Genetic risk factors in schizophrenia and neurodevelopmental disorders: association and epistatic analyses of Neuritin-1 gene and white matter related genes

Factores genéticos en esquizofrenia y enfermedades del neurodesarrollo: análisis de asociación y epistáticos en el gen Neuritina-1 en genes relacionados con la materia blanca

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List of abbreviations

Word List	Abbreviation
Schizophrenia	SZ
Bipolar Disorder	BPD
Major Depressive Disorder	MDD
Autism Spectrum Disorders	ASD
Genome-wide association studies	GWAS
Single Nucleotide Polymorphisms	SNPs
Copy number variants	CNVs
World health organization	WHO
Fractional anisotropy	FA
Years lived with disability	YLD
Disability adjusted life years	DALYs
Psychiatric Genetics Consortium	PGC
Central Nervous System	CNS
N-methyl-D-aspartate receptor	NMDAR
Neurotrophic Factors	NTF
Immediate early gene	IEG
Grey Matter	GM
White Matter	WM
Voxel-based morphometry	VBM
Neuregulin1-tyrosine kinase receptor ErbB4	NRG1-ErbB4
Genexgene interaction	gxg
Genexenvironment interaction	gxe
Multifactor dimensionality reduction	MDR
Main Genes mentioned in the Thesis	
Neuritin-1	NRN1
Brain-derived neurotrophic factor	BDNF
Dysbindin-1	DTNBP1
Oligodendrocyte/myelination related genes	OMR

1. Introduction

"Almost in the beginning was curiosity."
– Isaac_Asimov

1.1 **Neurodevelopment processes:** ***the etiological framework of schizophrenia and neurodevelopmental related disorders***

The brain is the most complicated organ comprised by a huge number of interconnections and permutations (~2×1010 neocortical neurons and ~1014 synapses) (Cook Jr and Scherer, 2008). Its development is a well-organized and dynamic process whose efficiency is essential for the adequate functioning of the whole brain. The functional capacity of the human brain and its organization depends on highly complex processes present during the ontogenetic development. These processes are genetically determined and epigenetically directed (Tau and Peterson, 2010). In addition, this highly dynamic sequence of processes is environmentally influenced, meaning that it is sensible to stressful and repetitive events occurring during vulnerability periods of brain development, which normally concur with prenatal, early childhood and adolescent stages. This highlights the concept of considering the brain as a highly sensitive system which enables behavioral flexibility in the face of dynamic environmental challenges (commonly known as *neuroplasticity*).

Therefore, both genetic and environmental inputs are involved in normal brain development, and the variability or disruption of any of them can fundamentally affect neural outcomes, potentially leading to the observed diversity in brain structure and function across individuals, from health to disease states. Accordingly, deviances in neurodevelopmental processes are thought to contribute to the variability in brain related phenotypes such as cognition, personality or affection and also, to the etiology of many psychiatric disorders (neurodevelopmental disorders) that manifest throughout the entire lifespan. In this sense, it is assumed that the effect and interaction of different factors involved in brain development can impact and modify different processes at different levels such as the synaptogenesis, synaptic transmission and plasticity, or myelination and connectivity, which could ultimately translate into changes in brain's ability to perceive and interpret the world and to make adaptive changes (Markham and Greenough, 2005). Thus, these changes would underlie the brain functioning variability in healthy individuals and the brain alterations related to the development of mental disorders.

To this regard, the *neurodevelopmental hypothesis* of schizophrenia (SZ) provided a valuable framework that allowed a condition that usually presents with frank disorder in adolescence or early adulthood to be understood at least in part as a consequence of

continuous events occurring along development (Fatemi and Folsom, 2009). Thus, the fact that several risk factors for SZ are related to prenatal or perinatal events, and that patients with SZ might exhibit various signs of impaired cognitive and social function during childhood, support the idea that developmental abnormalities might play an important role in shaping the vulnerability to SZ (Lewis and Levitt, 2002; Weinberger, 1987). This view can be also applied to several other conditions and disorders. Indeed, we cannot avoid that neurodevelopmental disorders represent a *continuum* of genetic and environmentally induced neurodevelopmental impairment, rather than a set of etiologically discrete entities. To this respect, the major clinical syndromes reflect in part the severity and predominant pattern of abnormal brain development and resulting functional abnormalities as well as the modifying effects of genetic and environmental factors.

In reference to the *neurodevelopmental continuum*, a growing body of research suggests that neuropsychiatric disorders such as schizophrenia-spectrum disorders (SSD), bipolar disorders (BPD), major depression (MD) and autism spectrum disorders (ASD), share several epidemiological, clinical and neurobiological characteristics. For instance, SSD and ASD both include deficits in social interaction and communication as primary symptoms and show impairments in similar cognitive domains (i.e. attention, memory and executive function) which make them to be placed in a pathophysiological *continuum* (Barch and Sheffield, 2014). Interestingly, the neurobiological similarities and dissimilarities between these major mental disorders can nowadays be investigated using neuroimaging techniques. Although there are some clear distinctions in the patterns of brain anatomical, white matter microstructure and functional abnormalities characterizing the specific conditions disorders, there are some similarities in neuroimaging findings detected across studies of psychiatric disorders such as SSD and BPD (Arnone *et al*, 2009; Ellison-Wright and Bullmore, 2010; De Peri *et al*, 2012). For example, substantial overlap in the localization of white matter fractional anisotropy (FA) reductions exists in both SZ and BPD (Sussmann *et al*, 2009). Also, even the complex nature of SSD and ASD, both disorders have structural connectivity alterations involving areas such as the corpus callosum and the superior longitudinal fasciculus, among others (Canu *et al*, 2014; Travers *et al*, 2012). Thus, these shared features across diagnoses raise important questions about the boundaries and distinctiveness among these psychiatric disorders. Some of these shared features are mirrored by many overlapping genetic mechanisms.

To this respect, in recent years, the field of molecular genetics has been uncovering evidence of an overlapping complex polygenetic architecture across several psychiatric disorders such as SZ, BPD, Major Depressive Disorder (MDD) or ASD (Consortium, 2013), which adds to the consideration of common pathophysiological mechanisms among these disorders. More in detail, recent findings from genome-wide association studies (GWAS) described overlapping mechanisms in neuronal development and synaptic plasticity between different psychiatric conditions in SZ, BPD and ASD (Cook Jr and Scherer, 2008; Guilmatre *et al*, 2009; Mitchell, 2015). In this sense, there is a common pool of risk genes affecting neurodevelopmental disorders and their function is highly associated with development of the nervous system and with synaptic plasticity.

Accordingly, the better knowledge of the genetic roots underlying these disorders could lead to elucidate their ethiopathological mechanisms, which could be translated to the identification of new possible therapeutic targets for treatment and therefore, could contribute to a better quality of life of the affected individuals. Then, genetic studies can contribute to reduce the economic and social burden associated to neuropsychiatric disorders.

In relation to the economic and social burden, nowadays, it is estimated that about 450 million people suffer from a mental or behavioural disorder in the world. According to World health organization (WHO)'s Global Burden of Disease 2001, 33% of the years lived with disability (YLD) are due to **neuropsychiatric disorders** (Figure 1). Moreover, four of the six leading causes of years lived with disability are due to neuropsychiatric disorders (depression, alcohol-use disorders, SZ and BPD). Neuropsychiatric conditions account for 13% of disability adjusted life years (DALYs).

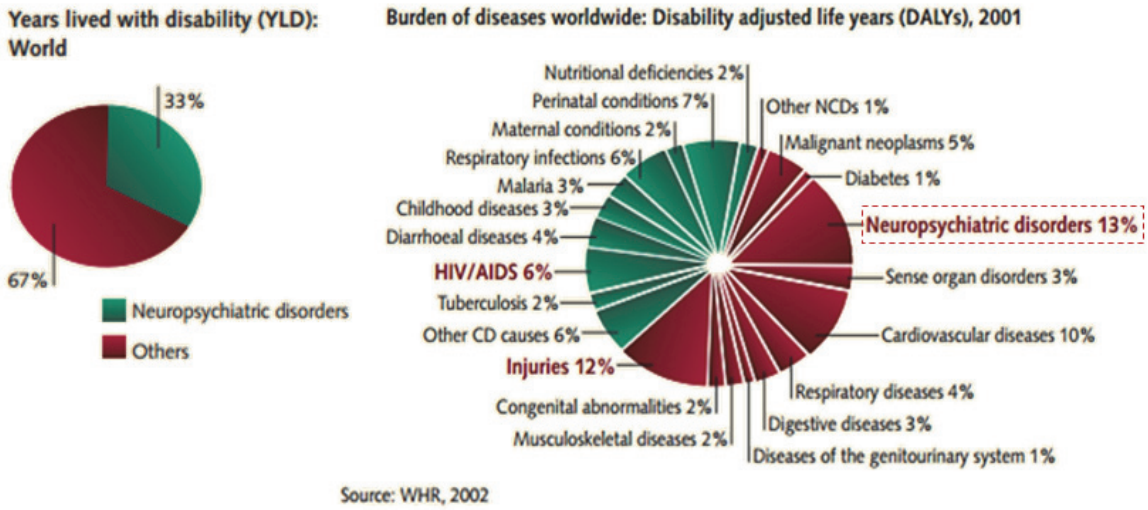

Figure 1. General burden of Neuropsychiatric Disorders: A) Years lived with disability in the world. **B)** Burden of diseases worldwide: Disability adjusted life years (DALYs) estimated for 2001. *Adapted source from World health report (WHO, 2002).*

Indeed, psychotic disorders remain one of the most mysterious and costliest mental disorders in terms of human suffering and social expenditure (Mueser and McGurk, 2004).

Much of the economic burden of mental illness is not the cost of care, but the loss of income due to unemployment, expenses for social supports, and a range of indirect costs due to a chronic disability that begins early in life. In more detail, in 2010, the global cost of mental disorders was estimated to be approximately US$2.5 trillion; and WHO estimates that by 2030 will go up to 240%, to US$6.0 trillion. Since mental disorders generate costs in terms of long-term treatment and lost productivity, it can be argued that such disorders contribute significantly to the individual and social economic burden. In this sense, mental health researchers and clinicians from around the world, clearly recognize many of the complexities of the "frequent chronicity" of mental disorders and their interplay with other chronic diseases, highlighting the importance in investing into research, treatment and prevention.

1.2 Schizophrenia and Schizophrenia-spectrum disorders

Psychotic disorders represent a group of severe mental disorders and their ethiopathogenic roots are nowadays largely unknown. The word *psychosis* derives from the Greek 'psyche', for **mind/soul**, and 'osis', meaning abnormal condition. It therefore refers to an abnormal condition of the mind, and is a psychiatric term for a mental state often described as involving a "loss of contact with reality". The word was originally used to distinguish what were regarded as disorders of the mind from neuroses, which were thought to stem from a disorder of the nervous system (Berrios, 1987). Thus, the psychoses became the modern equivalent of the old notion of madness, and subsequently, there has been much debate as to whether there is only one (unitary) or many forms of this disease (Berrios and Beer, 1994).

This debate is based on the fact that the definition of psychotic disorders includes those mental disorders involving psychotic symptoms such as hallucinations and delusions, accompanied by the inability to distinguish between subjective experience and reality. Identification of delusions and hallucinations is not difficult, but classifying all the disorders according to the emergence of these symptoms, it has not been easy. Indeed, psychotic features appear among various DSM-V diagnostic categories. (Table 1).

Table 1. Main DSM-V diagnosis categories including those that are presented with psychotic symptoms

Schizophrenia Spectrum and Other Psychotic Disorders
Schizophrenia
Schizophreniform Disorder
Schizoaffective Disorder
Brief Psychotic Disorder
Delusional Disorder
Schizotypal (Personality) Disorder
Substance/Medication-Induced Psychotic Disorder
Psychotic Disorder Due to another Medical Condition
Other Specified Schizophrenia Spectrum and Other Psychotic Disorder
Unspecified Schizophrenia Spectrum and Other Psychotic Disorder
Bipolar I Disorder with psychotic features
Major Depressive Disorder with psychotic features

Along with the clinical and cognitive deficits similarities among psychotic disorders, both epidemiological and molecular approaches have reported an important overlap in terms of their genetic liability. In particular, it has been reported that SZ diagnosis shows an important genetic overlap with other psychotic disorders, with recent studies describing nearly 40% of shared genetic factors between SZ and BPD (Lichtenstein *et al*,

2009a; Purcell *et al*, 2009). Interestingly, Lichtenstein and colleagues (2009) also reported that the comorbility between SZ and BPD was primarily (~63%) due to additive genetic factors. (Figure 2).

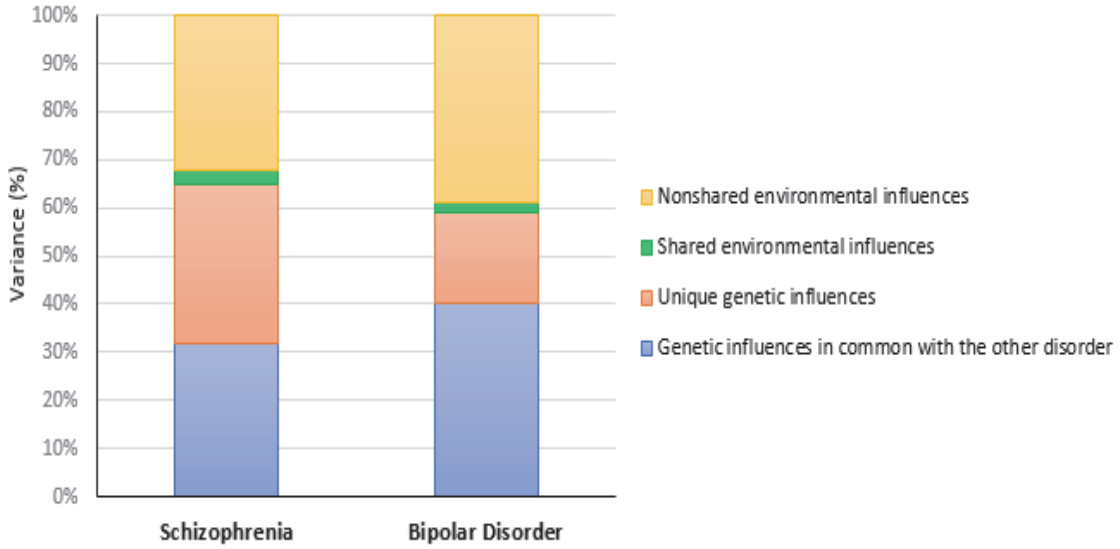

Figure 2. **Amount of variance explained by genetic, shared environmental and nonshared environmental influences for schizophrenia and bipolar disorder**. Note that according to this study unique genetic effects for SZ (not in common with bipolar disorder) accounted for ~48% of the genetic variance in SZ. *Adapted from Linchtenstein et al 2009.*

This evidence, have led to the notion that psychotic disorders represent different phenotypic manifestations of the same underlying processes; that is, these disorders have different thresholds on a single *continuum* of genetic-environmental liability (Esterberg and Compton, 2009; Jablensky, 2006). This group of disorders is also termed ***schizophrenia spectrum disorders*** **(SSD)** (Jablensky, 2006) (Figure 3) and they are considered as an evolving group of pathologies of the human brain that are shaped by several environmental stressors interacting with developmental trajectories.

From all diagnoses included within SSD, schizophrenia is the prototypical psychotic disorder, one that is characterized by prominent psychotic symptoms in the absence of medical disorders that would explain psychosis. Due to the central status of SZ as a diagnosis within the group of SSD, the following section will be mainly focused on the clinical and epidemiological characteristics of this diagnosis.

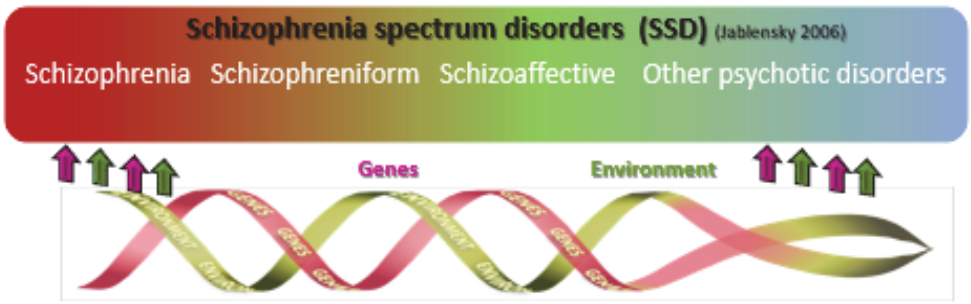

Figure 3. Schizophrenia spectrum disorders (SSD) continuum distribution, and the contribution of environmental and genetic factors: many genetic variants of small effects contribute to the manifestation of these disorders, along with many environmental factors. Specifically, the spectrum of SSD includes Schizophrenia, Schizophreniform disorder, Schizoaffective disorder and other psychotic disorders such as brief psychotic disorder or delusional disorder.

1.2.1 Clinical Characteristics and Epidemiology

Schizophrenia is a severe neurodevelopmental disorder of thought, and mind, which affect behavior and perception. This debilitating psychiatric disorder is characterized by a disruption in cognition and emotion along with negative (i.e. avolition, alogia, apathy) and positive (hallucinations, delusions) symptoms. Moreover, these symptoms can vary between patients, creating diverse symptom profiles, although one typically observes false perceptions (hallucinations), false beliefs of control or danger (delusions), disorganized speech and behavior (positive formal thought disorder, bizarre behavior) and impaired cognitive functioning (especially as regards the working memory and attention). The expression of signs and symptoms widely differs between patients and along time in the course of illness. Then, the phenotype description of SZ is highly heterogeneous, and up to date there is neither biological marker nor anatomopathological evidence for the disorder. Therefore, its identification is exclusively based on clinical observations regulated by the Diagnostic and Statistical Manual of Mental Disorder.

