNP, rs10056492).

Los resultados procedentes de los estudios de asociación caso-control son principalmente negativos. Con respecto a la muestra española, únicamente el SNP rs12656462 se asoció con la esquizofrenia diagnosticada según criterios DSM-IV (tablas C15 y C16), aunque al incluir en el análisis todos los pacientes psicóticos, el resultado dejaba de ser significativo (datos no mostrados).

Tabla C15. Resultados significativos del análisis de asociación caso-control para comparar las frecuencias alélicas de los SNPs del gen *PDE4D* en pacientes esquizofrénicos y controles de la muestra española.

Polimorfismo	Alelo	Frecuencia esquizofrénicos	Frecuencia controles	OR (95% IC)	χ^2	P[a]
rs12656462	T	0.918	0.9531	1	4.477	**0.03285** (0.309)
	A	0.08197	0.04687	1.815 (1.043 – 3.161)		

Los valores de *P* significativos (P < 0.05) se indican en negrita.
a. El valor de *P* corregido se indica entre paréntesis.
Abreviaturas: OR, *Odds Ratio*; IC, intervalos de confianza.

Tabla C16. Resultados significativos del análisis de asociación caso-control para comparar las frecuencias genotípicas de los SNPs del gen *PDE4D* en pacientes esquizofrénicos y controles de la muestra española.

Polimorfismo	Genotipo	Frecuencia controles	Frecuencia esquizofrénicos	OR (95% IC)	*P* [a]	AIC	Modelo
rs12656462	T/T	232 (90.6%)	153 (83.6%)	1.00	**0.028** (0.252)	595.6	N/A
	A/T	24 (9.4%)	30 (16.4%)	1.90 (1.07-3.37)			

a. El valor de *P* corregido se indica entre paréntesis.
Abreviaturas: OR, *Odds Ratio*; IC, intervalos de confianza; N/A, no aplicable.

Además, se detectaron tanto un haplotipo protector como un haplotipo de riesgo, ambos de dos marcadores, que diferían únicamente en el SNP rs12656462 (datos no mostrados). Sin embargo, todos los resultados significativos asociados con el SNP rs12656462 deben ser considerados con cautela puesto que la frecuencia de su alelo menor es bastante baja (0.047), por lo que el número de individuos homozigotos para ese alelo es especialmente bajo. Con respecto a los resultados obtenidos con la muestra de Alemania, los únicos resultados significativos fueron la asociación de 3 SNPs (rs10056492, rs4700316 y rs7714708) con el trastorno bipolar (tabla C17). Aunque la significación fue alta y resistió la corrección de Bonferroni secuencial, el número de individuos aquejados de este trastorno es demasiado bajo, por lo que sería conveniente realizar análisis adicionales en muestras de mayor tamaño. Además, puesto que el uso de muestras grandes es útil, se llevó a cabo un análisis de asociación con una muestra caso-control que combinaba a los controles y pacientes esquizofrénicos procedentes de Alemania y España. Sin embargo, los resultados fueron negativos (datos no mostrados), aunque este hecho podría estar relacionado con las diferencias en la estructura genética de las poblaciones española y alemana (Seldin *et al.*, 2006; Heath *et al.*, 2008). El uso de muestras más grandes, homogéneas y no estratificadas sería útil para ayudar a entender el papel de *PDE4D* en la vulnerabilidad a las enfermedades psiquiátricas.

Tabla C17. Comparación de las distribuciones genotípicas de los SNPs del gen *PDE4D* en pacientes con trastorno bipolar y sujetos control de la muestra alemana.

SNP	Genotipo	Frecuencia controles	Frecuencia pacientes bipolares	OR (95% IC)	*P* [b]	AIC	Modelo
rs17291089	A/A	466 (87.8%)	70 (82.3%)	1.00	0.18 (1)	496.6	Dominante
	C/A-C/C	65 (12.2%)	15 (17.6%)	0.65 (0.35-1.20)			
rs829259	T/T	184 (34.5%)	33 (37.9%)	1.00	0.53 (1)	506.8	Dominante
	A/T - A/A	350 (65.5%)	54 (62.1%)	1.16 (0.73-1.86)			
rs17719378	A/A	220 (41.2%)	37 (42%)	1.05 (0.74-1.48)[a]	0.79 (1)	511	Aditivo
	A/G	253 (47.4%)	42 (47.7%)				
	G/G	61 (11.4%)	9 (10.2%)				
rs10055954	G/G	365 (68.3%)	64 (72.7%)	1.26 (0.79-1.99)[a]	0.32 (1)	510.1	Aditivo
	C/G	155 (29%)	23 (26.1%)				
	C/C	14 (2.6%)	1 (1.1%)				

Tabla C17 (continuación). Comparación de las distribuciones genotípicas de los SNPs del gen *PDE4D* en pacientes con trastorno bipolar y sujetos control de la muestra alemana.

SNP	Genotipo	Frecuencia controles	Frecuencia pacientes bipolares	OR (95% IC)	*P* [b]	AIC	Modelo
rs10461656	G/G	232 (43.5%)	46 (52.3%)	1.32 (0.93-1.89)[a]	0.12 (0.840)	508.6	Aditivo
	A/G	244 (45.7%)	35 (39.8%)				
	A/A	58 (10.9%)	7 (8%)				
rs7713345	C/C	373 (69.8%)	63 (73.3%)	1.22 (0.77-1.94)	0.38 (1)	502.5	Aditivo
	G/C	145 (27.1%)	22 (25.6%)				
	G/C	16 (3%)	1 (1.2%)				
rs12656462	T/T	442 (83.1%)	68 (77.3%)	1.00	0.2 (1)	508.8	Dominante
	A/T - A/A	90 (16.9%)	20 (22.7%)	0.69 (0.40-1.20)			
rs10056492	C/C	268 (75.3%)	80 (90.9%)	3.23 (1.52-6.83)[a]	**0.0004 (0.004)**	433.7	Aditivo
	C/T	83 (23.3%)	8 (9.1%)				
	T/T	5 (1.4%)	0 (0%)				
rs4700316	C/C - C/G	339 (63.6%)	67 (76.1%)	1.00	**0.019** (0.152)	505.3	Dominante
	G/G	194 (36.4%)	21 (23.9%)	1.83 (1.08-3.07)			
rs7714708	C/C	211 (39.6%)	49 (55.7%)	1.00	**0.0049 (0.041)**	502.9	Dominante
	T/C - T/T	322 (60.4%)	39 (44.3%)	1.92 (1.22-3.02)			

Los valores de *P* significativos ($P < 0.05$) se indican en negrita.
a. El valor indicado corresponde a la diferencia que se le atribuye a cada alelo en el modelo aditivo.
b. El valor de *P* corregido se indica entre paréntesis.
Abreviaturas: OR, *Odds Ratio*; IC, intervalos de confianza.