The prevalence and incidence of SZ have shown variations according to how the disorder is defined. When SZ is considered in isolation, the lifetime prevalence and incidence are 0.30−0.66% and 0.10–0.22 per 1000 person-years, respectively (McGrath *et al*, 2008). However, a recent Finnish landmark study — allowing for a broad definition of psychotic disorder, including diagnostic categories such as delusional disorder, brief psychotic disorder, and the catch-all diagnostic category of psychotic disorder not otherwise specified — revealed a lifetime rate of SZ and related categories of 2.3% (Perala *et al*, 2007), rising to 3.5% if other psychotic disorders, such as BPD and substance-induced psychotic disorder, were included.

The existence of gender differences in the incidence of SZ has been a long subject to debate. Despite the fact that two independent systematic reviews have provided convergent evidence that the male-female ratio in SZ is 1.4:1 (Aleman *et al*, 2003; McGrath *et al*, 2004), a recent review suggests that there are no clear evidences of gender differences in the prevalence of the disorder (Ochoa *et al*, 2012).

As regards age at onset, a gender effect in age of onset is the most replicated finding in studies into gender differences in SZ (Galderisi *et al*, 2012; Goldstein *et al*, 1989; Gureje, 1991). In this sense, men usually tend to develop the illness at age 18–25, while in women, the mean age of onset is between 25–35 years.

Lastly, with respect with the age at onset of these disorders, it should be mentioned that the more severe clinical and cognitive profile associated to early onset forms has led to the definition of these cases as a group with more salient genetic factors and therefore, as a group of high interests to detect genetic factors associated with these disorders (Frangou *et al*, 2008).

In connection with the onset of the psychotic outbreak, SZ symptoms do not usually come totally out of the blue, and there are important changes that occur before the psychotic syndrome. Fragmentary psychotic symptoms, depression, changes in behaviour, attenuated general functioning, and other nonspecific features commonly occur in the weeks, months and sometimes years before the first psychotic breakdown. The period of subclinical signs and symptoms that precedes the onset of psychosis is referred to as the *prodrome* and is commonly characterized by accumulation of cognitive and functional impairments. This prodromal period can last from weeks to several years, and comorbid disorders are very common during this period (Rosen *et al*, 2006).

Premorbid functioning refers to the level of functioning prior to some pathological event. Furthermore, there are also other differences and abnormalities that occur before starting the risk period. This abnormal functioning that is preceding the occurrence of disease is known as *premorbid period*. Rather than being changes from the pre-existing state that herald the illness during the prodromal period, these *premorbid* features are more a long-standing part of the person, of his or her personality and early development. Premorbid and prodromal features may have theoretical importance because they seem to point toward early vulnerability or predisposing factors, rather than to events that occur as an illness is triggered or precipitated. The existence of premorbid abnormalities

and differences in those who will develop SZ some years later illustrates the longitudinal dimension of the illness (Figure 4).

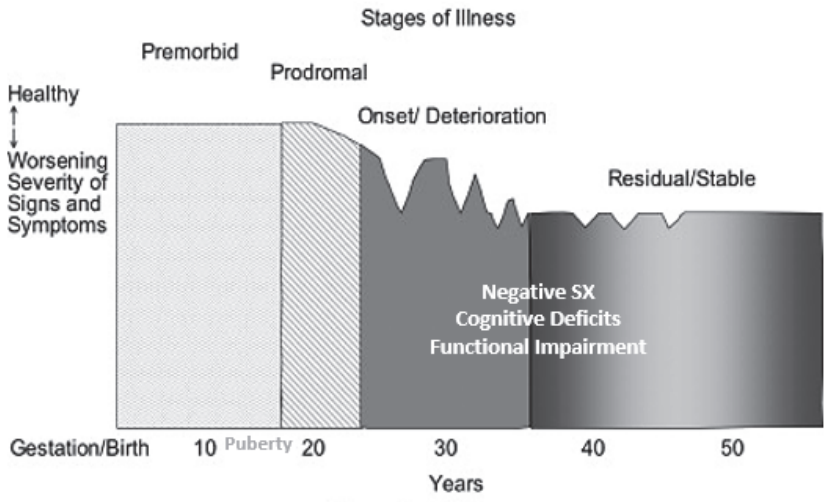

Figure 4. The natural course of schizophrenia evolution and its main stages of illness. Note that "Stage of Illness" describes all premorbid and morbid phases of the illness; increased severity of symptoms is represented along the *y* axis with worsening symptoms decreasing on this axis. *Adapted from* (Frankle *et al*, 2003).

Regarding the longitudinal course of the illness, SZ tend to follow a variable course. Some studies have suggested that cognitive function deteriorates over time (Bilder *et al*, 1991), whereas others have reported stability or even an improvement in some functions (Delisi *et al*, 1995; Rund, 1998). As an example, prospectively-designed outcome studies of first admission and first diagnosis of SZ with a follow-up of more than one year, have suggested that heterogeneity in outcome is common, with both good and poor outcomes being observed in less than 50% of patients. Therefore, the course and outcome of SZ is characterized mainly by unexplained heterogeneity rather than by uniformly poor outcome (Menezes *et al*, 2006), which apart from the clear clinical consequences, is also a reflection of the aetiological heterogeneity of this disorder.

1.2.2 Genetic factors

Research has mainly proceeded on the model of genetic heterogeneity and has been successful in defining rare genetic syndromes. However, research into psychiatric disorders is still a challenge with the purpose of trying to elucidate their genetic background. In this sense, our knowledge of the molecular mechanisms of psychotic disorders pathophysiology remains very incomplete due to the fact that their genetic architecture is enormously complex as compared with other genetic disorders.

Schizophrenia and other psychotic disorders belong to a group of pathologies known as complex genetic disorders by means of the complex mode of transmission, referring to that it is likely that several genes with weak to moderate effect jointly constitute a genetic basis for vulnerability to SZ. Moreover, epistatic interactions between genes and among their products, and interactions with environmental risk factors such as childhood maltreatment or cannabis exposure (Harley *et al*, 2010; Sideli *et al*, 2012), are considered highly plausible. In addition, current evidences suggest that the mutation frequency spectrum comprises a mix of common and rare mutations. All these concepts bring us an idea that is making strong inroads in the literature, referring to that complex disorders result from deviances in the function of individual genes, which finally converge to the dysfunction of entire molecular networks (Schadt, 2009).

Evidence from family, adoption and twin studies

Family, adoption and twin studies are different approaches for estimating the effect of familial and genetic influences on a disorder. Briefly, **family studies** seek to answer the question of whether a disorder of interest aggregates in families. In this sense, while evidence of familiality supports the possibility that genes are contributing to the disorder, family studies cannot determine the specific role of genes or estimate the magnitude of their influence. Secondly, **adoption studies** help to distinguish genetic and environmental influences on family resemblance by comparing rates of a disorder in biological family members (genetically related) to those in adoptive family members (environmentally related). In essence, adoption studies let us know that if genes influence the risk of a disorder, the biological family members will have more resemblance than do adoptive family members. Thirdly, **twin studies,** can help to assess the genetic and environmental contributions to the variance in liability to the disorder (Kendler, 2001). In other words, twin studies let us to estimate the *heritability* of the disorder which is an estimation of the proportion of phenotypic variance observed in the

population that can be attributed to genetic influences.

As it was mentioned, evidence on that schizophrenia and other schizophrenia-spectrum disorders run in families, led to a number of family, twin and adoption studies to elucidate the genetic background of the disorder. Thus, broadly speaking, **family studies** have confirmed an increased prevalence risk for SZ and related disorders in family members. By pooling the results of several studies, Gottesman showed the risks for developing SZ in first-degree, second-degree and third-degree relatives of patients with the disorder (Gottesman *et al*, 1991). This famous study showed that the individual risk depends on the number of shared genes between family members and their affected relative (Figure 5). Moreover, this study also highlights that the risk of a family member developing SZ markedly increases when two or more family members have the illness, with a lifetime expectancy for SZ of 17% for siblings with one sib and one parent with SZ and up to 46% in children of two parents with SZ.

Other family studies, using standardized methods of assessment, have reported lower lifetime risks for SZ among first-degree relatives than those found in earlier studies. Specifically, Kendler et al. (Kendler *et al*, 1985) reported a lifetime expectancy of only 3.7% among first-degree relatives when using DSM-III criteria for SZ. However, this must be compared with a lifetime risk among controls of 0.2%. Moreover, a review of family studies found a wide range (from 3.1% to 16.9%) of lifetime risks reported in first-degree relatives of probands with SZ (Gershon *et al*, 1988). The reason for these highly variable results appears to be the use of different diagnostic criteria.

Lastly, a recent study based on Swedish national registers (including a population-based cohort of 7.739.202 individuals of known relatedness) showed that the risk estimates for all relative types were strikingly similar to those reported in smaller and older studies (Lichtenstein *et al*, 2006). For example, the risk for first-degree relatives was around 10% (siblings=8.55, parents=9.43, offspring=10.3) (Figure 5). All these findings strongly support the familial nature of SZ, but they do not confirm a genetic over a familial environmental cause.

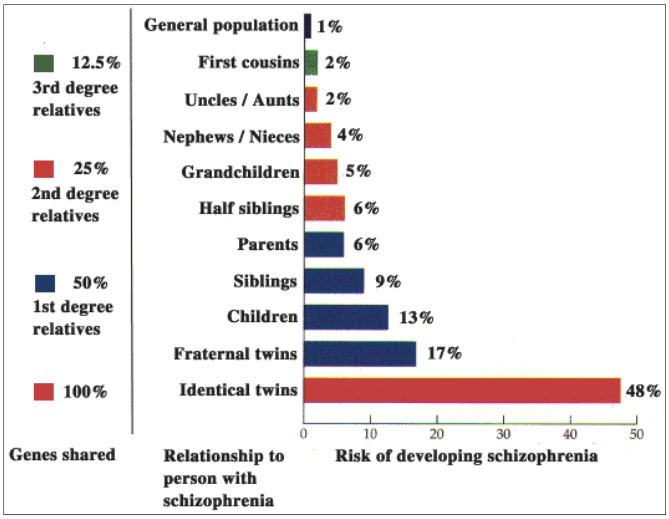

Figure 5. Average risks for developing Schizophrenia for the relatives of Schizophrenia patients. As an example, those who have a third degree relative with SZ are twice as likely to develop SZ as those in the general population. *Adapted from Gottesman, 1991.*

On the other hand, **adoption studies** have provided compelling evidence that SZ has an important genetic component. The first adoption studies compared the rate of SZ between adoptees with mothers with SZ and adoptees with well mothers. These studies showed that 4-10% of the offspring of parents with SZ developed schizophrenia themselves, compared with none of the controls (Kety *et al*, 1976). The most recent adoption study reported a SZ lifetime prevalence of 9.4% in the adopted-away offspring of parents with SZ and a lifetime prevalence in control adoptees of 1.2% (Tienari *et al*, 2000).

An alternative approach is to compare the rate of SZ among the biological and the adoptive relatives. With this strategy, it has been found that 20% of the biological parents of adopted-away SZ patients had schizophrenia-spectrum disorders, compared to 6% of adoptive parents and parents of control adoptees (Kety *et al*, 1976a).

Apart from family and adoption studies, **twin studies** constitute one of the most powerful methods used to disentangle genetic from environmental sources of resemblance between relatives (Boomsma *et al*, 2002; van Dongen *et al*, 2012). Briefly, the main strategy is to compare the disease concordance between members of monozygotic (MZ) twin pairs and members of dizygotic (DZ) twin pairs. As MZ twins are genetically identical, whereas DZ twins share on average 50% of their genes, greater MZ than DZ concordance will reflect genetic influence. Despite using a variety of methods, twin studies have been remarkably consistent in demonstrating high concordance rates for MZ twins (Farmer *et al*, 1987; McGuffin *et al*, 1984; Onstad *et al*, 1991). Rates have

averaged 48% in MZ as compared with 14% in DZ twins across multiple studies, with only one negative twin study (Tienari, 1963).

More recent studies, in which cases have been defined using operational criteria, have described concordance rates of 42-43% in MZ and 0-4% for DZ (Cardno and Gottesman, 2000; Cardno *et al*, 1999). However, the failure of MZ twins to be 100% concordant suggests that the genetic component may be necessary but is insufficient to cause SZ, and therefore, environmental factors are also important. At all events, and despite some variation in results, twin studies have reported high heritability estimates of ~80% (Cardno and Gottesman, 2000; Cardno *et al*, 1999; Plomin *et al*, 1994; Sullivan *et al*, 2003), which means that around 80% of the variation in liability is attributable to genetic factors.

Thus, it is clear from family, twin and adoption studies that there is a genetic contribution to SZ. Moreover, these studies have also shown that SZ shares an important familial liability with other psychotic illnesses (Kendler and Diehl, 1993), known collectively as the schizophrenia-spectrum disorders (SSD).

Evidence form molecular genetic studies

Epidemiological and quantitative genetics studies (family, twin, and adoptive) mentioned above have been attempted in a large number of psychiatric disorders. However, these studies do not provide information about what genes are involved in the disorder. In this regard, molecular studies are necessary to begin to elucidate these data. **Linkage and allelic association studies** are frequently methods used to find possible susceptibility genes (DNA susceptibility variants) involved in complex psychiatric disorders.

Firstly, **linkage studies** follow the idea of a hypothesis-neutral search for markers that statistically segregate with a disease, followed by fine mapping to identify the actual gene or genes. In this sense, main results of linkage studies in SZ seem to indicate that despite of the difficulty of finding consistent replication across studies, many candidate regions have been identified highlighting the regions including on chromosomes 1q, 2q, 5q, 6p, 8p, 13q, 10p, 10q and 22q (Craddock *et al*, 2005; Lewis *et al*, 2003; Ng *et al*, 2009) (Table 2).

Table 2. Brief summary of putative genetic linkage regions for SZ based on meta-analysis by Ng et al (2009) (adapted from SZGene database (www.szgene.org).

Chromosome	Location (Mb)
1p32.2-31.1	57.3-84.6
1p13.2-q23.3*	114.6-162.1
2q12.1-21.2*	103.3-134.0
2q21.2-31.1*	134.0-169.9
2q33.3-36.3	206.3-228.3
3p14.1-q13.32	71.6-120.2
5q31.3-35.1*	141.8-167.7
5q35.1-35.3	167.7-180.4
6p21.31-12.1	33.9-56.6
8p22-12*	15.7-32.7
10q26.12-26.3	123.1-135.1
16p13.12-q12.2*	13.2-51.5

Asterisks (*) indicate which regions were also associated with the previous Genome Search Meta-Analysis (GSMA) of Lewis *et al*, 2003.

Secondly, **association studies** basically ask whether particular variants of a candidate gene are more prevalent in patients than would be expected by chance. That is, unlike linkage, association studies have a higher spatial resolution and sufficient power to detect the small effect of common genetic variants (Mantripragada *et al*, 2010).

According to the SZGene database (www.szgene.org), a meta-analysis including 1727 association studies published in specialized articles, identified 43 candidate genetic variants ("top candidates") highly associated to SZ phenotypes (Allen *et al*, 2008). Moreover, a meta-analysis from Shi et al. (Shi *et al*, 2008) showed significant allelic associations across different populations in genes involved in the metabolism function of key neurotransmitters (e.g. *DAO, DRD4, PPP3CC*, serotonin transporter *SLC6A4*) as well as genes related to DNA methylation (e.g. *MTHFR*), apoptosis and neurodevelopment (e.g. *TP53*). In addition, other studies also found genes involved in the regulation of neurotransmitters implicated in the disorder, such as *COMT gene* (Chen *et al*, 2004; Shifman *et al*, 2004), *DTNBP1* (Maher *et al*, 2010; Straub *et al*, 2002), or *RGS4* (Chowdari *et al*, 2002; Talkowski *et al*, 2006) and functions related to neural development such as *NRG1* (Munafo *et al*, 2007; Stefansson *et al*, 2002) or *DISC1* (Pletnikov *et al*, 2008; Schumacher *et al*, 2009).

In connection with association studies, it has to be taken into account complementary strategies that try to face the number of challenges that arise from the genetic and

phenotypic complexity of mental disorders. Hence, strategies have been developed to identify elementary neurobiological components of schizophrenia-spectrum disorders that are susceptible to measurement and related to the genetic risk of the disease. These components or characteristics are called **endophenotypes** or intermediate phenotypes (Egan and Goldberg, 2003; Freedman *et al*, 1999; Gottesman and Gould, 2003). Thus, endophenotypes can improve our power to detect genes influencing risk of illness by being genetically simpler, closer to the level of gene action and also, by providing added statistical power through their ability to quantitatively rank people within diagnostic categories (Glahn *et al*, 2014). In this regard, neuroimaging (Glahn *et al*, 2007b) and cognitive endophenotypes (Glahn *et al*, 2007a; Gottesman and Gould, 2003), such as fMRI response of the dorsolateral prefrontal cortex (Callicott *et al*, 2003) and working memory performance (Egan *et al*, 2001), are examples of heritable traits that are proposed for genetic studies of neuropsychiatric diseases

On the other hand, the current genomics era provides new opportunities to explore the genetic etiology of psychiatric disorders at a whole genome level, using a hypothesis-free approach. In this sense, the main goal of psychiatric genetics is to discover loci that are robustly and repeatedly associated with a disorder and thereby gain insight into etiology. In this context, Genome-wide association studies (GWAS) assess Single Nucleotide Polymorphisms (i.e. SNPs) at several hundred thousand positions in the genome. The experimental paradigm of GWAS involves the identification of individual variants associated with case-control status (Manolio, 2010). Several such studies have been published across, which data could be pooled for meta-analysis (Purcell *et al*, 2009; Shi *et al*, 2008; Stefansson *et al*, 2009). The authors of these meta-analyses conclude that GWAS provide molecular genetic evidence for a substantial polygenic component to the risk for SZ, one involving thousands of common alleles of very small effect (Lee *et al*, 2012; Purcell *et al*, 2009). In addition, GWAS have also allowed identifying some specific genes involved in SZ. In this regard, a GWAS involving about 21,000 cases and 38,000 controls by the Psychiatric Genetics Consortium (PGC) (Ripke *et al*, 2013a), identified 22 loci which contain SNPs genome-wide significant association with SZ. Furthermore, a recent study also from the PGC on an enlarged sample (PGC2: almost 37000 cases and 113000 controls) has begun to reveal additional genes, identifying 108 loci (implicating about 600 genes) that are genome-wide significant for SZ (Ripke *et al*, 2014). In connection with this finding, some of the associations include the dopamine D2 receptor (*DRD2* – the ultimate SZ candidate gene) and several glutamate receptors (*GRIN2A,*

GRIA1, GRM3) highlighting the implication of the glutamatergic neurotransmission and synaptic plasticity as a potential therapeutic target. In addition, associations at *CACNA1C*, *CACNB2*, and *CACNA1I*, which encode voltage-gated calcium channel subunits, extend also previous findings implicating members of this family of proteins in SZ and other psychiatric disorders. However, popular associations in the literature before 2007 (e.g., *COMT, DRD3, HTR2A, NRG1, BDNF, DTNBP1* and *SLC6A4*) have generally not being found in GWAS studies (Collins *et al*, 2012).

Lastly, in addition to the evidence of the involvement of many common genetic variants which each make a small contribution to the risk, rare variants (i.e. copy number variants, (CNVs) or point mutations) have also been related with the risk for SZ (Malhotra and Sebat, 2012; Rees *et al*, 2014; Sullivan *et al*, 2012a). These variants seem to be rare (<0.1% in controls), potent (odds ratios of 4–20), and often non-specific risk factors for psychiatric disorders (Sullivan *et al*, 2012b). In this respect, it is important to highlight that much of the speculation about missing heritability from GWAS has been focused on the possible contribution of these variants. As common polymorphisms are unlikely to explain all the genetic risk for common disorders. In this sense, an evolutionary model of complex diseases (Pritchard, 2001) predicts roles for common SNPs and for multiple rare variants in some genes. Rare variants are expected to cause more detrimental effects on disease risk than the common DNA sequence variants. As shown in Figure 6, the occurrence of these events seems to be low, but the associated risk ratio for the development of a disorder is expected to be quite high.

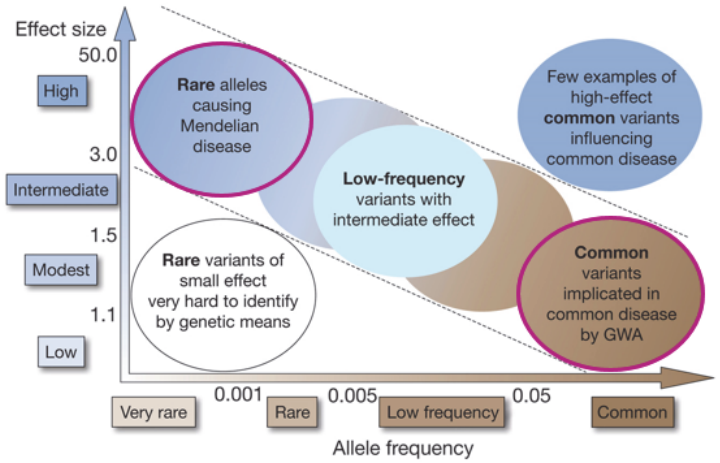

Figure 6. Feasibility of identifying genetic variants by risk allele frequency and strength of genetic effect (odds ratio). *Adapted from (Manolio et al, 2009).*

To this respect, rare variants typically show high, but incomplete, penetrance for SZ, and may also lead to other psychiatric disorders such as BPD, major depression, autism or attention deficit hyperactivity disorder, as well as to epilepsy and mental retardation (Mitchell and Porteous, 2011). These findings are consistent with studies showing increasing comorbidity in autism, BPD and epilepsy within SZ families (Cardno *et al*, 2002; Lichtenstein *et al*, 2009a; Qin *et al*, 2005) and provide additional evidence of etiological overlap.

In summary, the combined evidences of quantitative and molecular genetic studies strongly support the involvement of genetic risk factors. Although the mode of inheritance of SZ and related disorders is still unknown, technological advances are providing further clues to the genetic basis of the disease and enabling researchers to narrow the boundaries in models of genetic architecture that are consistent with the observed data. Then, data from current genome-wide approaches already reveal an important proportion of genetic variation in SZ and show that the underlying causal variants include common variants of small effect as well as rare variants of larger effect (Wray *et al*, 2011), which in combination with environmental stressors may lead to the development of the disorder.

1.2.3 Linking genes to the physiopathology

A common way in which candidate genes are identified for further association analyses is by combining linkage, functional data and genome-wide association studies to identify genes with a function plausibly related to the phenotype. As mentioned before, recent studies have shown an increased number of specific associated genes and gene pathways, giving new insights into the variants and genetic mechanisms underlying SSD. However, even many vulnerability genes have been identified, none have been conclusively linked to SSD (Pearlson and Folley, 2008). It has been postulated that the underlying pathophysiology of developing SZ is, at least in part, a progressive process where multiple pathways are involved. In this sense, identifying genes provides a guide to explore more about the biological processes through which genetic influences lead to psychopathology.

As has been indicated in the previous section (1.2.2), current evidences suggest that individuals with SZ carry risk genetic variants (including both, common and rare), which impact into their neurodevelopmental mechanisms, and subsequently results in inefficient or disturbed neuronal communication later in life (Pearlson and Folley, 2008). These genetic variants may ultimately lead to the manifestation of psychiatric disorders and/or to the modulation of their presentation (symptoms, cognitive performance, treatment response, etc), through small effects on neurotransmitter function, brain metabolism, cerebral structural and functional organization, or connectivity, as well as they interact with other non-genetic factors (environment) (Figure 7). However, one of the main challenges is to establish the biological mechanisms that connect DNA sequence variants with the variability in the expression of the phenotype; that is, understanding the specific role of a gene or a set of genes (either defined by candidate gene or GWAS approaches) in the pathophysiology of the disorder.

In this view, it is essential to disentangle the molecular genetics mechanisms involved in transcriptional and translational processes and how DNA sequence variability can generate deviances on them. Thereby, the use of technologies that probe the transcriptome and epigenome in post-mortem Central Nervous System (CNS) from subjects with SZ is a useful approach to attempt to identify changes in gene expression that are associated with the pathophysiology of the disorder. Gene expression profiling of human postmortem brains has shown alterations in the expression of genes associated with synaptic transmission in SZ patients (Mistry *et al*, 2013). In this sense, it is interesting

to note that, as an example, as genetic variability of *DTNBP1 (Dysbindin-1)* gene has been associated with reductions of the mRNA levels of *DTNBP1* in the dorsolateral prefrontal cortex of SZ patients as compared to controls (Tang *et al*, 2009). Similarly, when another candidate gene such as *BDNF* gene is considered, some SNPs have been associated with the risk for SZ and also with *BDNF* methylation differences between patients and healthy subjects (Rosa *et al*, 2006).

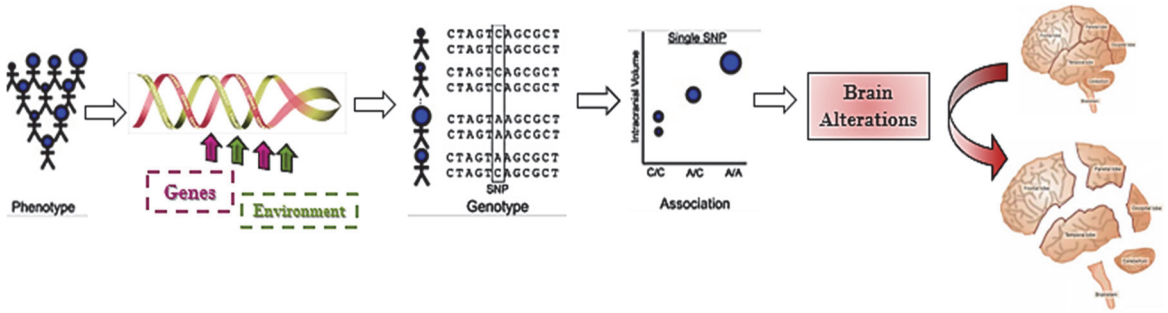

Figure 7. Schematic figure showing the link between genetic/environmental implication, and their pathophysiological consequences in the brain. For instance, genetic associations have been implicated with specific biological pathways affecting brain structure, such as connectivity.

In the same way, it is necessary to establish whether DNA sequence variants and deviances in the transcriptome and proteome observed in SZ do have an impact on brain structure and/or function.

On the one hand, many differences in brain structure and function have been reported in SZ patients as compared with controls, although there is considerable inter-individual heterogeneity. Interestingly, recent meta-analyses found that those people who are affected with SZ have smaller hippocampus, amygdala, thalamus, nucleus accumbens and intracranial volumes, along with larger pallidum and lateral ventricle volumes (van Erp *et al*, 2016; Haijma *et al*, 2013). Moreover, other neuroimaging studies also suggest that there are progressive changes in grey (Vita *et al*, 2012) and white matter (Mori *et al*, 2007), ventricular size (Saijo *et al*, 2001), the cortex (Palaniyappan, 2017), the hippocampus (Ho *et al*, 2017) and the deep brain nucleus (Wang *et al*, 2008) in SZ patients. Thus, linking the genes associated with SZ to these brain abnormalities related with SZ contributes to the understanding of the underlying biological mechanisms. Taking again the *DTNBP1* gene as an example, this gene has also been selected as an adequate candidate gene in a number of neuroimaging genetics studies. These studies have shown the influence of some *DTNBP1* SNPs on reduced occipital and prefrontal brain volumes (Donohoe et al., 2009), as well as on cortical thinning (Narr et al., 2009). Genetic variants at *DTNBP1* have also been associated with brain activation during

language production and working memory tasks in healthy subjects (Markov et al., 2009; Markov et al., 2010).

On the other hand, increasing data seems to indicate a complex picture of neurotramission balances/imbalances and synaptic signaling related to SZ etiology. As an example of a well-reported gene that has allowed researchers to establish the link from genetics to SZ pathophysiology through different study levels, there is the *COMT* gene (Catechol-*O*-methyl transferase, an enzyme involved in the degradation of cathecolamines). In detail, COMT-dependent dopamine degradation is of particular importance in brain regions with low expression of the presynaptic dopamine transporter (DAT), such as the prefrontal cortex (Matsumoto *et al*, 2003). Different studies have reported the association of a functional polymorphism ($COMT_{Val158Met}$) with either cognitive phenotypes, such as Executive function (Egan *et al*, 2001), brain expression (Bray *et al*, 2003; Shifman *et al*, 2002) or neuroimaging, relating this SNP to activation and de-activation in the prefrontal cortex (Pomarol-Clotet *et al*, 2010). Given the unequivocal effect of the Val158Met polymorphism on the functionality of COMT, and the evidence for a critical role of dopamine in the pathophysiology and treatment of psychosis, this gene has been placed near the top of a long list of plausible candidate genes for SZ.

Moreover, other relevant example to be mentioned comes from the last GWAS conducted by Ripke *et al* (2014), evidencing that one of the most significant locus associated with SZ is the Complement component 4 (C4), within the Major Histocompatibility Complex (MHC). C4 is present in the neuronal synapses, dendrites, axons and cell bodies. In this sense, a recent and interesting study that allows connecting genetic variability with neuronal phenotypes related with SZ is the one conducted by Sekar and colleagues (Sekar *et al*, 2016). This study show that a genetic variant of *C4* gene is related to an increased risk for SZ and also, with an increased expression level of the gene. Likewise, this study also show that, in animal model, there is a relationship between a higher expression of the gene and an increased synaptic pruning. Therefore, these findings enable researchers to establish a link from genetic variability of C4 gene to the reduction in the number of synapses associated with SZ.

In short, understanding the full extent and functional consequences of both, the molecular changes and brain alterations will be critical in gaining greater insight into the pathophysiology of SZ and SSD.

1.2.3.1 The role of synaptic plasticity: *NRN1*, as an example of candidate plasticity gene

The human brain undergoes continuous structural and functional remodeling in response to signals originating from inside and outside of the body. At a molecular level these changes can be very subtle and involve minor modifications of synaptic proteins. Specifically, **synaptic plasticity** refers to the biological processes by which specific patterns of synaptic activity result in changes in synaptic strength. In this sense, both pre-synaptic and post-synaptic mechanisms can contribute to the expression of synaptic plasticity.

A growing body of evidence has promoted the view of SZ as a disorder of synaptic plasticity (Hall *et al*, 2015). In this regard, the *disconnection hypothesis* supported by some authors, suggest that the core pathology of SZ is an impaired control of synaptic plasticity that manifests as abnormal functional integration of neural systems (i.e. dysconnectivity) (Frankle *et al*, 2003; Friston, 1998). In other words, the synaptic dysfunction has been hypothesized to result from a dysfunctional synaptic structure and transmission, affecting the adequate brain development and leading to an abnormal connectivity. Recently, this hypothesis was updated in the light of new experimental findings, particularly from genetic studies (Stephan *et al*, 2006).

Initial evidence for synaptic involvement in the etiology of SZ was indirectly, being substantially based on the efficacy of pharmacological agents to either improve or induce psychotic symptoms through their action on the neurotransmitter system. Firstly, the interest was focused on dopamine following the observation that all the effective medications in current use block dopamine D2 receptors. In this sense, the efficacy was correlated with D2 receptor affinity (Creese *et al*, 1976); and psychotic symptoms could be induced through repeated amphetamine use. Later, models based on the alteration of N-methyl-D-aspartate receptor (NMDAR) function were also developed (Javitt and Zukin, 1991; Kim *et al*, 1980; Olney and Farber, 1995). Moreover, theories concerning the role of other neurotransmitters have also proliferated, with c-aminobutyric acid (GABA)ergic, serotonergic, metabotropic glutamatergic, muscarinic and nicotinic signaling linked to various aspects of disease (Abi-Dargham and Guillin, 2007; Freedman, 2003; Lewis *et al*, 2005). Therefore, compelling data seems to indicate a complex picture of neurotramission balances/imbalances and synaptic signaling related to SZ etiology.

Besides evidences from neurotransmission systems, the positive findings from neuropathological studies show reasonable evidence about the existence of alterations in the cytoarchitecture of several brain areas, notably the hippocampus, the prefrontal cortex, and the dorsal thalamus where neurons, dendrites, synapses, and oligodendrocytes are affected (Owen *et al*, 2005). Taken together, the findings implying an alteration in cortical circuitry, may represent the anatomical basis of aberrant connectivity that has been inferred from neuropsychological and imaging studies.

In addition, functional coupling between brain areas can be abnormal because their anatomical connections are altered. Alternatively, functional coupling can be disturbed due to impairments in synaptic transmission. Furthermore, functional coupling has been related to be a function of experience-dependent synaptic plasticity (Zhang and Poo, 2001). Thus, pathological changes in synaptic plasticity have a direct impact into the synaptic computation which controls the information flow through the neural microcircuits responsible for the information processing necessary to drive adaptive behaviors.

Consistent with all the above mentioned, many of the genes that have been recently identified by the most recent GWAS in SZ (Ripke *et al*, 2014) are known to have functions in synaptic plasticity and neurotransmission. In addition, subsequent formal gene enrichment analyses have consistently shown the enrichment of synapse-related and neuronal signaling pathways in SZ (Network and Pathway Analysis Subgroup of Psychiatric Genomics Consortium, 2015). Also, gene expression profiling of human postmortem brains has shown alterations in the expression of genes associated with synaptic transmission in SZ patients (Mistry *et al*, 2013). In light of this knowledge, several genes associated with SZ are involved in both establishing long-range connections during development and also in regulating synaptic plasticity (i.e. *NRG1* or dysbindin) (Harrison and Weinberger, 2005). In this sense, researchers suggest that pathological alterations in synaptic plasticity mechanisms may critically underlie symptoms of SZ and other mental disorders.

A group of proteins called neurotrophins (or Neurotrophic Factors, NTF) are considered powerful molecular mediators of central synaptic plasticity. As they are important regulators of neural survival, growth, development, function, and plasticity (Huang and Reichardt, 2001), an inadequate neurotrophic support in the brain could lead to an inappropriate cortical circuitry and synaptic transmission in the developing brain

(Favalli *et al*, 2012). Moreover, NTFs and glutamate seem to interact to regulate developmental and adult neuroplasticity. For example, it is known that glutamate stimulates the production of brain-derived neurotrophic factor (*BDNF*) which, in turn, modifies neuronal glutamate sensitivity, Ca^{2+} homeostasis and plasticity. Thus, NTFs can modify glutamate signaling directly, by changing the expression of glutamate receptor subunits and Ca^{2+} (Frankle *et al*, 2003). In this sense, although the molecular and neuronal dysfunction that leads to psychosis and SZ remains poorly understood, there is a growing body of knowledge supporting theories of dysregulation of neurotransmitter systems including hyper-function of dopaminergic systems and hypo-function of glutamatergic systems (principally NMDA receptors) which have been proposed as candidates underlying altered synaptic plasticity (Baldessarini and Tarazi, 2005; Blum and Mann, 2002).

Interestingly, among the group of neurotrophins, brain-derived neurotrophic factor (BDNF) as well as neurotrophin-3 (NT-3) have emerged as having key roles in the neurobiological mechanisms related to psychiatric disorders and, more specifically, to learning and memory. Also in the group of NTFs, we could also highlight the nerve growth factor (NGF) and mainly, the protein Neuritin 1 (*NRN1*) also called candidate plasticity-related gene 15 (CPG15), which is part of the focus of the present thesis (for more information, see section Candidate gene approach 1.4.1).

Neuritin 1 (*NRN1*)

NRN1 gene is located at 6p25.1 about 6 Mb from the telomere. This gene encodes a small highly conserved protein (142-amino acids long; aa) and contains both, a predicted 27-aa secretory signal peptide at its N-terminus and a 27-aa glycosylphosphatidylinositol (GPI) anchor at its C-terminus (from aa 117 to aa 142). Specifically, Neuritin 1 is a soluble protein attached to the extracellular neuronal membrane by a GPI link and operates as an intercellular signal between neighboring neurons (Naeve *et al*, 1997). As a result of *in vitro* assays, it has been demonstrated that this protein promotes neurite outgrowth and arborisation, dendritic outgrowth, and axonal outgrowth (Fujino *et al*, 2011; Leslie and Nedivi, 2011; Loebrich and Nedivi, 2009; Nedivi *et al*, 1998), which suggests an important role in promoting neuritogenesis, a key process required during development of the brain whereby the differentiating neuron extends processes called neurites, which in turn extend into an axon and several dendrites. As will be discussed in detail below, like

other neurotrophic factors, *NRN1* has multiple roles during nervous system development although it has no sequence homology with traditional neurotrophin ligands.