Asimismo, además de estudiar el efecto de este gen sobre la vulnerabilidad a padecer algún tipo de psicosis, se analizó cómo este gen influía sobre diferentes variables clínicas. Como resultado de este estudio, se encontró que dos polimorfismos del gen *PDE4D* (rs17291089 y rs7713345) presentaban una distribución genotípica significativamente diferente dependiendo de la edad de inicio de la psicosis (datos no mostrados). Sin embargo, en este caso se debe volver a tener en cuenta que se contaba con una muestra de pacientes de inicio temprano muy reducida, por lo que el resultado, aunque sugerente, podría ser un falso positivo. Por otra parte, también es de especial interés el SNP rs17719378, ya que se ha relacionado con un número importante de variables clínicas (tabla C18).

Un dato interesante es el hecho de que este SNP se ha asociado principalmente con síntomas positivos, sugiriendo un posible papel en la severidad de estos síntomas, especialmente delirios. Además, la asociación de este SNP con el ítem de Ansiedad de la escala BPRS es también alentador, debido a la existencia de evidencias previas que han asociado el gen *PDE4D* con depresión y neuroticismo (Henkel-Tigges y Davis, 1990; Shifman *et al.*, 2008; Heck *et al.*, 2008).

Tabla C18. Resultados significativos del análisis de asociación entre el SNP rs17719378 (gen *PDE4D*) y algunos ítems de ciertas escalas clínicas.

SNP	Distribución genotípica	Puntuación media (SE)	Diferencia (95% IC)	Modelo	P[b]	BIC
KGV – Incoherencia del lenguaje						
rs17719378	A/A : 80 A/G : 93 G/G : 25	0.55 (0.1) 0.77 (0.12) 1.2 (0.26)	0.30 (0.08 - 0.52)[a]	Aditivo	**0.0089** (0.0712)	589.2
BPRS - Ansiedad						
rs17719378	A/A - A/G : 109 G/G : 12	3.09 (0.14) 4.08 (0.36)	0.00 0.99 (0.13 - 1.85)	Recesivo	**0.025** (0.2)	435.2
PANSS total						
rs17719378	A/A : 37 A/G - G/G : 42	59.32 (2.9) 67.26 (2.33)	0.00 7.94 (0.71 - 15.16)	Dominante	**0.034** (0.272)	676.8
PANSS G11 – Atención pobre						
rs17719378	A/A -A/G : 74 G/G : 5	2.03 (0.13) 3.6 (0.24)	0.00 1.57 (0.61 - 2.54)	Recesivo	**0.002** **(0.016)**	245.1
PANSS Positiva						
rs17719378	A/A : 37 A/G : 37 G/G : 5	13.49 (0.87) 17.38 (1.07) 11 (1.05)	0.00 3.89 (1.25 - 6.54) -2.49 (-7.90 - 2.93)	Codominante	**0.0057** **(0.0456)**	516.4
PANSS P1 - Delirios						
rs17719378	A/A : 37 A/G : 37 G/G : 5	2.03 (0.27) 3.3 (0.35) 1.2 (0.2)	0.00 1.27 (0.42 - 2.12) -0.83 (-2.56 - 0.91)	Codominante	**0.0046** **(0.0368)**	336.2
PANSS P3 – Comportamiento alucinatorio						
rs17719378	A/A : 37 A/G : 37 G/G : 5	2.16 (0.33) 3.27 (0.39) 1 (0)	0.00 1.11 (0.12 - 2.09) -1.16 (-3.18 - 0.85)	Codominante	**0.024** (0.192)	360.2
PANSS P5 - Grandiosidad						
rs17719378	A/A : 37 A/G - G/G : 42	1.54 (0.2) 2.33 (0.28)	0.00 0.79 (0.11 - 1.48)	Dominante	**0.026** (0.208)	304.3
PSYRATS AA – Grado de control						
rs17719378	A/A - A/G : 154 G/G : 22	2.09 (0.14) 1.27 (0.37)	0.00 -0.82 (-1.62 - -0.02)	Recesivo	**0.046** (0.414)	717.1
PSYRATS Delirios - Total						
rs17719378	A/A : 44 A/G - G/G : 54	4.14 (1.01) 7.69 (1.2)	0.00 3.55 (0.39 - 6.71)	Dominante	**0.03** (0.24)	695.8
PSYRATS Delirios – Convicción sobre el origen de los delirios						
rs17719378	A/A : 44 A/G - G/G : 54	0.84 (0.21) 1.52 (0.23)	0.00 0.68 (0.06 - 1.30)	Dominante	**0.035** (0.28)	376.9
PSYRATS Delirios – Duración de la preocupación						
rs17719378	A/A : 44 A/G - G/G : 54	0.77 (0.19) 1.37 (0.22)	0.00 0.60 (0.01 - 1.18)	Dominante	**0.048** (0.384)	364.8

Tabla C18 (continuación). Resultados significativos del análisis de asociación entre el SNP rs17719378 (gen *PDE4D*) y algunos ítems de ciertas escalas clínicas.

SNP	Distribución genotípica	Puntuación media (SE)	Diferencia (95% IC)	Modelo	P^b	BIC
PSYRATS Delirios - Repercusión						
rs17719378	A/A : 44	0.59 (0.15)	0.00	Dominante	**0.013** (0.104)	330.9
	A/G - G/G : 54	1.22 (0.19)	0.63 (0.14 - 1.12)			
PSYRATS Delirios – Intensidad de la ansiedad						
rs17719378	A/A : 44	0.57 (0.15)	0.00	Dominante	**0.018** (0.144)	329.8
	A/G - G/G : 54	1.17 (0.19)	0.60 (0.11 - 1.09)			

Los valores de *P* significativos (P < 0.05) se indican en negrita.
a. El valor de *P* corregido se indica entre paréntesis.
Abreviaturas: SE, error estándar; IC, intervalos de confianza.; AA, alucinaciones auditivas.

Genes *PLEKHB1* y *RAB6A*

Al igual que el gen *PDE4D*, estos dos genes adyacentes localizados en el cromosoma 11 fueron seleccionados por su posible relación con otro trastorno psiquiátrico (el trastorno por déficit de atención e hiperactividad o TDAH) en un estudio de búsqueda de variaciones en el número de copias realizado en la Universidad de Würzburg (Alemania). Se seleccionaron diferentes SNPs que capturaban toda la variabilidad de la región y, una vez eliminados aquellos que resultaron ser monomórficos o bien que se desviaban enormemente del equilibrio de Hardy-Weinberg, se analizaron 6 SNPs en la muestra española y 11 SNPs en la alemana.