Regarding its expression patterns, during embryonic neural development, neuritin is mainly expressed in brain regions that undergo a rapid proliferation of neuronal progenitor pools, suggesting a protective role of neuritin for differentiated neurons (Putz *et al*, 2005; Sato *et al*, 2012). Moreover, its expression level remains elevated after birth or even increases, especially in brain regions exhibiting high neural activity and synaptic plasticity, such as the hippocampus, visual cortex, and external granular layer of the cerebellum (Fujino *et al*, 2008; Lee and Nedivi, 2002; Naeve *et al*, 1997; Putz *et al*, 2005) (Figure 8). Thus, *NRN1* continues to be expressed in the adult brain (in postmitotic-differentiating neurons), where its expression is correlated with activity-dependent functional synaptic plasticity (Flavell and Greenberg, 2008; Harwell *et al*, 2005). At the neuronal level, NRN1 protein is concentrated in axon tracts (Nedivi *et al*, 2001) and is distributed inside the axon as well as on the axon surface (Cantallops and Cline, 2008).

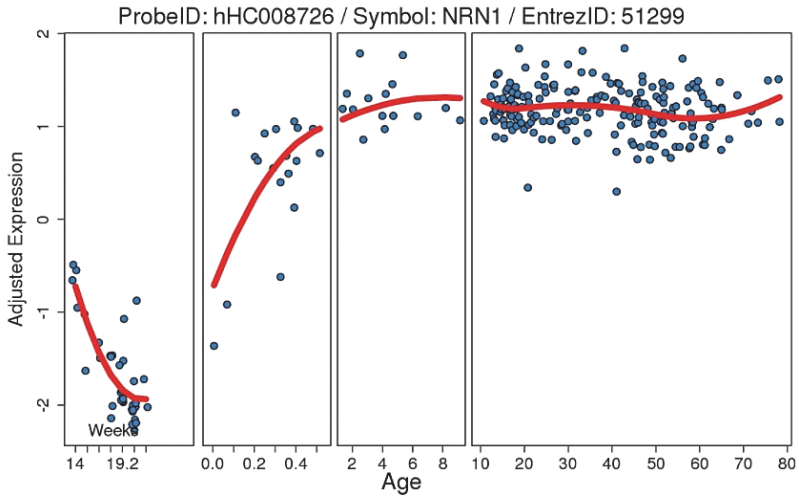

Figure 8. ***NRN1* expression pattern from early development to adult age in the human dorsolateral prefrontal cortex**. (Note that this is image has been generated using BrainCloud (http://braincloud.jhmi.edu/ (Colantuoni *et al*, 2011)), a freely-available, biologist-friendly application for exploring temporal dynamics and genetic control of transcription in the human prefrontal cortex across the lifespan).

Particularly, *NRN1* gene is considered an immediate early gene (IEG) which means that its expression is activated by a specific pathway that responds very quickly to regulatory signals (e.g. immune responses or cellular stress). Thus, the expression of this protein can be induced in response to neuronal activity and by neurotrophins such as *NGF*, *BDNF* and *NT3*. In this sense, it is well reported how the expression of *NRN1* gene is regulated by the neurotrophin *BDNF* (Naeve *et al*, 1997), which promotes the

differentiation and growth of developing neurons in central and peripheral nervous systems (Buckley *et al*, 2007).

However, *NRN1* is not only an activity-regulated gene that requires action potential activity for maintenance of synaptic plasticity of the adult brain but it is also expressed in an activity-independent manner during early brain development before circuit formation and maturation, suggesting that it may have different functions. In detail, during early embryonic development, *NRN1* acts as a survival factor for neural progenitors and differentiated neurons (Putz *et al*, 2005). Later in development, *NRN1* promotes growth and stabilization of axonal and dendritic arbors along with synapse formation and maturation (Harwell *et al*, 2005) (Figure 9).

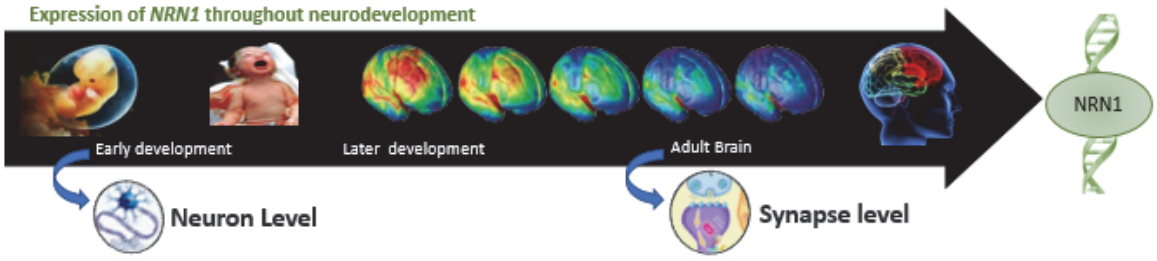

Figure 9. *NRN1* expression throughout neurodevelopment. Note that during the early development has a function mainly located at the neuron level while in the adult brain has also a role in synapse formation and maturation.

Furthermore, the transcriptional activation of Neuritin is a key link between neuronal activity and long-term synaptic plasticity. In this light, the specific mechanisms of activation of this gene are related with the fact that *NRN1* is an immediate early gene (IEG). As an immediate early gene, is induced by Ca2+ influx through N-Methyl-Daspartate receptors (NMDA) and L-type voltage-sensitive calcium channels. Neuritin expression requires convergent activation of the Ca2+/calmodulin-dependent protein kinase and mitogen-activated protein kinase pathways. Although activation of protein kinase A is not required for activity-dependent expression, neuritin is induced by cAMP in active neurons. cAMP response element-binding protein binds the neuritin promoter *in vivo* and partially regulates its activity-dependent expression (Fujino *et al*, 2003). In that connection, Cantallops et al. (2000) showed that *NRN1* expression significantly increased the growth rate of retinal axons and also promoted synaptic maturation by recruitment of functional AMPA receptors to synapses. The same study, also showed that while NMDA receptor-mediated remained unchanged in response to increased *NRN1* levels, AMPA receptors were significantly increased. Then, this gene is playing a

role in the glutamatergic signaling through NMDA receptors, whose hypofunction in limbic brain structures has been associated with the neurobiology of SZ involving the postsynaptic NMDA and/or AMPA subtypes of glutamate receptors (Rubio *et al*, 2012).

Interestingly, as a candidate gene implicated with synaptic plasticity, *NRN1* gene has already been involved in the risk for mental disorders and associated phenotypes. On the one hand, previous studies have reported the effect of *NRN1* polymorphic variation on the risk for developing SZ and on general cognitive performance (Chandler *et al*, 2010).

As a gene sensitive to environment, a recent epigenetic study has found that *NRN1* is differentially methylated in SZ patients compared to controls. Particularly, by means of genome-wide quantification of DNA methylation in prefrontal cortex, Pidsley and colleagues have found that 29 adjacent CpG sites spanning the gene body of *NRN1* appear consistently hypomethylated in SZ patients compared with controls (Pidsley *et al*, 2014a). Then, this finding of methylation differences suggests that *NRN1* could also be differentially expressed in patients.

On the other hand, from animal model based studies, there is evidence of *NRN1* relationship with depressive symptoms. Specifically, a study led by Son et al (2012) found that chronic unpredictable stress decreases neuritin expression in the hippocampus. Moreover, a Neuritin-1 knockdown model results in depressive-like behaviors (Son et al., 2012). Other study showed that electroconvulsive therapy, which is one of the most robust gene inducer among all antidepressant treatments (Segi-Nishida, 2011), induces changes in both *NRN1* and *BDNF* expression (Dyrvig *et al*, 2014). Therefore, it seems that Neuritin mRNA is regulated by electroconvulsive seizure therapy (Newton *et al*, 2003). Third, fluoxetine increases the level of *NRN1* and *BDNF* specifically in the prefrontal cortex, hippocampus and dentate gyrus (Alme *et al*, 2007), which suggest that antidepressant treatment promotes gene expression responses linked to *NTFs* signaling and synaptic plasticity. Thus, these studies demonstrate a function of neuritin in models of stress and depression and a role for neuroplasticity in the response to antidepressant treatment and related behaviors. In this sense, developing strategies to target neuritin or related signaling pathways could be an interesting approach for improved antidepressant treatment

In short, *NRN1* gene can be considered to have pleiotropic effects due to its ability to affect multiple phenotypes mainly related with neurodevelopment and synaptic

plasticity (Figure 10). However, it should be noted that the neuritin receptor has not been identified yet and therefore the physiological functions of neuritin through its receptor and the related intracellular signal transduction mechanisms remain still unclear.

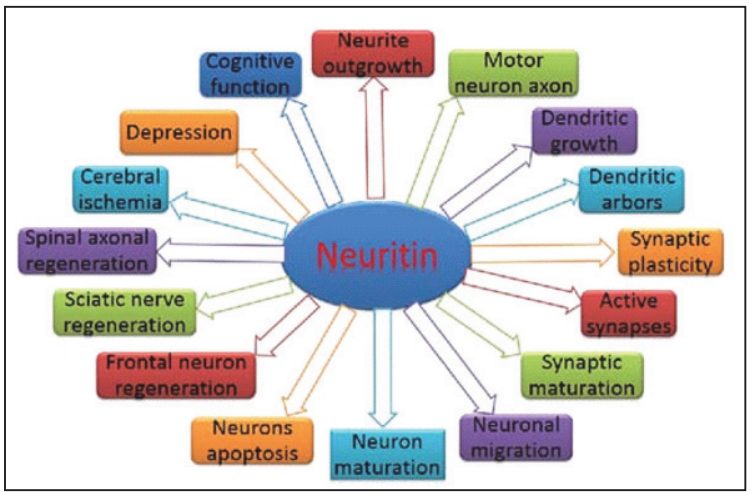

Figure 10. Pleiotropic effects of Neuritin on nervous system physiology and pathology *(Adapted from Zhou and Zhou (2014))*

Considering both, the important role that NTFs have in SZ and in neurodevelopmental related disorders, and also due to all the above-mentioned evidences, *NRN1* gene has been considered to be a strong candidate to be studied in the present thesis (for a candidate approach information: see section 1.4).

1.2.3.2 The role of white matter: The involvement of white matter related genes across neurodevelopmental disorders

It is well known that the central nervous system (CNS) consists of a central cavity surrounded by grey and white matter**. Grey matter (GM)** is a major component of the CNS, consisting of neuronal cell bodies (un-myelinated neurons), glial cells, dendrites and axon terminals, so it is where all synapses are located. Thus, grey matter contains most of the brain's neuronal cell bodies. Then, there is the **White matter (WM)**, which contains the connections between specialized processing regions and comprises approximately 50% of the human brain (Arai and Lo, 2009; Harris and Attwell, 2012). In detail, WM is composed of bundles myelinated axons connecting different parts of grey matter regions to each other and carries nerve impulses between neurons. Myelin, the lipid that forms a thin layer, around the axons providing electrical insulation is white in colour, giving rise to the name white matter. (Figure 11). To this respect, myelination refers to a developmental process, with white matter showing a clear linear increase throughout childhood and adolescence. The maximum WM volumes often reached as late as the third decade of life (Pfefferbaum *et al*, 1994). Interestingly, developmental processes for the ulterior brain function are taking place when most psychiatric disorders have their age of onset (Paus *et al*, 2008). The main function of the WM is to transmit information to and from the grey matter, while the grey matter is more related to process information in the brain.

It should be noted that apart from neurons, **glial cells** are the most abundant cell types in the central nervous system. The glial cells, also known as supportive cells, include oligodendrocytes, astrocytes, ependymal cells, Schwann cells, microglia and satellite cells. Unlike neurons, glial cells do not conduct electrical impulses, and are broadly defined as non-neuronal cells that maintain homeostasis or the local environment. In this sense, considering also that one of the functions of the glial cells is to transport nutrients and energy to the neurons, it is not wrong to think that abnormalities in these cells can lead to influence in how well the neurons function and communicate, affecting the white-grey matter dialogue or interconnection.

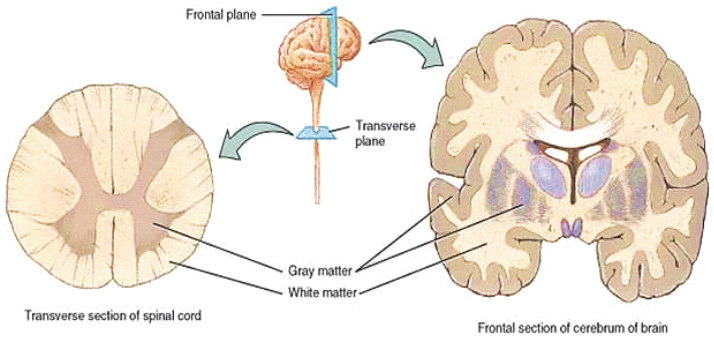

Figure 11. Location of grey and white matter in a brain graph section

The functional significance of grey and white matter has been established by extensive lesion and anatomic studies. To this respect, last decades, neuropathological and neuroimaging studies in psychotic disorders have largely confirmed grey and white matter structural brain abnormalities, some of which are disparate in terms of proximity, but are highly functionally related (see review: Shenton et al., 2001). On the one hand, most of the studies investigating volumetric differences between SZ and control subjects have detected grey but not white matter volume reductions (see review: Shenton et al., 2001). More in detail, this review described differences between SZ and control subjects, highlighting a ventricular enlargement in most of the 80% reviewed, frontal lobe abnormalities (59% of studies reviewed), particularly prefrontal GM and orbitofrontal regions and also, specific GM volume reductions that are especially prominent in the superior temporal gyrus and in medial temporal lobe brain regions (amygdala, hippocampus, and parahippocampal gyrus). In addition, a voxel-based morphometry (VBM) meta-analyses identified in adults with SZ, consistent reductions in the GM in the temporal lobe, the prefrontal cortex, the anterior cingulate cortex, the parahippocampal gyrus and the hippocampal region (Ellison-Wright and Bullmore, 2010).

However, two other restudies from the last decade, both report WM volume deficits, within the temporal and frontal regions on the left side (Sigmundsson *et al*, 2001), and within the frontal lobes bilaterally (Paillere-Martinot *et al*, 2001). To this respect, lack of compelling evidence for white matter abnormalities in SZ is not surprising, given that white matter appears fairly uniform and homogeneous on conventional MR scans, where the orientation, density, and asymmetry of the fiber tracts cannot be visualized or quantified.

Interestingly, in favour of findings pointing to WM abnormalities, a recent study with the largest diffusion-imaging sample of SZ patients to date, have identified a widespread

and diffuse reduction in FA, involving white matter in all cerebral lobes (Klauser *et al*, 2016). In detail, they found that more of the 50% of cortico-cortical and cortico-subcortical WM fiber bundles comprising the connectome were disrupted in SZ. Thus, this study concludes that white matter disruptions in SZ are widespread, affecting all cerebral lobes and the cerebellum, leading to disruptions in the majority of the brain's fiber bundles.

Overall, all these WM abnormalities in SZ research have been interpreted in terms of disturbed connectivity of neural networks (*"disconnectivity hypothesis")* (Friston and Frith, 1995), affecting prefrontal–temporolimbic connections (Ford *et al*, 2002; Wolf *et al*, 2007), cortico–cerebellar–thalamic–cortical circuits (Schmitt *et al*, 2011) and interhemispheric connectivity (Crow, 1998). To this respect, there are functional consequences that this disruption of connectivity could have in SZ. As an example, it can be mentioned the auditory hallucinations, which provide an example of combined hodological and topological hyperfunction, with increased activation of Broca's, Wernicke's and Geschwind's territory (Lennox *et al*, 2000; Shergill *et al*, 2000) and indirect, diffusion tensor tractography evidence of increased anatomical connectivity between these regions (Hubl *et al*, 2004). Moreover, WM alterations can also be linked with deficits in neurocognitive performance in SZ (Phillips et al., 2009; Spoletini et al., 2011; Szeszko et al., 2008; Knöchel et al., 2016). For example, reductions in WM FA have been associated with cognitive flexibility (Pérez-Iglesias *et al*, 2010).

On the other hand, abnormalities at the neuronal cytoarchitecture level have been found in microscopic post-mortem studies of SZ. In this sense, neuropathological studies suggest that astrocytes and oligodendrocytes, glial cells may be abnormal in SZ (Uranova *et al*, 2001), supporting the oligodendrocyte/myelin dysfunction hypothesis of SZ (Höistad, 2009). Since glial cells also play an important role in neuronal migration and in synaptic function (including glutamatergic and NMDA regulation), a reduction in the cortical glial cell numbers could be responsible for some of the pathological changes found in psychiatric disorders (Cotter *et al*, 2001), including reduced neuronal size, reduced levels of synaptic proteins and abnormalities of cortical neurotransmission, all related with SZ (Harrison, 1999).

With these aspects mentioned, it is widely known the importance of intact white matter for the correct brain functionality as well as its implication in a numerous of psychiatric diseases, such as SZ or ASD (e.g., (Catani and ffytche, 2005)).

Interestingly, among the spectrum of psychiatric disorders, SZ and ASD have found to share common deficits in connectivity and synaptic plasticity (Cheung *et al*, 2010; de Lacy and King, 2013), which could be related to the WM abnormalities also observed in both disorders (Dennis and Thompson, 2013; Wheeler and Voineskos, 2014). To this respect, both disorders have structural connectivity deficits regarding long-distance and interhemispheric bundles in: the corpus callosum, the superior longitudinal fasciculus, the inferior fronto-occipital fasciculus and the inferior longitudinal fasciculus (Ford *et al*, 2002; Friston and Frith, 1995). In detail, a recent study has found that patients affected by either ASD or SZ exhibited similar WM alterations in the left fronto-occipital inferior fasciculus with a decrease in generalized fractional anisotropy compared with controls. As regards grey matter, ASD group presented bilateral prefrontal and anterior cingulate increases in contrast with prefrontal and left temporal reductions in SZ (Katz *et al*, 2016).

Moreover, individuals with SZ and autism also show alterations in the density of dendritic spines in cortical pyramidal cells, suggesting than those genes related with myelin structure, oligodendrocyte development, synaptic functions and axonal regeneration can be considered as logical putative candidate genes for both SZ and ASD (Penzes *et al*, 2011).

As mentioned throughout this thesis, polygenic models of inheritance and linkage analysis studies have postulated that several genes confer susceptibility to SZ (Owen, 2000). Interestingly, recent genetic studies have also pointed to the presence of oligodendrocyte abnormalities in SZ (Karoutzou et al., 2008; Hakak et al., 2001). In light of the hypothesis that oligodendroglial dysfunction is related with subsequent abnormalities in myelin maintenance (Davis *et al*, 2003a), a review by Karoutzou et al (2008) highlights the implication of linkage and association studies reporting the implication of this oligodendrocyte-related genes in SZ.

Considering all the evidences mentioned above and also, highlighting the important role that grey and white matter exert in brain function, there is therefore a strong rationale to target for genetic analysis in **oligodendrocyte/myelination related (OMR) genes**. In detail, genes such as the neuregulin1-tyrosine kinase receptor ErbB4 (NRG1-ErbB4) gene

system and OMR genes are of high interest to be related with SZ. Of further note, ErbB4 has been related to a coordinated expression with OMR genes oligodendrocyte transcription factor-2 (OLIG2) and 2',3, cyclic nucleotide 3'-phosphodiesterase (CNP), in postmortem brain (Georgieva *et al*, 2006). OMR genes are mainly expressed in oligodendrocytes and are involved in the myelination (Davis *et al*, 2003b), trophic support (Segal *et al*, 2007) and axon-glial interactions (Pernet *et al*, 2008). It is known that *NRG1* plays an important role in cortico-cortical myelination during neurodevelopment (Chen *et al*, 2006), and disruption of the NRG1-ErbB4 pathway in oligodendrocytes in animal models leads to an alteration of the myelin sheath of white matter tracts, reduced conduction velocity, and cognitive changes (Roy *et al*, 2007).

In that regard, it is worth to point out that there is a genetic evidence derived from candidate gene association studies or genome-wide approaches that support oligodendroglial and OMR abnormalities in SZ. As an example, *ERBB4* – the *NRG1* receptor – has been suggested as a candidate susceptibility gene and has been related with positive epistatic interactions with *NRG1* gene in SZ (Norton *et al*, 2006).

Additionally, considering the largest published SZ GWAS data set (PGC-SWE; https://pgc.unc.edu/; Ripke et al., 2013), although no OMR genetic association reached genome-wide significance, a set of OMR genes (*ANK3, ERBB4,* and *NRG1*) were related with a suggestive association ($P < 5 \times 10^{-5}$). Along these lines, other recent study proposed WM volume as an excellent candidate endophenotype to be linked to SZ-associated SNPs (Terwisscha Van Scheltinga *et al*, 2013). Particularly, these authors reported an effect of GWAS-identified SZ risk variants, when compiled to polygenic scores, on total brain and WM volumes in SZ and even more pronounced in healthy individuals Terwisscha Van Scheltinga et al., 2013. However, these results have not been replicated in an independent study, suggesting caution in interpreting studies based on polygenic risk scores without replication samples (Papiol *et al*, 2014). Finally, other interesting recent study reported that astrocyte and oligodendrocyte gene sets, but not microglia gene sets, are associated with an increased risk for SZ (Goudriaan *et al*, 2014), suggesting that genetic alterations are underlying specific glial cell type functions which increase susceptibility to SZ. Thus, WM volume is an excellent candidate endophenotype to be linked to SZ-associated SNPs.

Considering all evidences mentioned above, and also, the importance of the integrative effects of a number of genes contributing to functional pathways related with WM related functions, and the implication of this structure in the continuum SZ-ASD, in the present thesis, a set of **white matter related genes** has been explored.

1.3 Genetic approaches of the present thesis

Understanding human genetics disorders requires both experimental and statistical research involvement. The existence of molecular genetic variation among complex diseases bring us the choice to reconstruct the lack of knowledge among them by analyzing multilocus genotypes on chromosomes observed in either healthy or affected people and their inheritance within families. In this sense, nowadays there are various genetic approaches used as a tool to dissect complex biological processes that have been developed in parallel with the development of genomic technologies.

One commonly used strategy to identify genetic risk factors for complex disorders is the **candidate gene approach**, which directly tests the effects of genetic variants of a potentially contributing gene in an association study. The basic essence of the Candidate gene approach is to assess the association between a particular allele (or set of alleles) of a gene that may be involved in the disease (the candidate gene) and the disease itself. In other words, this approach tries to answer the question: *"Is one allele that is present in a candidate gene more frequently seen in subjects with the disease than in subjects without the disease?"*. The rationale of candidate gene approaches lies in that a major component of quantitative genetic variation of phenotype under investigation is caused by functional variants of putative gene.

The clue in this kind of approaches is to choose potential candidate genes which are generally selected based on known biological, physiological or functional mechanisms underlying the disease in question (i.e., disease pathophysiology). In addition, the proposed gene can be selected due to both, its position in the genomic map (*positional candidate gene, defined by Linkage genetic studies*) and/or due to the functional related properties of the coding protein (*functional candidate gene*). Candidate gene approach has been widely applied for gene-disease research, genetic association studies, biomarker and drug target selection in many organisms from animals to humans (Zhu and Zhao, 2007). Interestingly, unlike linkage genetic studies, candidate gene studies do not require large families with both affected and unaffected members, but can be performed with unrelated cases and control subjects or with small families (e.g., a proband and parents). In addition, candidate gene studies are better suited for detecting genes underlying common and more complex diseases where the risk associated with any given candidate gene is relatively small (Collins *et al*, 1997; Risch and Merikangas, 1996). In turn, this approach has been proven to be highly powerful for studying the genetic architecture of

complex traits and is a far more effective and economical method for direct gene discovery.

However, the practicability of candidate gene approaches is limited due to its reliance on existing knowledge about the well-known biology, physiology or biochemical pathways involved in the phenotype under investigation, which is generally finite or sometimes not available at all. In addition, even this approach is recognized because its good strategy and application, in some cases it has also been criticized due to low replication of results and its limited ability to include all possible causative genes.

On the other hand, in the last years a novel approach called *genome-wide association study* (GWAS) has been developed. GWAS is a non-candidate-driven approach and involves the examination of a genome-wide set of genetic variants covering the whole genome. Thereby GWA studies examine common variation across the entire genome, and as such detect new region/s of interest that is/are in or near to potential candidate gene/s. Thus, unlike candidate gene approaches, one of the major advantages of GWA studies is that facilitates a hypothesis-free approach to genetic epidemiological investigations.

To conclude, in recent years, integrative approaches combining multiple data sources have been widely used to identify susceptible genes in complex disorders such as SZ (Sun *et al*, 2009). In this sense, the focus of *candidate gene pathway approach* is to work with different candidate genes related to multiple cellular pathways (Figure 12). Moreover, due to the complexity present in complex disorders, several authors have suggested that genexgene (gxg) interaction is also important in order to elucidate the etiology of these disorders. As such, in the present thesis these two approaches (candidate gene and gxg) will be combined in order to better understand the pathological processes involved in neurodevelopmental disorders.

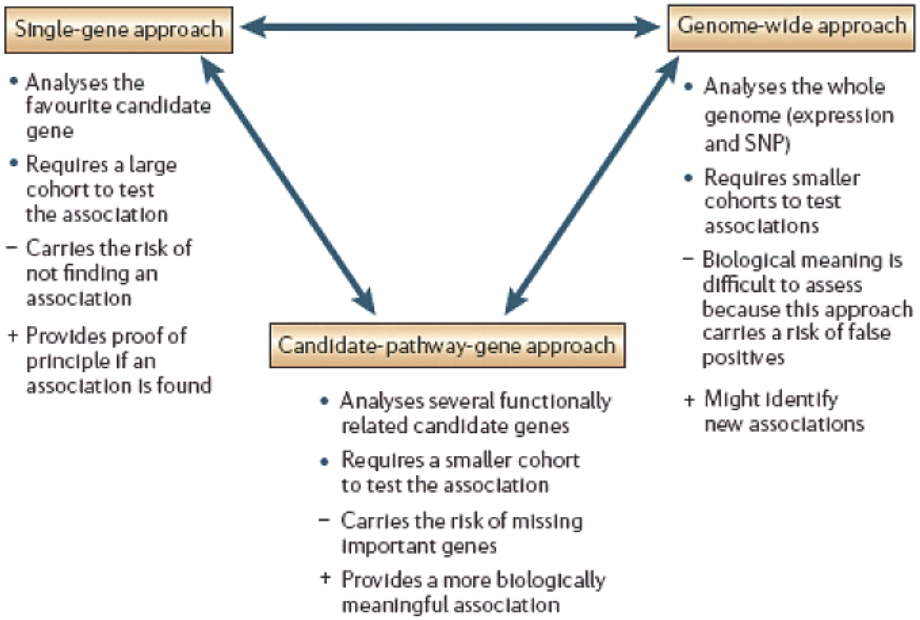

Figure 12. Scheme comparison of single-gene approach, candidate-pathway-gene approach and genome – wide approach. The main characteristics (*), disadvantage (-) and advantage (+) of each approach are indicated. The connection between the three approaches indicates that any approach can lead to another and that a combination of approaches might be considered.

1.3.1 Candidate gene approach

Last years, candidate gene studies have been a major focus in schizophrenia research with the SZGene database listing >1400 studies since 1965 (Allen *et al*, 2008). In this regard, linkage data have provided positional evidence implicating the short arm of chromosome 6 in the risk for SSD and also in their associated cognitive deficits (Hallmayer *et al*, 2005; Straub *et al*, 1995). The most studied gene included in this chromosomal region is Dysbindin-1 gene (*DTNBP1,* 6p22.3), which has been consistently associated with SSD and BPD (Schwab and Wildenauer, 2009a) as well as with age at onset and cognitive deficits (Fatjó-Vilas *et al*, 2011; Wessman *et al*, 2009). Also in this region, and far less explored, there is the Neuritin 1 gene (*NRN1,* 6p25.1) also called *candidate plasticity-related gene 15* (cpg15) (Nedivi *et al*, 1993) (Figure 13). As mentioned before in the present thesis, we have investigated the role of *Neuritin 1* gene (*NRN1*) in the risk for SSD and for related phenotypes, using a *candidate gene approach*. (for *NRN1* function see section 1.2.3.1).

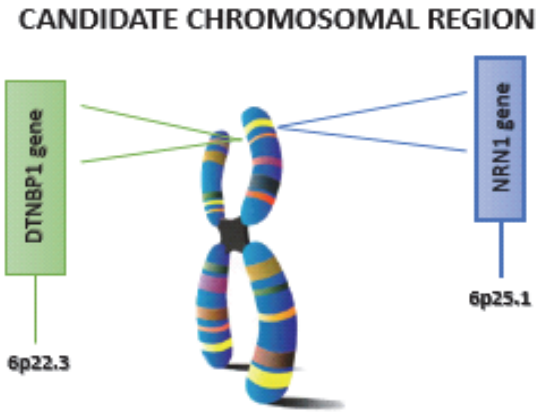

Figure 13. **The candidate chromosomal region 6p25-p22**: ***NRN1*** **and** ***DTNBP1*** **genes.**

1.3.2 Gene-gene interaction approach

In the last years, the psychiatric field has been revolutionised by the success of GWAS which have identified a number of genetic risk variants associated with different disorders. However, GWAS do not count for the underlying existence of interactions between loci. Moreover, most of the variants that achieve genome-wide significance have a small effect size, and can only explain a small proportion of the overall heritability. For this reason, there is increasing interest in studying *gene x gene interaction (gxg)*, also known as **epistasis**, which may play an important role in explaining the missing heritability in complex psychiatric diseases. It can also be defined as a logical interaction between two or more genes that affects the phenotype of organisms and it is currently described as a ubiquitous component of the genetic architecture of common human diseases (Moore, 2003). Thus, the ultimate goal of the study of gene-gene interactions is to recognize gene functions, to identify pathways and their relationship with pathophysiological processes. For instance, if a genetic factor operates primarily through a complex mechanism involving multiple other genes, and maybe possible environmental factors, the obstacle is that the effect will be missed if one examines it in isolation, without allowing for its potential interactions with these other unknown factors. In other words, studying gxg interactions is particularly important in complex disorders, as the effect of a gene on an individual phenotype is depending on more than one additional gene.

Analysis of gxg interactions can be conducted by means of different statistical approaches. It is well reported that logistic regression is considered as the workhorse of modern epidemiology (Gilbert-Diamond and Moore, 2011). Commonly, for two or more known hypothetic genetic factors that have an influence in the disease risk, maybe the most natural way to test for statistical interaction on the log odds scale is to fit a logistic regression model. This model normally includes the main effects and relevant interaction terms. The advantage of logistic regression is that interactions can be implemented relatively easily and be applied in almost any statistical analysis packages after construction of the required genotypes.

Beyond the traditional parametric statistical methods other approaches are better suited for testing interactions among multiple polymorphisms, in which there are multilocus genotype combinations (e.g. two SNP with three genotypes each one, there are nine two-locus genotype combinations). If we consider the interaction between three SNPs, there

will be 27 three-locus genotype combinations. In other words, as each additional SNP is considered, the number of multilocus genotype combinations increases exponentially. Thus, this added dimensionality will result in the requirement of larger sample sizes to have enough data in order to estimate the interaction effects and to avoid increasing type II errors and decrease in power (Gilbert-Diamond and Moore, 2011). With these reasons in mind, several methods and software packages have been developed considering statistical interactions between loci, when analysing data from genetic association studies (Chung *et al*, 2007a; Gayán *et al*, 2008; Hahn *et al*, 2003; Moore, 2004; Purcell *et al*, 2007; Zhang and Liu, 2007).

A complementary or an alternative method to logistic regression is the multifactor dimensionality reduction (MDR) (Cordell, 2009a). Briefly, this non-parametric method was developed as a genetic model-free (no genetic model is assumed) data mining strategy for identifying combinations of discrete genetic and environmental factors (Chung *et al*, 2007b; Ritchie *et al*, 2003). Unlike other methods, MDR was designed to detect interactions in the absence of detectable main effects and thus, to complement approaches such as logistic regression. Its basic idea is to pool multilocus genotypes into two groups: high-risk vs a low-risk group.

As noted in the articles of the present thesis, we used the MB-MDR approach proposed by Calle et al. (2008) which was found to have higher power for detecting the causal interacting pair(s) than MDR. In addition, MB-MDR was the first method implemented and used for case-control studies (i.e. binary traits), but later was extended to quantitative traits and also censored traits (Van Lishout *et al*, 2013). In the case of MB-MDR it is highlighted that this method only merges genotype combinations that show significant evidence of high or low risk. The rest of combinations with no evidence or insufficient sample size, are merged into a third category. In this way, MB-MDR can avoid noise from combinations that are not important for the association effect such as due to power issues from small or low effect size, or because the null is true.

Specifically, MB-MDR was first used as a data-driven for detecting gxg interaction and then, the detected interaction was complemented by a logistic regression which allows analysing the magnitude of the interaction effect and obtaining the corresponding graphical representation of the interaction. It should be also noted that even the interpretation of gxg interaction results must be done with caution and more studies in order to better demonstrate the biological mechanisms underlying the detected epistatic

effect are needed, this approach can help in the way of detecting and characterizing the biological and biochemical pathways underpinning mental disorder

Finally, in the context of the present thesis, a gxg interaction approach has been used in order to explore if the effect of *NRN1* on the phenotype was modulated by other two genes (*BDNF* and *DTNBP1*) which are related with synaptic plasticity and neurodevelopmental processes. Firstly, considering the important role that NTFs play in SZ and as the expression of *NRN1* is regulated by the brain-derived neurotrophic factor (*BDNF*), we analyzed the statistical epistasis between *NRN1* and *BDNF genes,* as a proxy analysis of their involvement in common biological pathways. Secondly, we also explored the epistasis between *NRN1* and *DTNBP1*, based on that both: (i) are implicated in plasticity processes, (ii) are expressed in hippocampus and cortical neurons, and (iii) play a role in the glutamatergic signaling through NMDA receptors, whose hypofunction has been associated with the neurobiology of SZ (Schwab and Wildenauer, 2009b; Sodhi *et al*, 2008) (Figure 14. A).

In addition, considering the genetic liability overlap present in the psychotic disorders (Craddock *et al*, 2005; Lichtenstein *et al*, 2009b), and as mentioned in the previous section, due to the high interest of studying OMR and WM-related genes, a gxg multidimensionality approach has been also tested with the purpose of better understanding the combined effect of multiple genetic variants in a set of **oligodendrocyte/myelination related (OMR) genes** and **WM-related genes** on the risk for developing either SSD or ASD (Figure 14. B).