Con respecto al análisis de asociación caso-control, un SNP localizado en el gen *PLEKHB1*, rs663303, se asoció con psicosis y psicosis con alucinaciones auditivas en la muestra española: (ver tabla C19 para los datos de las distribuciones genotípicas). Además, se detectaron un haplotipo de riesgo y uno protector que implicaban a los marcadores rs663303, rs940828 y rs3741147, en coherencia con los estudios de SNPs individuales (datos no mostrados). Desafortunadamente, casi ninguno de estos hallazgos (con la excepción del haplotipo protector), resistieron la corrección estadística para comparaciones múltiples. Con respecto a la muestra alemana, algunos SNPs se asociaron con esquizofrenia (tabla C20) o desorden bipolar (tabla C21), aunque los polimorfismos diferían de los de la muestra española. Concretamente, un SNP del gen *PLEKHB1* (rs940828) se asoció con esquizofrenia y desorden bipolar. Curiosamente, este marcador está localizado en la misma región que el SNP rs663303, uno de los SNPs asociados con psicosis en la muestra española, aunque el desequilibrio de ligamiento entre ambos polimorfismos es moderado (D’ = 1; $r^2 = 0.436$). Además, se encontró que otros dos SNPs del gen *PLEKHB1* (rs4944850 y rs6592527) estaban asociados con riesgo de padecer desorden bipolar, mientras que el polimorfismo rs7127066 del gen *RAB6A* se asoció con esquizofrenia y desorden bipolar. Finalmente, los resultados fueron completamente negativos cuando se usó una muestra combinada de individuos españoles y alemanes (datos no mostrados).

Tabla C19. Comparación de las distribuciones genotípicas de los SNPs de los genes *PLEKHB1* y *RAB6A* en pacientes psicóticos y sujetos sanos de la muestra española.

SNP	Genotipo	Frecuencia controles	Frecuencia pacientes	OR (95% IC)	*P* [b]	AIC	Modelo
rs663303	C/C	223 (82.3%)	171 (74.3%)	1.57 (1.05-2.34)[a]	**0.025** (0.15)	690.2	Aditivo
	T/C	46 (17%)	55 (23.9%)				
	T/T	2 (0.7%)	4 (1.7%)				
rs940828	T/T	161 (61.9%)	138 (60.3%)	1.07 (0.78-1.47)[a]	0.68 (1)	679.8	Aditivo
	G/T	91 (35%)	83 (36.2%)				
	G/G	8 (3.1%)	8 (3.5%)				
rs3741147	T/T	187 (65.8%)	163 (71.5%)	0.75 (0.55-1.04)[a]	0.085 (0.425)	704.7	Aditivo
	G/T	83 (29.2%)	60 (26.3%)				
	G/G	14 (4.9%)	5 (2.2%)				
rs12274970	T/T - T/C	254 (87.6%)	205 (89.1%)	1.00	0.59 (1)	717.6	Recesivo
	C/C	36 (12.4%)	25 (10.9%)	0.86 (0.50-1.48)			
rs11235876	G/G - G/A	209 (73.3%)	177 (77%)	1.00	0.34 (1)	711.2	Recesivo
	A/A	76 (26.7%)	53 (23%)	0.82 (0.55-1.23)			
rs7127066	G/G	167 (60.3%)	133 (57.8%)	1.10 (0.82-1.47)[a]	0.51 (1)	702.1	Aditivo
	C/G	96 (34.7%)	83 (36.1%)				
	C/C	14 (5%)	14 (6.1%)				

Los valores de *P* significativos (P < 0.05) se indican en negrita.
a. El valor indicado corresponde a la diferencia que se le atribuye a cada alelo en el modelo aditivo.
b. El valor de *P* corregido se indica entre paréntesis.
Abreviaturas: OR, *Odds Ratio*; IC, intervalos de confianza.

Tabla C20. Comparación de las distribuciones genotípicas de los SNPs de los genes *PLEKHB1* y *RAB6A* en pacientes esquizofrénicos y sujetos sanos de la muestra alemana.

SNP	Genotipo	Frecuencia controles	Frecuencia pacientes	OR (95% IC)	*P* [b]	AIC	Modelo
rs663303	C/C	408 (76.4%)	191 (78.6%)	1.00	0.5 (1)	969	Dominante
	T/C - T/T	126 (23.6%)	52 (21.4%)	0.88 (0.61-1.27)			
rs4944850	A/A	255 (72%)	188 (78%)	1.00	0.099 (0.891)	804.5	Dominante
	C/A - C/C	99 (28%)	53 (22%)	0.73 (0.50-1.07)			
rs591804	A/A	162 (45.6%)	119 (49%)	0.87 (0.68-1.12)[a]	0.28 (1)	810.8	Aditivo
	A/G	155 (43.7%)	104 (42.8%)				
	G/G	38 (10.7%)	20 (8.2%)				
rs6592527	G/G - C/G	341 (96.1%)	226 (93%)	1.00	0.1 (0.891)	809.2	Recesivo
	C/C	14 (3.9%)	17 (7%)	1.83 (0.89-3.79)			
rs940828	T/T - G/T	510 (96%)	223 (92.5%)	1.00	**0.045** (0.495)	958.6	Recesivo
	G/G	21 (4%)	18 (7.5%)	1.96 (1.02-3.75)			
rs3741147	T/T	422 (79%)	196 (80.7%)	0.90 (0.63-1.28)[a]	0.56 (1)	969.1	Aditivo
	G/T	106 (19.9%)	45 (18.5%)				
	G/G	6 (1.1%)	2 (0.8%)				
rs12274970	T/T	315 (59%)	153 (63%)	1.00	0.29 (1)	968.4	Dominante
	T/C - C/C	219 (41%)	90 (37%)	0.85 (0.62-1.16)			

Tabla C20 (continuación). Comparación de las distribuciones genotípicas de los SNPs de los genes *PLEKHB1* y *RAB6A* en pacientes esquizofrénicos y sujetos sanos de la muestra alemana.