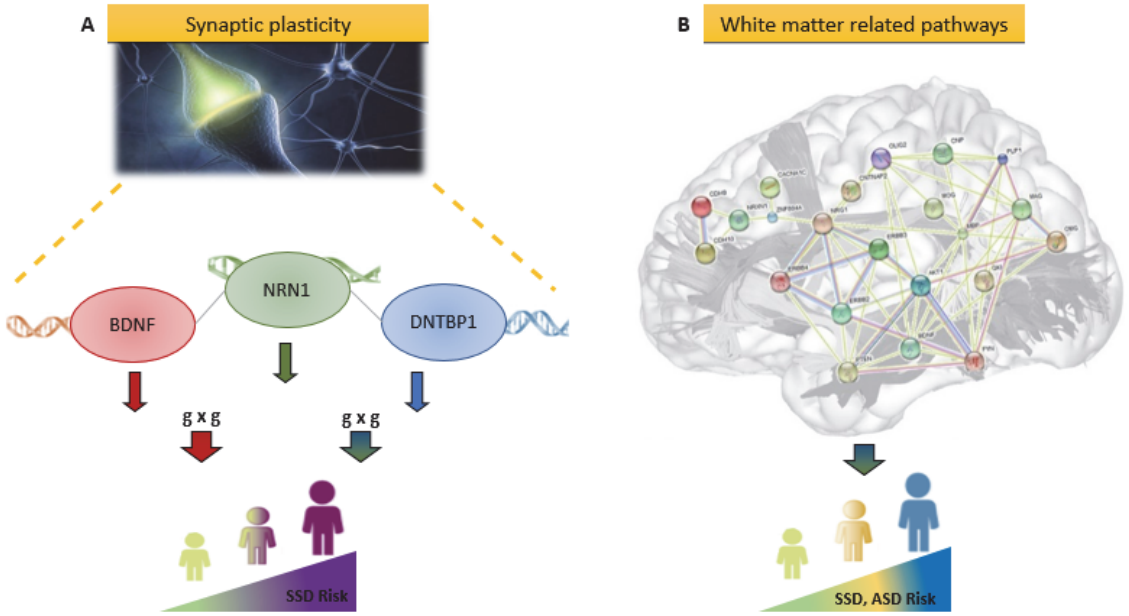

Figure 14. Scheme showing genexgene interactions explored in the present thesis. Epistatic interactions have been explored among this selected set of genes: **A) Synaptic plasticity genes:** *NRN1xBDNF and NRN1xDTNBP1; on the SSD risk.* **B) White matter related genes:** *MAG, MBP, PLP1, MOG, CNP, PTEN, AKT1, FYN, OMG, CDH10, QKI, OLIG2, NRG1, ErbB2, ErbB3, ErbB4, NRXN1, CNTNAP2, SPON1, CACNA1C, CACNB2, ZNF804,; on the SSD and ASD risk.*

2. Hypotheses and Objectives

"The important thing is to never stop questioning"
— Albert Einstein

Based on the background described in the Introduction, the **main hypothesis** guiding the present thesis was the following:

According to the substantial genetic component of schizophrenia (SZ) and schizophrenia-spectrum disorders (SSD), understanding the genetics involved may provide a way to dissect the biology of the disease and, ultimately, allow developing novel therapies and better treatment strategies that will improve quality of life of patients. In that sense, molecular genetic studies are particularly relevant because, unlike RNA and protein, primary genomic DNA sequences are generally impervious to extrinsic influences. Consequently, establishing the genetic association between SZ and a gene or set of interacting genes directly indicates its/their involvement in the etiology of the disorder.

A growing body of evidence has established that connectivity and synaptic plasticity, modulated by neuronal activity, is an inherent feature of brain function during both development and adulthood. Indeed, from a biological pathway perspective, it has been shown that some of the genes linked to SZ through GWAS studies converge in complex and identifiable molecular pathways related to synaptic plasticity, neurotransmision and connectivity processes.

Therefore, we hypothesized that genetic variability of genes involved in either synaptic plasticity (*NRN1*, *BDNF* and *DTNBP1*) and/or white matter related pathways (and their interactions) would be associated with SSD. In addition, due to the clinical, cognitive, neuroimaging and genetic overlap observed across different psychiatric disorders, we also hypothesized that the studied genetic variability would be also associated with other neurodevelopmental psychiatric disorders such as Autism Spectrum Disorders (ASD) and Bipolar Disorders (BPD), which can be placed in a pathophysiological continuum.

Two specific hypotheses can be drawn from above:

Specific hypothesis A [*NRN1*, candidate plasticity gene]: Genetic variability of Neuritin-1 gene (*NRN1)* will be associated with SSD and BPD, and also with some clinical and cognitive phenotypes both in patients and in healthy subjects from the general population. Moreover, *NRN1* action will be modulated by other genes such as *BDNF* and *DTNBP1*.

Specific hypothesis B [*White matter related genes*]: Integrative effects of a set of white matter related genes will contribute to both SSD and ASD. Moreover, we hypothesize that similar epistatic interactions will be contributing to both disorders.

In relation to these hypotheses, this PhD thesis has been focused in two objectives (A and B):

Main Objective A

First, to study the role of the genetic variability in a neurodevelopment and synaptic plasticity candidate gene called Neuritin-1 gene, on the risk for Schizophrenia-Spectrum Disorders and on clinical and cognitive phenotypes of interest. **S**econd, to investigate whether the role of *NRN1* is modulated by the interaction with other plasticity genes (*BDNF* and *DTNBP1*).

The specific objectives drawn from the main Objective A were:

- **Objective A.1**

Since synaptic plasticity alterations have been suggested to be present both in SSD and BPD, and also considering the important role that Neurotrophins (such as *NRN1*) have as molecular mediators of central synaptic plasticity, we aimed to develop a genetic association study in a sample of 954 SSD-BPD patients and 668 healthy subjects in order to:

i) investigate the implication *of NRN1* in the etiology of these disorders,

ii) analyze the role of *NRN1* in age at onset and general cognitive performance.

In addition, as the expression of *NRN1* is regulated by the brain-derived neurotrophic factor (BDNF) we also aimed to analyze the statistical epistasis between *NRN1* and *BDNF genes,* as a proxy analysis of their involvement in common biological pathways.

- **Objective A.2**

The chromosome region 6p25-p22, in which *NRN1* is located, has been recurrently linked to schizophrenia and intelligence and it also includes one of the most studied and significantly associated genes with SZ, the Dysbindin-1 gene (*DTNBP1*). Both *NRN1* and *DTNBP1*: (i) are implicated in plasticity processes, (ii) are expressed in hippocampus and cortical neurons, and (iii) play a role in the glutamatergic signaling through NMDA receptors, whose hypofunction has been associated with the neurobiology of schizophrenia. Considering the common pathway in which both genes are involved, we

aimed to study whether these genes work in concert and collectively contribute to increase the risk of SSD in a sample comprised 388 SSD patients and 397 healthy subjects. More specifically, we aimed to examine whether the effect of three functional *DTNBP1* SNPs (related with changes in gene expression) on the risk for SSD were moderated by *NRN1 risk* haplotype (previously described in Objective A1).

- **Objective A.3**

As the study of genotype-phenotype relationships in healthy individuals is considered a useful framework to investigate the etiology of brain dysfunctions, we aimed to investigate in a non-clinical sample comprised of 410 non-clinical subjects from the general population, whether *NRN1* gene contributes to the psychopathological profile, with a particular focus on the clinical dimensions previously related to *NRN1* gene (i.e. depressive and psychotic). Furthermore, we aimed to analyze:
i) the role of *NRN1* on executive functions,
ii) whether the association between either *NRN1*-psychopathological profile or *NRN1*-cognitive performance is moderated by the *BDNF* gene.

Main Objective B

To study the involvement of the epistatic effects of a selected set of white matter related genes on the risk for neurodevelopmental disorders, including SSD and ASD.

The specific objectives drawn from the main Objective B were:

- **Objective B.1**

Considering the signature framework of white matter dysconnectivity of psychiatric disorders and also, due to the genetic liability overlap among different neurodevelopment disorders such as SSD and ASD (along with the clinical similarities among them), we aimed to explore the involvement of a selected set of white matter related genes across the continuum SSD and ASD. Specifically, in a sample comprised of i) 91 ASD patient-parents' trios, ii) 187 SZ patient-parents' trios, and iii) 915 SZ case-control sample (451 cases and 464 controls), we aimed to investigated 108 SNPs across a set of 22 candidate genes related to white matter related pathways and its association with psychotic and autistic spectrum disorders. Furthermore, we also aimed to explore

the epistatic effect between these selected WM genes on the risk for developing SSD or ASD.

3. Supervisor's Reports on Articles

"The only guarantee for failure is to stop trying"
— John C. Maxwell

UNIVERSITAT DE BARCELONA

Dos campus d'excel·lència internacional:
B:KC Barcelona Knowledge Campus HUBC Health Universitat de Barcelona Campus

Dr Fañanás and Dr Fatjó-Vilas
Anthropology Unit,
Department of Evolutionary Biology, Ecology
and Environmental Sciences
Faculty of Biology

Supervisor's Report on Articles

The doctoral thesis **"Genetic risk factors in Schizophrenia and Neurodevelopmental disorders: association and epistatic analyses of Neuritin-1 gene and white matter related genes"** is based on the original results obtained by Claudia Prats. These results have been published or will be submitted to international peer reviewed journals. The impact factors of these journals demonstrate the quality of the research conducted, and are as following:

- **Involvement of *NRN1* gene in schizophrenia-spectrum and bipolar disorders and its impact on age at onset and cognitive functioning**, published in *The World Journal of Biological Psychiatry*. This Journal is the official journal of The World Federation of Societies of Biological Psychiatry and aims to serve as a major forum for the publication and wide dissemination of high quality research in biological psychiatry in the basic, clinical and interface domains. *The World Journal of Biological Psychiatry* is indexed in Science Citation Index, Science Citation Index Expanded (SciSearch), PubMed/Medline, among others. The Impact Factor of the Journal at the time of online publication = 4.159 (Journal Citation Reports 2015, Thomson Reuters), classified in the first quartile of the area of Psychiatry (ranking: 29/142).

- **Evidence of an epistatic effect between Dysbindin-1 and Neuritin-1 genes on the risk for Schizophrenia Spectrum Disorders**, published in *European Psychiatry*. This journal is the official journal of the European Psychiatric Association (EPA), the largest international association of psychiatrists in Europe. This journal supports the mission of the EPA and publishes articles on topics relevant to all mental health clinicians, researchers and neuroscientists. The wide scope of the journal is aimed at

i) encouraging the exchange of ideas and research within Europe, and ii) establishing within the international psychiatric. *European Psychiatry* is indexed in Journal Citation Reports (Science Edition 2015) with a current impact factor of 3.912 and classified in the first quartile of the area of Psychiatry (ranking: 32/142).

- **Neurotrophins role in depressive symptoms and executive function performance: Association analysis of *NRN1* gene and its interaction with *BDNF* gene in a non-clinical sample**, published in the *Journal of Affective Disorders*. This journal publishes papers concerned with affective disorders in the widest sense: depression, mania, anxiety and panic. It is interdisciplinary and aims to bring together different approaches for a diverse readership. *Journal of Affective Disorders* publishes articles focused in several aspects of affective disorders including neuroimaging, cognitive neurosciences, genetics, molecular biology, experimental and clinical neurosciences. It is indexed in Journal Citation Reports (Science Edition) with a current impact factor of 3.570 and classified in the first quartile of the area of Clinical Neurology (ranking: 45/193) and in the second quartile of the area of Psychiatry (ranking 37/142).

- **Genetic variability of a set of white matter related genes: Association and epistatic analysis in Schizophrenia and Autism spectrum disorders.** Statistical analyses associated to this article have been conducted in Aarhus University, during the research PhD stay at Aarhus University, under the supervision of Dr. Demontis. The manuscript is currently in preparation.

Accordingly, we confirm the quality of the published and submitted articles.

Dr. Lourdes Fañanás and Dr. Mar Fatjó-Vilas

Barcelona, June 1st 2017

4. Results – Publications

"Nothing in life is to be feared, it is only to be understood.
Now is the time to understand more, so that we may fear less."
– Marie Curie

4.1

Involvement of *NRN1* gene in schizophrenia-spectrum and bipolar disorders and its impact on age at onset and cognitive functioning.

M Fatjó-Vilas*, **C Prats***, E Pomarol-Clotet, L Lázaro, C Moreno, I González-Ortega, S LeraMiguel, S Miret, M Muñoz, I Ibáñez, S Campanera, M Giralt, MJ Cuesta, V Peralta, G Ortet, M Parellada, A González-Pinto, PJ Mckenna, L Fañanás.

***** *Joint first authorship*.

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ORIGINAL INVESTIGATION

Involvement of NRN1 gene in schizophrenia-spectrum and bipolar disorders and its impact on age at onset and cognitive functioning

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ABSTRACT

Objectives Neuritin 1 gene (*NRN1*) is involved in neurodevelopment processes and synaptic plasticity and its expression is regulated by brain-derived neurotrophic factor (BDNF). We aimed to investigate the association of *NRN1* with schizophrenia-spectrum disorders (SSD) and bipolar disorders (BPD), to explore its role in age at onset and cognitive functioning, and to test the epistasis between *NRN1* and *BDNF*. **Methods** The study was developed in a sample of 954 SSD/BPD patients and 668 healthy subjects. Genotyping analyses included 11 SNPs in *NRN1* and one functional SNP in *BDNF*. **Results** The frequency of the haplotype C-C (rs645649–rs582262) was significantly increased in patients compared to controls ($P = 0.0043$), while the haplotype T-C-C-T-C-A (rs3763180–rs10484320–rs4960155–rs9379002–rs9405890–rs1475157) was more frequent in controls ($P = 3.1 \times 10^{-5}$). The variability at *NRN1* was nominally related to changes in age at onset and to differences in intelligence quotient, in SSD patients. Epistasis between *NRN1* and *BDNF* was significantly associated with the risk for SSD/BPD ($P = 0.005$). **Conclusions** Results suggest that: (i) *NRN1* variability is a shared risk factor for both SSD and BPD, (ii) *NRN1* may have a selective impact on age at onset and intelligence in SSD, and (iii) the role of *NRN1* seems to be not independent of *BDNF*.

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Introduction

Schizophrenia and bipolar disorder are psychiatric disorders characterised by a prevalence of ~2–3%, which increases up to 3.5% when other affective and non-affective psychotic disorders such as schizoaffective or schizophreniform disorders are also included (Perala et al. 2007). A growing body of research suggests that schizophrenia-spectrum disorders (SSD) and bipolar disorders (BPD) share several epidemiological, clinical, neurobiological and genetic characteristics, raising important questions about the boundaries and distinctiveness of these psychiatric disorders.

On the one hand, they have a number of symptoms in common particularly in acute episodes, with regard to the presence of psychotic symptoms; their age at onset is quite similar; and, although there must be neurochemical differences, several findings emphasise the likelihood of dopamine dysregulation in both (Murray et al. 2004). Available evidence also supports that a generalised deficit is present across SSD and BPD, even though

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quantitative differences may exist (Hill et al. 2013). In view of these similarities, the integration of categorical and dimensional approaches has been suggested of particular interest to the complete understanding of psychotic disorders (Peralta and Cuesta 2007).

On the other hand, an important genetic overlap between SSD and BPD has been classically reported by both epidemiological (Gottesman 1991; Lichtenstein et al. 2006) and molecular studies (Owen et al. 2007). More recently, genome-wide approaches have evidenced a substantial shared polygenic contribution involving thousands of common genetic variants of small effect to the aetiology of these disorders (Lee et al. 2013).

These shared genetic risk factors, along with the clinical and cognitive similarities, have led to the notion that these severe mental disorders can be placed in the same aetiopathological continuum, probably representing different phenotypic manifestations of common underlying processes.

In the search for specific genetic factors related to these disorders, studies face a number of challenges that arise from the genetic and phenotypic complexity of these disorders. To this respect, it has been recently indicated that combining disorders with similar genetic risk profiles improves power to detect shared risk loci (Ruderfer et al. 2014). Similarly, genotype–phenotype-based approaches and the use of features with strong aetiological significance have been suggested as a useful strategy to reduce heterogeneity and to identify specific genetic factors associated with such traits (Rasetti and Weinberger 2011; Swerdlow et al. 2015). Then, the observed variability on traits such as cognitive impairments and age at onset among patients may reflect differences in the distribution of aetiological factors and possibly also differences underlying vulnerability. To this respect, heritability estimates indicate that genetic factors contribute significantly to age at onset of psychotic symptoms (Hare et al. 2010) and to general cognitive functioning (Deary et al. 2009). Moreover, cognitive impairments are present in 70% of the patients with schizophrenia (Palmer et al. 1997) and twin studies have shown that a large genetic overlap underlies the observed comorbidity between these two phenotypes (Toulopoulou et al. 2007, 2010). Also, the earlier forms of these disorders usually present severe clinical and cognitive expression, high incidence of treatment refraction and poor outcome (Rapoport et al. 2005; Joseph et al. 2008). Accordingly, cognitive and clinical traits associated to age at onset may provide leads for recognising and studying biological differences across diagnostic boundaries (Ongur et al. 2009).

Linkage data have provided positional evidence implicating the short arm of chromosome 6 in the risk for SSD and also in their associated cognitive deficits (Straub et al. 1995; Schwab et al. 1995; Hallmayer et al. 2005). The most studied gene included in this chromosomal region is Dysbindin-1 gene (*DTNBP1,* 6p22.3), which has been consistently associated with SSD and BPD (Schwab and Wildenauer 2009) as well as with age at onset and cognitive deficits (Wessman et al. 2009; Fatjo-Vilas et al. 2011). Also in this region, and far less explored, there is the Neuritin 1 gene (*NRN1,* 6p25.1), also called candidate plasticity-related gene 15 (cpg15) (Nedivi et al. 1993). During early embryonic development, *NRN1* is expressed in multiple brain regions and acts as a survival factor for neural progenitors and differentiated neurons (Putz et al. 2005). Later in development, *NRN1* promotes growth and stabilisation of axonal and dendritic arbours along with synapse formation and maturation (Cantallops et al. 2000; Javaherian and Cline 2005). *NRN1* continues to be expressed in the adult brain, where its expression is correlated with activity-dependent functional synaptic plasticity (Corriveau et al. 1999; Harwell et al. 2005; Flavell and Greenberg 2008). Furthermore, the expression of *NRN1* is regulated by neurotrophins such as brain-derived neurotrophic factor (*BDNF*, 11p13) (Naeve et al. 1997; Karamoysoyli et al. 2008). BDNF promotes the differentiation and growth of developing neurons in central and peripheral nervous systems (Buckley et al. 2007) and its intracellular distribution and activity-dependent secretion is altered by the Met variant of a functional polymorphism in the *BDNF* gene, which consists of a valine (Val) substitution for methionine (Met) at codon 66 (Val66Met). Interestingly, *BDNF* gene polymorphisms have been associated with clinical characteristics – such as age at onset – and cognitive functioning in both SSD and BPD (Krebs et al. 2000; Rybakowski et al. 2006).

According to all the above mentioned, *NRN1* was already defined as a candidate gene for neurodevelopment disorders by Chandler et al. (2010), who reported the effect of *NRN1* polymorphic variation on general intelligence impairments in patients with schizophrenia. We considered the interest of investigating the implication of *NRN1* in the aetiology not only of schizophrenia, but also across the SSD and BD continuum. Moreover, we also aimed to extend the previous study on the relationship of *NRN1* with cognitive impairments by testing the effect of this gene on age at onset, a characteristic that is related to cognitive performance.

Since synaptic plasticity alterations have been suggested to be present both in SSD and BPD (Craddock et al. 2006), we hypothesised that sequence variability of the gene would be related to the risk for developing any of these disorders. Considering the described involvement of *NRN1* in cognitive processes, we also hypothesised that *NRN1* gene could exert its effect not only by

modulating general cognitive functioning, but also age at onset. Finally, given that *NRN1* is a BDNF-regulated gene, we explored the statistical epistasis between *NRN1* and *BDNF* as a proxy analysis of their involvement in common biological pathways.

Materials and methods

Sample

The patients' sample comprised 954 individuals of Spanish Caucasian origin. They were drawn from consecutive admissions to three Child and Adolescent Psychiatry Units and four Adult Psychiatric Units, and were evaluated by experienced psychiatrists. All of them met the DSM-IV-TR diagnosis criteria: 73% SSD (49% schizophrenia, 11% schizophreniform disorder, 8% schizoaffective disorder, 5% psychotic disorder NOS) and 27% bipolar disorder I or II. Exclusion criteria included: age above 65 years, major medical illnesses that could affect brain functions, substance-induced psychotic disorder, neurological conditions, history of head trauma with loss of consciousness and having at least one parent not from Spanish Caucasian origin. Patients were diagnosed based on the following schedules: KSADS (Kaufman et al. 1997) for patients up to 17 years of age, and SCID (First et al. 1997) or CASH (Andreasen et al. 1992) for adult patients. Age at onset of the first episode was determined by means of these clinical schedules and/or the SOS inventory (Perkins et al. 2000).

The control sample consisted of 668 Spanish Caucasian unrelated adult healthy individuals. They met the same exclusion criteria as patients. They were recruited from university students and staff, and their acquaintances, plus independent sources in the community. They were interviewed and excluded if they reported a history of mental illness and/or treatment with psychotropic medication.

All participants provided written consent after being informed about the study procedures and implications. In the case of patients below the age of 18, written informed consent was also obtained from their parents or legal guardians. The study was performed in accordance with the guidelines of the institutions involved and was approved by the local ethics committee of each participating centre. All procedures were carried out according to the Declaration of Helsinki.

Neurocognitive assessment

The general cognitive performance was evaluated in 607 patients and in 476 healthy subjects. Intellectual quotient (IQ) was estimated using the Block Design and Vocabulary or Information subtests of the WAIS-III (Wechsler 1997) or WISC-IV (Wechsler 2004), in accordance with the method suggested by Sattler (2001). Cognitive assessment was carried out by experienced neuropsychologists. In patients, the cognitive evaluation was conducted when stabilisation of symptoms and readiness for cognitive evaluation was decided by the clinical team.

Molecular analyses

Genomic DNA was extracted from peripheral blood cells or from buccal mucosa using standard methods: the Real Extraction DNA Kit (Durviz S.L.U., Valencia, Spain) or the BuccalAmp DNA Extraction Kit (Epicentre® Biotechnologies, Madison, WI, USA).

Coverage of *NRN1* genomic sequence and ~10 kb upstream and downstream was achieved by including 11 tag SNPs (Table 1). The optimal set of SNPs that contained maximum information about surrounding variants was selected by using SYSNPs (http://www.sysnps.org/) with a minor allele frequency (MAF) > 5%, using pairwise option tagger (threshold of r^2=0.8). The SNPs included in the study by Chandler et al. (2010) were also considered. The SNP rs6265 (Val66Met) at *BDNF* gene was also genotyped. Genotyping was performed using a fluorescence-based allelic discrimination procedure (Applied Biosystems Taqman 5′-exonuclease assays). Standard conditions were used. The genotyping call rate for all SNPs was higher than 94.2% and all were in Hardy–Weinberg equilibrium.

Statistical analyses

All data were processed using SPSS 21.0 software (SPSS IBM, New York, USA). Haploview v4.1 (Barrett et al. 2005) was used to estimate the Hardy–Weinberg equilibrium and the linkage disequilibrium (LD) between *NRN1* SNPs (Supplementary Figure S1 available online). By means of using the Solid Spine criteria three haplotype blocks were identified (Block 1: SNP1-SNP3, Block 2: SNP4-SNP5 and Block 3: SNP6-SNP11) and a sliding window analysis was conducted within each block.

The genetic power was calculated using Epi-info-v3.5.1 (Dean et al. 1991) by assuming an additive model, a disease prevalence of 3% and considering the minor allele frequencies observed in our sample. All markers had an 80% power to detect a genetic effect with an OR ≥ 1.2.

Case–control associations were analysed using the Unphased-v3.1.4 (Dudbridge 2003), using a cut-off threshold for rare haplotypes of 1%. A 10,000-permutations procedure was applied to all tests to limit type II error. The odds ratios (OR) were estimated from the absolute number of alleles/haplotypes estimated in patients and controls (EpiInfo-v3.5.1).

Table 1. SNPs genotyped in Neuritin 1 gene (*NRN1*, chromosome 6p25.1, from 598233 to 6007633 bp).

	SNP	Position	Region	Distance from SNP1	Distance from previous SNP	Alleles[a]	MAF[b]
SNP1	rs2208870	5992490	Intergenic			A/G	0.333
SNP2	rs12333117	5994992	Downstream	2502	2502	C/T	0.402
SNP3	rs582186	6001381	Intronic	8891	6389	A/G	0.393
SNP4	rs645649	6004959	Intronic	12469	3578	C/G	0.356
SNP5	rs582262	6007991	Upstream	15501	3032	G/C	0.273
SNP6	rs3763180	6009848	Upstream	17358	1857	G/T	0.437
SNP7	rs10484320	6010437	Upstream	17947	589	C/T	0.236
SNP8	rs4960155	6010539	Upstream	18049	102	T/C	0.492
SNP9	rs9379002	6012391	Intergenic	19901	1852	T/G	0.27
SNP10	rs9405890	6012721	Intergenic	20231	330	T/C	0.309
SNP11	rs1475157	6017169	Intergenic	24679	4448	A/G	0.176

The table includes the dbSNP number, the genomic and gene position and the alleles of the 11 SNPs genotyped along the gene (UCSC Genome Browser on Human Mar. 2006 Assembly (hg18), http://genome.ucsc.edu/cgi-bin/hgTracks).
[a]The less frequent allele (minor allele) is placed second.
[b]MAF refers to Minor Allele Frequency observed in the 1000 Genomes project (Abecasis et al. 2012).

Additive models as implemented in Plink 1.07 (Purcell et al. 2007) were used to conduct lineal regression analyses to explore the relationship between *NRN1* and age at onset and IQ. First, the relationship between the *NRN1* and age at onset was tested in the complete patients' sample (including gender and diagnosis group as covariates) and also separately in each group (adjusted by gender). Second, the relationship between the *NRN1* and IQ was tested in the complete patient's sample (including age at onset, months of evolution and diagnosis group as covariates) and also separately in SSD, BPD (adjusted for age at onset and months of evolution) and controls. PLINK's max(T) permutation procedure with 10,000 iterations was performed.

The effect of *NRN1* and *BDNF* interaction was tested on: (i) the risk for developing SSD or BPD, (ii) age at onset (adjusted for sex and diagnosis) and IQ (adjusted for age at onset and months of evolution), in patients. Epistasis was explored using the model-based multifactor dimensionality reduction (MB-MDR) approach by applying 'mbmdr' R-package (Calle et al. 2010). This method merges multi-locus genotypes in order to overcome the dimensionality problem and to increase the power to detect gene interactions associated with disease or phenotype. It also allows adjusting for confounding effects and correcting for multiple testing by 1000 permutations approach. In all analyses, the significance cut-off was established at *P* value of 0.05.

Results

Sample characteristics

Table 2 shows the main sociodemographic and clinical data of the sample. Variables that showed differences between groups were used as covariates when appropriate (see Statistical analyses section).

Association analysis of NRN1 *and schizophrenia-spectrum and bipolar disorders*

There were no differences between sampling groups as regards the genotypic distribution of each polymorphism (data not shown), and genotype frequencies showed no gender differences within groups (patients and controls; data not shown).

SNP1 (G allele), SNP4 (C allele) and SNP5 (C allele) were significantly more frequent among patients compared to controls (χ^2=4.81 $P = 0.028$, χ^2=5.05 $P = 0.024$ and χ^2=8.04 $P = 0.004$, respectively). After multiple correction adjustment only the association of SNP5 remained significant (OR(95%CI) = 1.27(1.07–1.49), empirical *P* value = 0.044).

Haplotypes associated with SSD and BPD are given in Table 3. The frequency of the haplotype G-C (Block 1: SNP1-SNP2) and haplotype C-C (Block 2: SNP4-SNP5) was significantly increased in patients than in controls. The result in Block 2 remained significant after permutation procedure; then, this haplotype was considered a risk haplotype for SSD and BPD. On the contrary, the haplotype T-C-C-T-C-A (Block 3: SNP6-SNP11) had higher frequencies in controls. Results in Block 3 also remained significant after multiple testing and could be interpreted as reflecting a protective effect of this haplotype. Note that other haplotypes included in the haplotype in Block 3 were also detected (Supplementary Table S1 available online). These results remained essentially unchanged when only SSD patients and controls were included.

NRN1 *and age at onset of the disorders*

Patients carrying two copies of the T allele at SNP2 (15.33%) presented a lower age at onset than those not carrying this allele ($\beta = -0.772$ $P = 0.029$). Patients homozygous for the C allele of SNP10 (7.80%) also showed later age at onset than those not carrying this allele ($\beta = 0.918$

Table 2. Sample description and statistical comparisons between patients and controls.

	All Patients (n=954)	SSD (n=697)	BPD (n=257)	Controls (n=668)
Male (%)	65.6%	71.2%	50.6%**	46.7%*
Age at interview	32.33 (13.10)	31.79 (12.83)**	33.9 (13.71)**	27.05 (9.99)*
Years of education	10.13 (4.06)	9.58 (3.82)	11.98 (4.29)**	13.87(2.87)*
Age at onset	21.54 (6.47)[a,b]	20.72 (5.33)[a]	23.88(8.53)[a,**]	–
Months of evolution	146.24 (137.6)	140.35 (140.07)	162.93 (129.25)	–
Current IQ	89.80 (15.26)[c]	89.02 (15.37)[c]	92.86 (14.48)[c,**]	99.48 (13.64)[c,*]

Proportion (%) or mean scores (standard deviation) are given. SSD, schizophrenia-spectrum disorders; BPD, bipolar disorders.
[a]Information about age at onset was available for the 73.5% of patients (74.3% SSD and 71.2% BPD).
[b]35.29% were classified as early-onset (first psychotic episode occurred before 18 years of age).
[c]Information about IQ was available for 63.6% of patients (69.4% SSD and 47.8% BPD) and 71.25% of healthy subjects.
*Controls differed significantly from patients ($P<0.001$).
**BPD patients differed significantly from SSD patients ($P<0.03$).

Table 3. *NRN1* most significant haplotypes associated to the risk for schizophrenia-spectrum and bipolar disorders (grey boxes). Frequency estimates (%) in patients and controls, significance levels and OR of the case-control comparison are given.

SNP1	rs2208870	G		
SNP2	rs12333117	C		
SNP3	rs582186			
SNP4	rs645649		C	
SNP5	rs582262		C	
SNP6	rs3763180			T
SNP7	rs1048432			C
SNP8	rs4960155			C
SNP9	rs9379002			T
SNP10	rs9405890			C
SNP11	rs1475157			A
Case Freq[a]		34.3	25.9	0.1
Control Freq[b]		30.7	21.4	1.5
χ^2		4.26	7.99	17.45
OR (CI 95%)[c]		1.18 (1.01–1.37)	1.28 (1.08–1.51)	0.09 (0.02–0.37)
Global P value		0.11	0.038	0.001
Individual haplotype P value		0.037†	0.0043*	0.000031**

[a]Case Freq refers to each haplotype frequency within cases.
[b]Control Freq refers to each haplotype frequency within controls.
[c]Chi-squared tests and Odds ratio (OR) were estimated from the absolute number of observed haplotypes in cases and controls.
†Not significant after performing 10,000 permutations, adjusted P value from permutation test $P=0.1748$.
*Significant adjusted level based on 10,000 permutations, adjusted P value from permutation test $P=0.0219$.
**Significant adjusted level based on 10,000 permutations, adjusted P value from permutation test $P=0.002$.

$P=0.016$). The haplotype C-A (SNP10-11) was associated with age at onset: ($\beta=0.956$ $P=0.015$) and also several haplotypes within Block 3 (all including the C-A haplotype) (Supplementary Table S2 available online).

When the same analysis was conducted only including SSD patients, the results for SNP10 and haplotype SNP10-11 remained significant while SNP2 did not (Supplementary Table S3 available online). In an additive way, carrying two copies of the haplotype C-A was associated with later SSD age at onset (Figure 1A). However, these results were not significant after permutation procedure. No association was detected within BPD patients' group.

NRN1 *and cognitive functioning*

In SSD patients, the same haplotypes within Block 3 contributed to IQ scores (Supplementary Table S4 available online). A linear trend was detected between the number of copies of these haplotypes and higher IQ scores (Figure 1B), meaning that subjects carrying these haplotypes showed better general cognitive performance than non-carrier subjects. However, after permutation analyses these results did not remain significant. No significant association with IQ was detected between these polymorphisms either in the whole patients' sample, in BPD or in healthy subjects.

Epistasis between NRN1 and BDNF

Two order gene–gene interaction models were developed and revealed that the combination of the *BDNF* Val/Val genotype with different *NRN1* variants (SNP1 (GG: $\beta=0.654$ $P=0.001$), SNP3 (AA: $\beta=0.514$ $P=0.003$) and SNP9 (TG: $\beta=0.457$ $P=0.0004$)) was related to an increased risk for developing both SSD and BPD. In contrast, *BDNF* Met/Met was associated with a lower risk

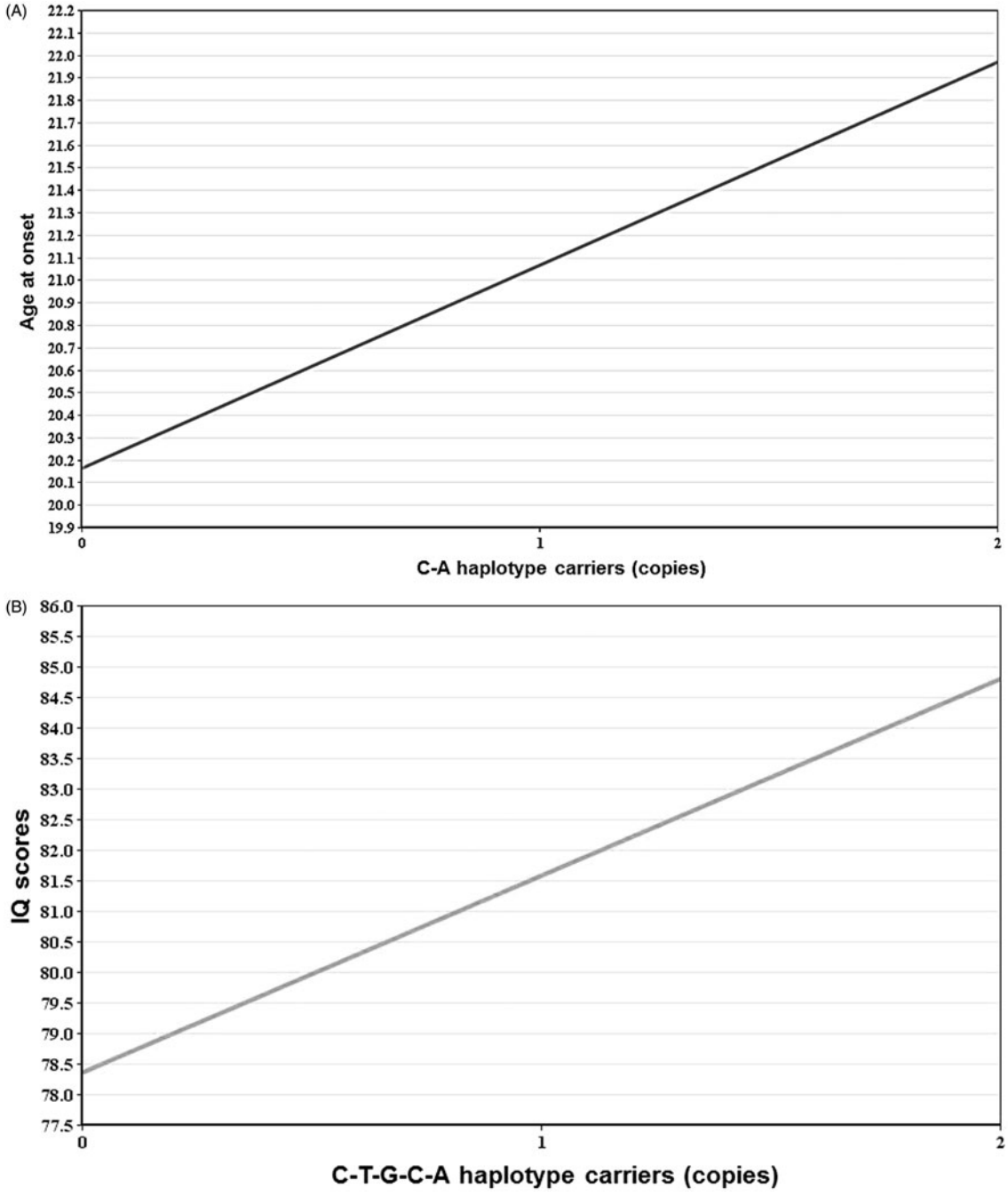

Figure 1. Relationship between *NRN1* and age at onset and IQ in SSD patients. Linear regression graphs showing the relationship between SSD patients' *NRN1* haplotypes and: (A) age at onset, (B) IQ. For illustration purposes, the haplotype dump option was used to estimate individual haplotype phases. Considering only those haplotypes estimated with a probability ≥95%, each subject was defined according to its haplotype dose. (A) The haplotype C-A (SNP10-11) was selected to represent graphically the described association between NRN1 and age at onset ($\beta = 0.89$ P=0.019). SSD patients were classified as: 47.01% non-carriers (0), 45.41% one-copy carriers (1) and 7.58% two-copy carriers (2). (B) The haplotype C-T-G-C-A (SNP7-11) was selected to represent graphically the detected association between *NRN1* variability and IQ within SSD patients ($\beta = 4.02$ P=0.022). SSD patients were classified as: 82.2% non-carriers (0), 16.9% one-copy carriers (1) and 0.9% two-copy carriers (2).

in combination with *NRN1* SNP2 (TT: $\beta = -2.185$ $P = 0.0052$). After permutation analysis, the interaction $BDNFxNRN1_{SNP9}$ remained significant ($P = 0.005$). No significant epistatic effect was detected on age at onset and IQ after permutation.