SNP	Genotipo	Frecuencia controles	Frecuencia pacientes	OR (95% IC)	P [b]	AIC	Modelo
rs10736793	C/C - C/A	346 (97.5%)	240 (98.4%)	1.00	0.45 (1)	813.1	Recesivo
	A/A	9 (2.5%)	4 (1.6%)	0.64 (0.20-2.10)			
rs11235876	G/G - G/A	447 (83.7%)	199 (81.9%)	1.00	0.53 (1)	969.1	Recesivo
	A/A	87 (16.3%)	44 (18.1%)	1.14 (0.76-1.69)			
rs11235880	C/C - C/A	343 (96.3%)	229 (93.8%)	1.00	0.16 (1)	812.8	Recesivo
	A/A	13 (3.6%)	15 (6.2%)	1.73 (0.81-3.70)			
rs7127066	G/G - C/G	506 (94.8%)	221 (91%)	1.00	**0.051** (0.51)	965.6	Recesivo
	C/C	28 (5.2%)	22 (9.1%)	1.80 (1.01-3.21)			

Los valores de *P* significativos (P < 0.05) se indican en negrita.
a. El valor indicado corresponde a la diferencia que se le atribuye a cada alelo en el modelo aditivo.
b. El valor de *P* corregido se indica entre paréntesis.
Abreviaturas: OR, *Odds Ratio*; IC, intervalos de confianza.

Tabla C21. Comparación de las distribuciones genotípicas de los SNPs de los genes *PLEKHB1* y *RAB6A* en pacientes con desorden bipolar y sujetos sanos de la muestra alemana.

SNP	Genotipo	Frecuencia controles	Frecuencia pacientes	OR (95% IC)	P [b]	AIC	Modelo
rs663303	C/C - T/C	525 (98.3%)	85 (96.6%)	1.00	0.32 (1)	510.1	Recesivo
	T/T	9 (1.7%)	3 (3.4%)	0.49 (0.13-1.83)			
rs4944850	A/A - C/A	344 (97.2%)	81 (92%)	1.00	**0.04** (0.36)	441	Recesivo
	C/C	10 (2.8%)	7 (8%)	0.34 (0.12-0.91)			
rs591804	A/A - A/G	317 (89.3%)	73 (83.9%)	1.00	0.18 (0.9)	440.6	Recesivo
	G/G	38 (10.7%)	14 (16.1%)	0.63 (0.32-1.21)			
rs6592527	G/G - C/G	341 (96.1%)	78 (89.7%)	1.00	**0.027** (0.27)	437.5	Recesivo
	C/C	14 (3.9%)	9 (10.3%)	0.36 (0.15-0.85)			
rs940828	T/T	325 (61.2%)	57 (64.8%)	1.00	**0.024** (0.264)	504.8	Codominante
	G/T	185 (34.8%)	22 (25%)	1.47 (0.87-2.49)			
	G/G	21 (4%)	9 (10.2%)	0.41 (0.18-0.94)			
rs3741147	T/T	422 (79%)	62 (71.3%)	1.00	0.11 (0.77)	504.7	Dominante
	G/T - G/G	112 (21%)	25 (28.7%)	0.66 (0.40-1.09)			
rs12274970	T/T	315 (59%)	51 (58%)	1.00	0.86 (1)	511.1	Dominante
	T/C - C/C	219 (41%)	37 (42%)	0.96 (0.61-1.51)			
rs10736793	C/C	243 (68.5%)	58 (65.9%)	1.00	0.65 (1)	445.5	Dominante
	C/A - A/A	112 (31.6%)	30 (34.1%)	0.89 (0.54-1.46)			
rs11235876	G/G - G/A	447 (83.7%)	68 (77.3%)	1.00	0.15 (0.9)	509	Recesivo
	A/A	87 (16.3%)	20 (22.7%)	0.66 (0.38-1.15)			
rs11235880	C/C - C/A	343 (96.3%)	82 (94.2%)	1.00	0.4 (1)	442.2	Recesivo
	A/A	13 (3.6%)	5 (5.8%)	0.62 (0.22-1.79)			
rs7127066	G/G - C/G	506 (94.8%)	79 (89.8%)	1.00	0.089 (0.712)	508.2	Recesivo
	C/C	28 (5.2%)	9 (10.2%)	0.49 (0.22-1.07)			

Los valores de *P* significativos (P < 0.05) se indican en negrita.
a. El valor indicado corresponde a la diferencia que se le atribuye a cada alelo en el modelo aditivo.
b. El valor de *P* corregido se indica entre paréntesis.
Abreviaturas: OR, *Odds Ratio*; IC, intervalo de confianza.

Es importante remarcar que, durante la realización de los análisis de asociación caso-control, se encontraron diferentes asociaciones que implicaban principalmente al gen *PLEKHB1*. Sin embargo, solamente se puede referir como una replicación parcial, ya que los SNP asociados con psicosis varían en cada muestra. Sin embargo, es arriesgado establecer una relación causal entre este gen y los desórdenes mentales. En primer lugar, la significación no ha sido muy alta. Además, el papel de este gen en el comportamiento o en la fisiopatología de los desórdenes mentales no ha sido explorado todavía. Por lo que sabemos, éste es el primer estudio que ha intentado relacionar este gen con una patología psiquiátrica. Se sabe poco sobre la función de este gen, aunque sí se ha sugerido que la proteína PLEKHB1 podría tener un papel en la modificación de los procesos de transducción de señales que tienen lugar en las células fotorreceptoras (Xu *et al.*, 2004). De acuerdo con esto, se puede hipotetizar que las alteraciones en el gen *PLEKHB1* podrían tener un papel en el desarrollo de alucinaciones auditivas asociadas a la psicosis. Además, la expresión de este gen es particularmente alta en áreas cerebrales que parecen tener un papel importante en la fisiopatología de la esquizofrenia, como el lóbulo temporal, el córtex prefrontal, el tálamo y el hipotálamo, entre otros. Estas evidencias, junto con los resultados obtenidos en nuestro análisis de asociación caso-control, nos llevaron a estudiar más en profundidad la relación de esta región del cromosoma 11 y la psicosis.

Se estudió el impacto que la variación genética de los genes *PLEKHB1* y *RAB6A* tenía sobre diferentes rasgos de la enfermedad, evaluados en la muestra de pacientes españoles. Como resultado de este análisis, se encontraron un número bastante elevado de asociaciones, que implicaban a los 6 SNPs genotipados. Sin embargo, muchos de estos hallazgos son posiblemente falsos positivos, debido a la reducida frecuencia del alelo menor y al bajo número de sujetos homozigotos para ese alelo. A pesar de esto, algunos resultados son especialmente interesantes, ya que resistieron la corrección de Bonferroni secuencial (tabla C22). Son:

- La asociación del SNP rs12274970 (localizado en la región intergénica entre *PLEKHB1* y *RAB6A*) y el ítem de la PANSS Trastorno de la Volición.
- La asociación del SNP rs11235976 (localizado en el gen *RAB6A*) y los siguientes ítems de la PANSS: Trastorno de la Volición, Retraimiento Social y Hostilidad.

Los rasgos que parecen estar influidos por la variación genética son de naturaleza diversa. Por lo tanto, es difícil elaborar una hipótesis sobre el verdadero significado de estas asociaciones. Además, ninguno de los polimorfismos implicados parece ser funcional, de acuerdo a los resultados obtenidos mediante el análisis con PupaSuite (Conde *et al.*, 2006). Es también posible que estos SNPs estén en fuerte desequilibrio de ligamiento con otras variantes funcionales. Por último, a pesar de que se hipotetizó que el gen *PLEKHB1* podría estar implicado en la vulnerabilidad a padecer alucinaciones, no se ha encontrado ninguna asociación entre el gen *PLEKHB1* y las dimensiones de las alucinaciones auditivas.

Tabla C22. Resultados más significativos (resistieron la corrección para comparaciones múltiples) del análisis de asociación entre los SNPs de los genes *PLEKHB1* y *RAB6A* y diferentes escalas clínicas.

Marcador	Distribución genotípica	Puntuación media (SE)	Diferencia (95% IC)	Modelo	P^b	BIC
PANSS P7 - Hostilidad						
rs11235876	G/G - G/A : 60	1.33 (0.18)	0.00	Dominante	**0.0094 (0.047)**	242.7
	A/A :19	2.08 (0.14)	0.75 (0.20 - 1.30)			
PANSS N4 – Pasividad / Retraimiento social						
rs11235876	G/G : 18	2.06 (0.33)	0.68 (0.18 - 1.18)[a]	Aditivo	**0.0093 (0.0465)**	304.2
	G/A : 42	3.02 (0.25)				
	A/A :19	3.42 (0.34)				
PANSS G13 – Trastorno de la volición						
rs12274970	T/T : 67	1.83 (0.19)	0.00	Dominante	**0.0065 (0.0325)**	275.9
	C/C : 12	2.65 (0.21)	0.82 (0.25 - 1.39)			
rs11235876	G/G : 18	1.67 (0.21)	0.67 (0.26 - 1.08)[a]	Aditivo	**0.0021 (0.0126)**	273.8
	G/A : 42	2.21 (0.21)				
	A/A :19	3 (0.31)				

Los valores de *P* significativos ($P < 0.05$) se indican en negrita.
a. El valor indicado corresponde a la diferencia que se le atribuye a cada alelo en el modelo aditivo.
b. El valor de *P* corregido se indica entre paréntesis.
Abreviaturas: SE, error estándar; IC, intervalo de confianza.

En resumen, las evidencias a favor de la implicación de los genes *PLEKHB1* y *RAB6A* en diferentes aspectos de las psicosis son débiles, por lo que necesitarían ser confirmadas en otras muestras adicionales.

Limitaciones del estudio

A pesar de los interesantes resultados, se han de tener en cuenta una serie de consideraciones y limitaciones. En primer lugar, se ha usado un importante número de variables clínicas (correspondientes a diferentes escalas) con el propósito de evaluar la existencia de correlaciones con las variaciones genéticas. Sin embargo, en algunos casos los pacientes evaluados han sido pocos. Asimismo, se ha especulado sobre la poca validez biológica de estas medidas. A pesar de ello, pensamos que pueden ayudar a detectar si determinado gen o polimorfismo influye en un determinado tipo de síntomas. En segundo lugar, somos también conscientes de que el tamaño de muestra puede ser considerado pequeño, especialmente en el caso de algunos grupos, como el de pacientes alucinadores. Asimismo, aunque los pacientes y controles fueron elegidos para evitar estratificaciones, no podemos descartar que no exista estratificación de la muestra.

Otro aspecto importante es que el elevado número de SNPs analizados y de variables estudiadas puede aumentar peligrosamente la aparición de falsos positivos (error de tipo I). Por este motivo, se aplicó la corrección de Bonferroni secuencial (Rice, 1989), que es menos restrictiva que la corrección de Bonferroni, Actualmente, no hay consenso sobre si es necesario aplicar correcciones en los estudios de asociación, o sobre qué corrección es más

apropiada. En nuestro caso, se consideró que el ajuste de Bonferroni secuencial era el más idóneo.

Por último, cabe reseñar la importancia de tener un adecuado tamaño muestral en los estudios de neuroimagen. Aunque en el caso del análisis realizado sobre la muestra española, el tamaño ha sido reducido, se espera aumentar el número de individuos para próximos estudios. Otras variables, como la edad, el sexo o la lateralidad se tuvieron también en cuenta para estos análisis.

Hacia un modelo general de vulnerabilidad

Cuando se estudia una enfermedad común y compleja, como la esquizofrenia, que se caracteriza por un patrón de herencia no mendeliano y una alta prevalencia, una de las hipótesis más plausibles es la Hipótesis "Enfermedad Común – Variante Común" (CDCV por sus siglas en inglés). Esta hipótesis plantea que el espectro de variantes alélicas que confieren susceptibilidad a cierta enfermedad compleja se compone de variantes comunes con un efecto moderado sobre el riesgo de padecer la enfermedad y compartidas por diferentes subpoblaciones. Esta hipótesis es de hecho nuestro marco de trabajo y, por esta razón, este trabajo se ha centrado en el estudio de diferentes polimorfismos genéticos localizados en varios genes candidatos. Al inicio de este estudio se esperaba que la contribución de cada uno de estos genes candidatos fuera pequeña y los resultados obtenidos apoyan esta idea y permiten la elaboración de un posible modelo integrativo de vulnerabilidad (figura C7).

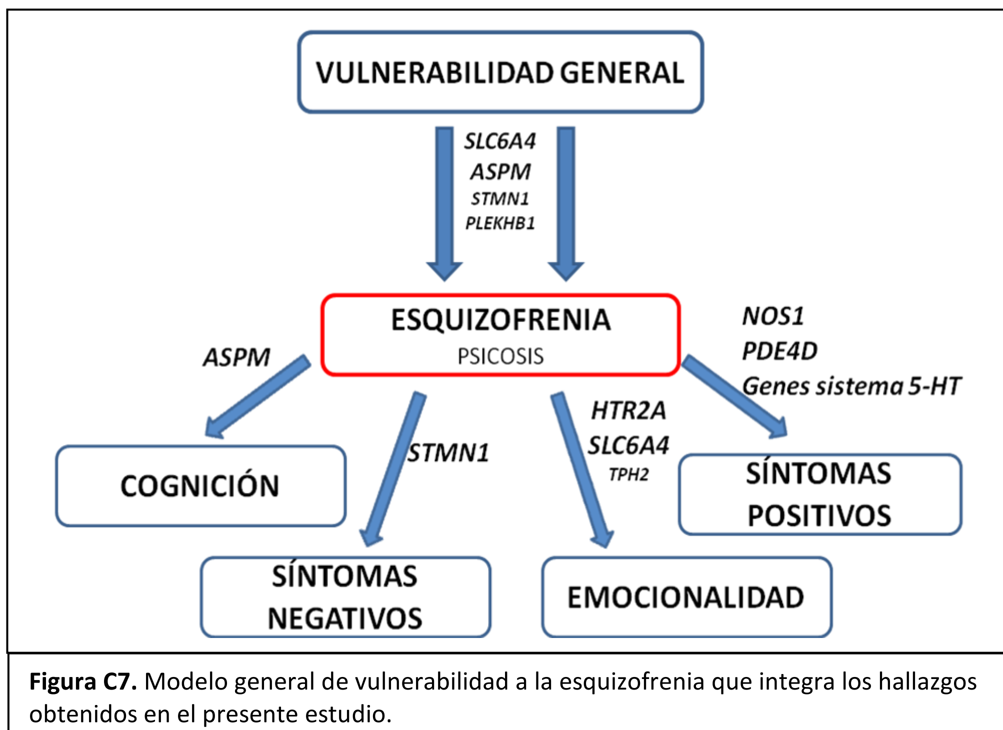


Figura C7. Modelo general de vulnerabilidad a la esquizofrenia que integra los hallazgos obtenidos en el presente estudio.

En primer lugar, existe una vulnerabilidad general a los trastornos psicóticos, que estaría influenciada por un cierto número de factores de origen genético y no genético. Entre estos factores, los genes *SLC6A4* y *ASPM* pueden tener un papel importante. De acuerdo con este

escenario, las proteínas codificadas por estos genes podrían influir sobre aquellos sistemas neurales que están implicados en la aparición de los síntomas psicóticos y esquizofrénicos. Asimismo, otros genes, como *STMN1* y *PLEKHB1*, podrían tener también un papel sobre la susceptibilidad a la esquizofrenia, aunque en este caso las evidencias son menores.

En segundo lugar, los resultados de este estudio sugieren que hay variantes polimórficas con un efecto sobre la severidad de los síntomas (figura C7). Un claro ejemplo es el efecto de la variación del gen *STMN1* sobre la severidad de los síntomas negativos. Además, ligeras evidencias sugieren un posible papel de otros genes como *NOS1* y *PDE4D* sobre los síntomas positivos. Estos resultados no son sorprendentes, sobre todo si consideramos el modelo presentado por Fanous y Kendler (2005). Según este modelo, algunos genes incrementarían la susceptibilidad a padecer esquizofrenia sin alterar sus características clínicas asociadas (genes de susceptibilidad), mientras que otros genes afectarían a esas características clínicas sin modificar la susceptibilidad a padecer la enfermedad.

El presente estudio también contribuye al estudio de la vulnerabilidad a las psicosis desde una nueva óptica, llamada “deconstrucción del síntoma”, que permite el estudio de los sistemas neurobiológicos implicados en los síntomas más representativos de la esquizofrenia, como las alucinaciones auditivas, el deterioro cognitivo y el deterioro emocional. Con respecto a los aspectos cognitivos, se ha encontrado un efecto de la variación del gen *ASPM* sobre diferentes habilidades cognitivas y variables neuroanatómicas (figura C7), lo que sugiere que este gen puede tener un importante papel durante el desarrollo de aquellas áreas cerebrales implicadas en la patogénesis de la esquizofrenia, tales como el córtex prefrontal, el córtex anterior cingulado o los ganglios basales. Se puede también argumentar que el gen *ASPM* tiene un papel clave en la cognición normal y puede ser también considerado un interesante candidato para entender la evolución del cerebro humano. En conclusión, el papel activo de la proteína ASPM en la evolución del cerebro humano puede haber desencadenado la aparición de los déficits cognitivos y alteraciones mentales asociadas a la esquizofrenia.

Además, la relación entre la variación genética del sistema serotoninérgico y las alteraciones emocionales son de especial interés. Nuestros resultados sugieren que la alteración en el funcionamiento de ciertos elementos clave de este sistema, como el transportador de serotonina o el receptor 5-HT_{2A} y, en menor medida, la triptófano hidroxilasa 2, lleva a un estado emocional alterado en los pacientes aquejados de esquizofrenia. Además, la proteína stathmin, codificada por el gen *STMN1*, podría estar también implicada en la patogénesis de las alteraciones afectivas asociadas a la esquizofrenia, a través de su implicación en los síntomas negativos. Sin embargo, en este último caso las evidencias son menores.

Finalmente, este estudio apoya la implicación de diferentes variantes polimórficas en la patogénesis de las alucinaciones auditivas. En realidad, este estudio forma parte de un proyecto más ambicioso cuyo principal objetivo es desentrañar, desde un punto de vista dimensional, los mecanismos moleculares que son responsables de la vulnerabilidad a las alucinaciones auditivas. Como consecuencia de nuestros resultados en este campo, tanto los presentados en este estudio como en otros estudios, hemos desarrollado un modelo que integra los diferentes hallazgos (figura C8), permitiendo dar una explicación sobre la etiopatogénesis de las alucinaciones auditivas. De acuerdo con este modelo, se puede

considerar que hay dos mecanismos principales implicados en la vulnerabilidad genética a las alucinaciones auditivas en pacientes psicóticos. El primer mecanismo está relacionado con la vulnerabilidad a trastornos del lenguaje, que implicaría al gen *FOXP*2, entre otros. En segundo lugar, hay una importante dimensión en la neurobiología de las alucinaciones auditivas: la vulnerabilidad a respuestas emocionales anormales. Tenemos razones para apoyar el importante papel del procesado emocional en la patofisiología de las alucinaciones auditivas, ya que, de igual manera que hemos detectado un efecto de la variación genética sobre el estado emocional del paciente, se ha detectado también un importante efecto de los genes serotoninérgicos sobre la respuesta emocional a las alucinaciones auditivas (Sanjuán *et al.*, 2006b), las cuales pueden ser entendidas como un elemento estresante para el paciente. Por último, los factores ambientales tampoco pueden ser olvidados en este modelo. Particularmente, los aspectos culturales pueden tener un efecto sobre el contenido y el ajuste social de las voces (Sanjuán *et al.*, 2005; Sanjuán, 2006; Aleman y Larøi, 2008).

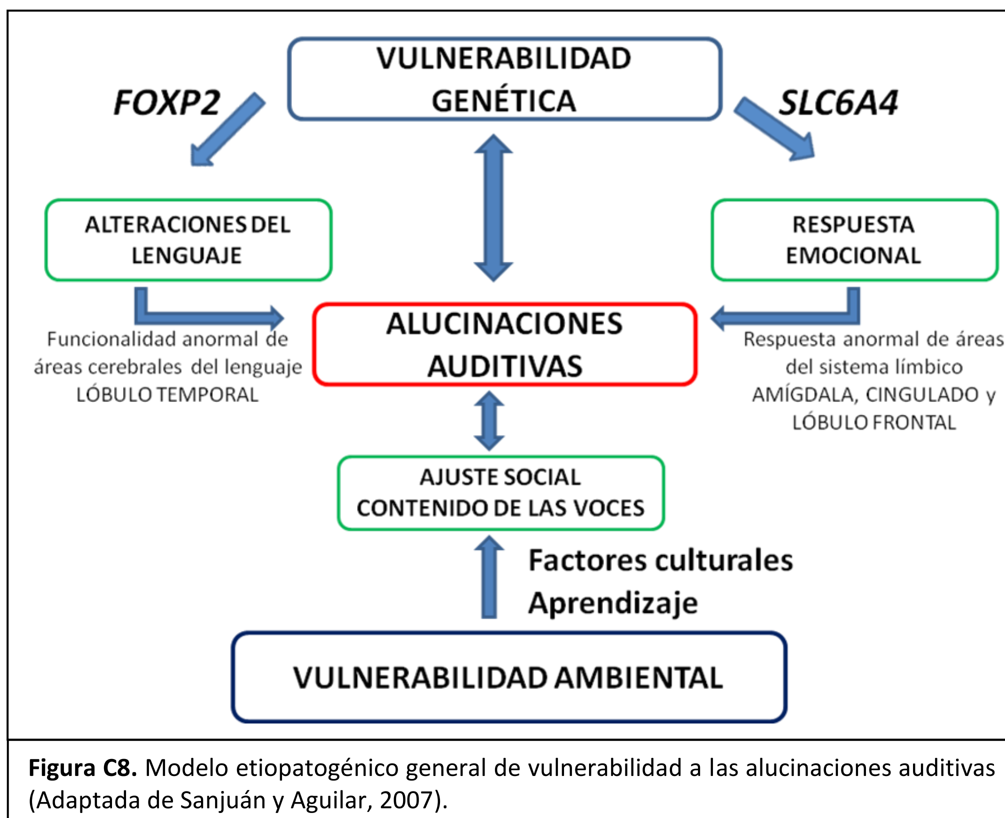


Figura C8. Modelo etiopatogénico general de vulnerabilidad a las alucinaciones auditivas (Adaptada de Sanjuán y Aguilar, 2007).

Sin embargo, a pesar de que éste y otros estudios han conseguido desenmascarar algunos elementos clave en la patogénesis de la esquizofrenia, muchos otros elementos no han sido descubiertos todavía: existe todavía mucha incertidumbre y un importante número de factores genéticos, epigenéticos y ambientales esperan a ser descubiertos y entendidos. Sin embargo, a la luz de los más recientes descubrimientos, los estudios de asociación (tanto clásicos como de genoma amplio) parecen ser una herramienta limitada, al menos para los tamaños muestrales y las herramientas estadísticas actuales. Nuevas aproximaciones, como el uso de modelos animales, estudios epigenéticos y estudios de interacción gen x ambiente, son necesarias para desentrañar los mecanismos neurofisiológicos y etiopatogénicos responsables de una de las más graves enfermedades mentales.

En resumen, los datos de este estudio permiten obtener las siguientes conclusiones:

1. El análisis caso-control ha permitido detectar asociación entre el SNP rs2020936 del gen del transportador de serotonina y la psicosis. También se han encontrado varios haplotipos protectores que eran más frecuentes en el grupo control que en el grupo de pacientes esquizofrénicos.

2. Los datos del presente estudio apoyan la relación entre la variación en el polimorfismo 5-HTTLPR y la respuesta emocional del paciente esquizofrénico. Concretamente, el alelo s se ha asociado con una mayor puntuación en los ítems emocionales de la escala PSYRATS, una mayor activación de la amígdala ante palabras de contenido emocional y una menor densidad de materia gris en determinadas áreas del sistema límbico (córtex anterior cingulado en pacientes esquizofrénicos; hipocampo y parahipocampo en sujetos sanos).

3. Los estudios de asociación han puesto de manifiesto el efecto del gen *HTR2A* sobre la emocionalidad de los pacientes psicóticos, evaluada mediante diferentes ítems de escalas psicopatológicas: PANSS, BPRS y PSYRATS. El gen *TPH2* también se ha asociado con diversos ítems de la escala PSYRATS de alucinaciones auditivas, si bien en este caso la significación es menor.

4. La realización de análisis de interacción ha permitido la detección de efectos epistáticos que implican a los genes *SLC6A4*, *HTR2A* y *TPH2.* Estas epistasias parecen modular diferentes parámetros relacionados con el procesado emocional y, especialmente, la respuesta emocional a las alucinaciones auditivas.

5. Se ha detectado un efecto del VNTR del gen *NOS1* sobre la puntuación en ciertos parámetros de la PANSS, tres del componente depresivo y dos ítems positivos.

6. El gen *STMN1* se ha asociado débilmente con el riesgo de esquizofrenia, así como con diferentes ítems de escalas psicopatológicas relacionados principalmente con la sintomatología negativa/afectiva de la esquizofrenia.

7. A través de estudios caso-control y basados en familias realizados en dos muestras independientes, el gen *ASPM* se ha asociado con el riesgo de psicosis, así como con diferentes parámetros de tipo cognitivo relacionados principalmente con la memoria de trabajo. Concretamente, un SNP, rs10922163 se ha asociado con el riesgo de psicosis en ambas muestras estudiadas. Asimismo, se ha observado un efecto de la variación en el gen *ASPM* sobre la activación del cortex anterior cingulado durante la realización de un ejercicio de memoria de trabajo. Finalmente, el gen *ASPM* también influye en la densidad de materia gris de determinadas áreas cerebrales relacionadas con la patogénesis de la esquizofrenia, como son el cerebelo, el córtex prefrontal, el cortex anterior cingulado subgenual y los ganglios basales.

8. Un SNP del gen *PDE4D* se ha asociado débilmente con el riesgo de esquizofrenia en la muestra española, mientras que en el caso de la muestra alemana se ha encontrado

una asociación de diferentes SNPs con el trastorno bipolar. Este gen también se ha asociado con la severidad de diversos síntomas, principalmente de tipo positivo.

9. Se ha encontrado asociación débil entre diferentes SNPs del gen *PLEKHB1* y el riesgo de esquizofrenia y/o desorden bipolar.

10. Todos estos resultados permiten proponer un modelo de vulnerabilidad, mediante el cual ciertos genes tendrían un mayor efecto sobre la vulnerabilidad a sufrir esquizofrenia, mientras que otros genes tendrían un efecto sobre la severidad de ciertos síntomas. En cuanto a las alucinaciones auditivas, los resultados del presente estudio apoyan la existencia de una vulnerabilidad genética a respuestas emocionales aberrantes, que podría estar modulada por la variación en diferentes genes, por ejemplo el transportador de serotonina.

ABBREVIATIONS

ABBREVIATIONS AND GENE SYMBOLS

5-HIAA, 5-HydroxyIndole Acetic Acid

5-HT, 5-HydroxyTryptamine or serotonin

5-HTT, 5-HydroxyTryptamine or serotonin transporter

5-HTTLPR, 5-HTT Linked Polymorphic Region

ACC, Anterior Cingulate Cortex

ADHD, Attention-Deficit Hyperactivity Disorder

AH, Auditory Hallucinations

AIC, Akaike's Information Criterion

ApoE4, Apolipoprotein ε4 allele

ASPM, Abnormal Spindle-like Microcephaly-associated

BDNF, Brain-Derived Neurotrophic Factor

BIC, Bayesian Information Criterion

BLA, Basolateral Amygdala

BOLD, Blood Oxygenation Level Dependent

bp, base pairs

BPD, Bipolar Disorder

BPRS, Brief Psychiatric Rating Scale

BSA, Bovine Serum Albumin

cAMP, cyclic Adenosine Monophosphate

CBDB, Clinical Brain Disorders Branch

CDCV, Common Disease - Common Variant

CEPH, Centre d'Etude du Polymorphisme Humain. (In the HapMap Project, it refers to DNA samples of Utah residents with ancestry from northern and western Europe).

cGMP, cyclic guanosine monophosphate

CH domain, Calponin-homology domain

CNS, Central Nervous System

CNV, Copy Number Variation

*COM*T, Cathecol-O-methyl Transferase

CPT, Continuous Performance Task

DA, Dopamine

DAAO, D-amino Acid Oxidase

DAT, Discordant Allele Test

DAT, Dopamine Transporter

DISC1, Disrupted-in-schizophrenia 1

DLPFC, Dorsolateral Prefrontal Cortex

DMSO, Dimethyl Sulfoxide

DNA, Deoxyribonucleic Acid

DSM-IV, Diagnostic and Statistical Manual of Mental Disorders, 4th edition.

DZ, Dizygotic

EEG, Electroencephalogram

EF, Electrophoresis

EM, Expectation-Maximization algorithm

EMBL-EBI: European Molecular Biology Laboratory-European Bioinformatics Institute

ER, Endoplasmic Reticulum

ERP, Event-related Potential

FBAT, Family Based Association Test

FDR, False Discovery Rate

fMRI, functional Magnetic Resonance Imaging

GM, Gray Matter

GWA, Genome-Wide Association

HOR, high order repeat

HPA, hypothalamic-pituitary-adrenal

HTR2A, Serotonin Receptor 2A

HWE, Hardy-Weinberg Equilibrium

indel, insertion-deletion

IP, Inositol Phosphate

IQ, Intelligence Quotient

IQ domain, Isoleucine-glutamine domain

IUBMB, International Union of Biochemistry and Molecular Biology

IUPAC, International Union of Pure and Applied Chemistry

Ka, rate of non-synonymous substitutions

Ks, rate of synonymous substitutions

kb, kilobase

KGV, Krawiecka, Goldberg & Vaughan (psychiatric assessment scale)

LA, Lateral nucleus of the amygdala

LD, Linkage Disequilibrium

LOD, Logarithm of Likelihood

LSD, Lysergic Acid Diethylamide

LTP, Long-Term Potentiation

MAF, Minor Allele Frequency

MALDI-TOF, Matrix-Assisted Laser Desorption/Ionization - Time-Of-Flight

MAO, Monoamine Oxidase

MCPH, Microcephaly, Primary Autosomal Recessive

MGB, Minor Groove Binder

MNI, Montreal Neurological Institute

MR, Magnetic Resonance

MRI, Magnetic Resonance Imaging

mRNA, Messenger Ribonucleic Acid

MRS, Magnetic Resonance Spectroscopy

MS, Mass Spectrometry

MT, Microtubules

MTL, Medial Temporal Lobe

MZ, Monozygotic

NAA, N-Acetyl Aspartate

NET, Norepinephrine Transporter

NFQ, Non-Fluorescent Quencher

NIMH, National Institute of Mental Health

NO, Nitric Oxide

NOS, Nitric oxide synthase

NRG1, Neuregulin 1

OCD, Obsessive-Compulsive Disorder

OR, Odds Ratio

PANSS, Positive and Negative Syndrome Scale

PCP, Phenylcyclidine

PCR, Polymerase Chain Reaction

PDE4D, phosphodiesterase 4D

PET, Positron Emission Tomography

PFC, Prefrontal Cortex

PH, Pleckstrin homology

PI3K, PhosphatidylInositide 3-Kinase

PSYRATS, Psychotic Symptom Rating Scales

RELN, Reelin

REM, Rapid Eye Movement

RFLP, Restriction Fragment Length Polymorphism

RGS4, Regulator of G protein signaling 4

ROI, Region of Interest

rt, room temperature

SAP, Shrimp Alkaline Phosphatase

SBE, Single Base Extension

SD, Standard Deviation

SE, Standard Error

SERT, Serotonin Transporter

SLC6A4, solute carrier family 6 (neurotransmitter transporter, serotonin) – serotonin transporter

sMRI, Structural Magnetic Resonance Imaging

SNP, Single Nucleotide Polymorphism

SPECT, Single-Photon Emission Computed Tomography

SSCP, Single Strand Conformation Polymorphism

SSRI, Selective Serotonin Reuptake Inhibitor

S-TDT, Sib Transmission/Disequilibrium Test

STG Superior Temporal Gyrus

STin2, Serotonin Transporter intron 2 VNTR

SVZ, Subventricular Zone

TDT, Transmission/Disequilibrium Test

Tm, Melting Temperature

TM, Transmembrane

TPH, Tryptophan Hydroxylase

TPH2, Tryptophan hydroxylase 2

UCR2, Ultra Conserved Region 2

UCSC, University of California Santa Cruz

UTR, Untranslated Region

VBM, Voxel-Based Morphometry

VCFS, Velocardiofacial Syndrome

VNTR, Variable Number of Tandem Repeats

WCST, Wisconsin Card Sorting test

WHO, World Health Organization

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