Discussion

This case–control based approach adds to the only one previous Neuritin 1 gene association study developed by Chandler et al. (2010) in a sample of 336 patients with schizophrenia and 172 controls. Unlike Chandler and

collaborators, in our sample of 954 patients and 668 healthy subjects we report that *NRN1* sequence variability accounts for a modest proportion of the risk for these disorders. On the one hand, we have identified a two SNP haplotype (SNP4-SNP5: C-C) that is associated with the risk for these disorders. As expected, due to the polygenic architecture of the studied disorders, the effect of this haplotype is small although significant (OR = 1.28). On the other hand, we have observed haplotypes in the 5 upstream region that have a protective effect. Although significance for these associations persisted after permutation procedure, the low frequency of the protective haplotypes in the population has to be considered when evaluating the attributable risk associated to these genetic variants.

The present study also provides new evidence of interest as regards understanding the heterogeneity in age at onset and cognitive performance of SSD and BPD. Our results suggest that *NRN1* variability has a role in SSD age at onset, pointing towards a specific effect on modifying neurodevelopment processes related to the time of emergence of these disorders. Although these results should be interpreted cautiously because they are only significant at an uncorrected level, it is interesting to note that the C allele of SNP10, which is included in the above described protective haplotype, is associated with a later age at onset of SDD. Then, taking into account that the 51% of SSD patients are carriers of this allele (358 C carriers vs 339 TT), together with the particularly poor prognosis associated to schizophrenia in childhood and adolescence in contrast to the adult manifestation (Clemmensen et al. 2012), this modulatory effect is of non-dismissible potential clinical interest.

Our study also shows the association between this gene and intelligence in SSD. This selective impact of *NRN1* on intelligence may suggest its involvement in processes underlying cognitive functioning, which are described to be more quantitatively impaired in SSD (Hill et al. 2013). Again, although results did not reach significance after permutation, it is of interest that the haplotypes identified in the present study contain the same haplotype that Chandler et al. (2010) described to be associated with better fluid intelligence in schizophrenia patients and not in healthy subjects (SNP10-SNP11: C-A).

In all, our results suggest in a convergent manner that allelic variants in Block 3 of *NRN1* could represent a protective factor, not only due to their association to a reduction of the risk for SSD and BPD, but also because within SSD patients, these variants are related to a later of age at onset and a better cognitive performance. This lends support to the notion that specific genetic variability could play a role in defining illness subgroups and points towards the interest of understanding the pathways from genotype to clinical phenotype, which will be crucial for new classification systems and for the development of novel therapeutic strategies.

In further interpreting these results, it is necessary to consider the results obtained by whole genome approaches. To our knowledge, *NRN1* has not appeared as a significant locus in the published GWAS for schizophrenia and bipolar disorders. However, these negative results could be influenced, for example, by the small effect attributable to common variants or by the heterogeneity of the samples. It should also be considered that *NRN1* could be exerting its effect by means of modifying more specific traits associated with psychotic disorders. In this regard, a genome-wide scan for intelligence conducted in a general population sample revealed suggestive linkage for IQ on 6p25.3–21.31 and already highlighted *NRN1* as a positional candidate gene (Posthuma et al. 2005). Moreover, a subtype of schizophrenia characterised by pervasive cognitive deficit was also linked to 6p25–p22 region (Hallmayer et al. 2005). More recently, a GWAS has established that common variants (SNPs) may account for 40–50% of intelligence variance (Davies et al. 2011) and a GWAS-based pathway analysis has reported that general fluid intelligence appears to be characterised by genes affecting quantity and quality of neurons and therefore neuronal efficiency (Christoforou et al. 2014). Among the genes included in the top pathways identified in this study, there was the *BDNF,* a regulator of *NRN1* expression. According to all these data and given the described gradual increase in heritability of IQ from childhood to late adolescence (Deary et al. 2009; Bouchard 2013) and the reported early occurrence of intellectual impairment even years before the onset of the psychotic symptoms (Cannon et al. 2002), it is plausible that those genes that influence brain development, as *NRN1*, may be modulating illness traits, as IQ and age at onset, and ultimately influencing the risk for these disorders.

Although the connection between the *NRN1* sequence variability and the risk for SSD and BPD is still unclear, the consideration of the putative effects of the analysed polymorphic sites on gene expression regulatory mechanisms represents a valuable resource to provide additional meaning and importance to our association data. Recent data has revealed the importance of intronic and intergenic variants as regulatory elements of gene expression (Dunham et al. 2012). The impact of non-coding variants of the *NRN1* SNPs can be examined using HaploReg (Ward and Kellis 2012), which is a tool that uses LD information from the 1000 Genomes Project to provide data on the predicted chromatin state of the queried SNPs, their sequence conservation mammals,

and their effect on regulatory motifs. As an example, SNP2 (rs12333117), associated with age at onset in the present study, is located in a downstream region, in a DNAse region (T-47D) and it is predicted to alter several motifs that overlap the recognition sequences of transcription factors such as AP-1/Jun, suggesting possible factor–factor interactions. There is also evidence that this SNP could modify the promoter histone mark H1, which plays an active role in the formation of epigenetic silencing marks (Yang et al. 2013). Another example refers to the SNP4 (rs645649), included in the identified risk haplotype and that is located in an intronic region where two proteins bound: SUZ12 (involved in methylation processes leading to transcriptional repression of the affected target genes) and ZNF263 (implicated in basic cellular processes as a transcriptional repressor). Furthermore, several resources provide information about the correlation between genotype and tissue-specific gene expression levels, which may help in the interpretation of molecular genetics association studies (GTEx Project, www.gtexportal.org (Lonsdale et al. 2013); BrainCloud, http://braincloud.jhmi.edu/ (Colantuoni et al. 2011)). In this regard, variations in NRN1 expression have been associated with SNPs along the gene. Therefore, although functional studies are needed, the association of *NRN1* sequence variants with SSD and BPD phenotypes could be linked to the final availability or functionality of the protein which, in turn, could dysregulate NRN1 role on neurite outgrowth and arborisation and/or on neuronal processes associated with plasticity.

Finally, based on the analyses of epistasis between *NRN1* and *BDNF,* our data suggest that the interaction between the Val/Val genotype (*BDNF*) and the TG genotype (*NRN1*, *SNP9:* rs9379002) could modulate the risk for SSD and BPD. Despite the fact that evidence of a statistical interaction as we report here does not necessarily map directly onto biological interaction, it is of note that it is based on a previously described effects of BDNF on NRN1 regulation (Naeve et al. 1997). Then, it could be hypothesised that the reported functional effects of the *BDNF* Val66Met polymorphism could impact on NRN1 availability or function, explaining therefore the gene–gene interaction on the risk for developing SSD and BPD and contributing to understand the controversial results associated to single gene analyses. To this respect, some studies have implicated the *BDNF* Val allele in these disorders and, as the Val allele is associated with increased synaptic plasticity and growth (Egan et al. 2003), it has been suggested that this allele could promote increased synaptic connections between certain brain regions that underpin common symptoms. However, recent meta-analyses have failed to confirm the direct association of Val66Met polymorphism with the risk for schizophrenia (Zhao et al. 2015) or bipolar disorder (Gonzalez-Castro et al. 2014). On the other hand, taking into account that BDNF exerts a direct impact on neuronal growth and plasticity in the limbic system (Conner et al. 1997; Rattiner et al. 2004), it should be contemplated that G allele carriers of rs9379002 (SNP9, *NRN1*) show higher *NRN1* expression than TT homozygotes in the hypothalamus (GTEx Project). Then, we could speculate that higher expression of both *BDNF* and *NRN1* could be underlying the detected epistatic risk effect. To this respect, it is remarkable that a case-report study suggested the relationship between a duplication of *NRN1* gene (i.e. increased gene dosage) and the white matter and neurocognitive abnormalities observed in one patient (Linhares et al. 2015). Accordingly, we would have expected to detect the association not only with the heterozygous TG genotype but also with the GG. This lack of significant interaction could be explained by the low frequency of GG genotype (7%) and the corresponding low frequency of the combination of Val/Val x GG (*BDNFxNRN1*$_{SNP9}$). Therefore, although further studies are needed, these results are in line with recent trends in the field of molecular genetics, which consider the importance of testing gene networks rather than isolated gene effects for better understanding the gene–phenotype relationship in complex disorders (Gilman et al. 2012). Nonetheless, the fact that the SNP9 is included in the protective haplotype while it is detected to exert a risk effect when interacts with Val/Val genotype could suggest that the effect of this SNP may differ depending on the genetic background in which the alleles are present (Moore 2003). Moreover, beyond gene–gene interactions, the effect of environmental factors should also be studied. In this regard, the fact that *NRN1* is classified as an immediate early gene (Loebrich and Nedivi 2009), meaning that it can be rapidly induced by extracellular stimuli and act as a transcription factor on downstream targets, highlights the interest of analysing the combined effect of *NRN1* and *BDNF* in gene-environment studies.

Some limitations of this study must be acknowledged. First, the controls' age range is partially overlapped with the age range of incidence of SSD and BPD. However, due to the fact that personal psychiatric history and treatment was discarded, the percentage of false negatives would be very low and should not interfere with the obtained results. Second, the polygenic nature of mental disorders and the minor effect of the common genetic variants limit the power of our sample size, especially in the case of the analyses split by diagnosis. In line with this, although the use of features with strong aetiological significance has

been suggested as a useful strategy to increase the power to detect genetic effects, the power of the analyses targeting age at onset and neurocognition is reduced due to the non-availability of data in all subjects. This statistical power reduction could be related with the loss of significant effects after permutation procedures. Third, the antipsychotic treatment was not specified and, therefore, cognitive analyses, although covaried by age at onset and months of evolution, were not adjusted by treatment type or duration. Fourth, in spite of the interest of the selected polymorphism at *BDNF* due to its functional effects, future studies should include other genetic variants along this gene. Lastly, although the permutation procedures have been applied, if multiple testing is addressed for the overall analyses not all the findings would remain significant. Then, although results cannot be dismissed completely, since they come from a directed hypothesis and they are partially in line with a previous study (Chandler et al. 2010), their interpretation should be conducted with caution and replication studies are needed.

Overall, our results contribute, from a biological approach, to the understanding of the genetic mechanisms involved in SSD and BPD and also of the relationship between genetic variability and the clinical heterogeneity of these disorders. Then, our findings suggest the role of Neuritin 1 gene as a mixed susceptibility/modifier gene (Fanous and Kendler 2008), which increases the susceptibility to these disorders and modifies certain presentations. However, new studies should be developed to further acknowledge the involvement of *NRN1* and its interaction with other genes in the aetiology of mental disorders.

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Statement of interest

None to declare.

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Advisors' report on the contribution of the PhD. candidate to the article:

Dr. Fañanás (Associate Professor) and Dr. Fatjó-Vilas (Assistant Professor) from the Department of Evolutionary Biology, Ecology and Environmental Sciences of the Faculty of Biology (University of Barcelona), both supervisors of the present doctoral thesis by Claudia Prats, hereby certify that the participation of the PhD applicant in the article "**Involvement of *NRN1* gene in schizophrenia-spectrum and bipolar disorders and its impact on age at onset and cognitive functioning"**, included the following tasks:

- Participation in the conception and design of the manuscript
- Laboratory tasks
- Statistical analysis and interpretation of data
- First drafting of the manuscript
- Critical revision of the article for intellectual content

Signed by Dr. Lourdes Fañanás and Dr. Mar Fatjó-Vilas

Barcelona, June 1st 2017

4.2

Evidence of an epistatic effect between Dysbindin-1 and Neuritin-1 genes on the risk for Schizophrenia Spectrum Disorders.

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Original article

Evidence of an epistatic effect between Dysbindin-1 and Neuritin-1 genes on the risk for schizophrenia spectrum disorders

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ABSTRACT

Background: The interest in studying gene–gene interactions is increasing for psychiatric diseases such as schizophrenia-spectrum disorders (SSD), where multiple genes are involved. Dysbindin-1 (*DTNBP1*) and Neuritin-1 (*NRN1*) genes have been previously associated with SSD and both are involved in synaptic plasticity. We aimed to study whether these genes show an epistatic effect on the risk for SSD.
Methods: The sample comprised 388 SSD patients and 397 healthy subjects. Interaction was tested between: (i) three *DTNBP1* SNPs (rs2619537, rs2743864, rs1047631) related to changes in gene expression; and (ii) an haplotype in *NRN1* previously associated with the risk for SSD (rs645649-rs582262: HAP-risk C-C).
Results: An interaction between *DTNBP1* rs2743864 and *NRN1* HAP-risk was detected by using the model based multifactor dimensionality reduction (MB-MDR) approach ($P = 0.0049$, after permutation procedure), meaning that the risk for SSD is significantly higher in those subjects carrying both the A allele of rs2743864 and the HAP-risk C-C. This interaction was confirmed by using a logistic regression model ($P = 0.033$, OR (95%CI) = 2.699 (1.08–6.71), $R^2 = 0.162$).
Discussion: Our results suggest that *DTNBP1* and *NRN1* genes show a joint effect on the risk for SSD. Although the precise mechanism underlying this effect is unclear, the fact that these genes have been involved in synaptic maturation, connectivity and glutamate signalling suggests that our findings could be of value as a link to the schizophrenia aetiology.

1. Introduction

Complex genetic disorders with a polygenic architecture such as schizophrenia involve multiple genes of small effect, which interact with different social and environmental factors. Several genes combine with other genes influencing the control of a trait variation. In this sense, the interest in studying gene–gene interactions is increasing because of the enhanced power to elucidate the biological processes underlying mental disorders [1].

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In regard to these biological processes, a growing body of evidence has established that synaptic plasticity is an inherent feature of brain function during both development and adulthood. Recent studies highlight the convergence of several genes associated with schizophrenia-spectrum disorders (SSD) onto a coherent biological pathway at the synapse, with a specific role in plasticity [2]. Therefore, focusing on genes and their interactions implicated in synaptic plasticity may provide better understanding of SSD pathogenesis.

Dysbindin-1 gene (*DTNBP1*) is described as a modulator of the synaptic transmission homeostasis [3] due to its pre- and post-synaptic roles. Dysbindin-1 interacts pre-sinaptically with proteins involved in vesicular trafficking and exocytosis regulating the neurotransmitters release [4]. Additionally, Dysbindin-1 regulates the formation and function of the postsynaptic density (PSD), a set of proteins that interacts with the postsynaptic membrane to provide structural and functional regulatory elements for neurotransmission and for NMDA receptors [5]. Schizophrenia patients show lower levels of Dysbindin-1 mRNA in dorsolateral prefrontal cortex and hippocampus compared to healthy subjects [6,7]. This reduction in Dysbindin-1 expression has been related to a decreased glutamate output [5] and to an increased dopamine release [8]. Moreover, it has been reported that *DTNBP1* expression levels are modulated by common DNA sequence changes (Single Nucleotide Polymorphisms, SNPs) [9,10]. Several studies based on SNP analyses have shown an association between *DTNBP1* and SSD (i.e. [11–13]).

Interestingly, the chromosome region 6p25-p22, in which *DTNBP* is located, has been recurrently linked to schizophrenia and intelligence [14–16] and it also includes the Neuritin-1 gene (*NRN1*, also called candidate plasticity gene 15). *NRN1* is an immediate early gene that is induced by synaptic activity through activation of NMDA receptors [17] and it is regulated by brain-derived neurotrophic factor (*BDNF*) and neurotrophin-3 [18]. As reviewed by Zhou and Zhou [19], *NRN1* is involved in neurodevelopment and synaptic plasticity, and also in promoting processes such as dendritic and axonal growth, neurite outgrowth, neuronal migration, and the maturation of synapses. Basic activity-related changes in the central nervous system are thought to depend on neuritin-mediated modification of synaptic transmission, especially in the hippocampus and neocortex [20]. Two previous studies have analyzed common variants along *NRN1* sequence and have shown its association with intelligence and SSD [21,22], suggesting that certain allelic variants at this gene modulate the risk for developing SSD and the general cognitive performance.

Based on the above mentioned, both *DTNBP1* and *NRN1* are: (i) implicated in plasticity processes, (ii) expressed in hippocampus and cortical neurons, and (iii) playing a role in the glutamatergic signalling through NMDA receptors, whose hypofunction has been associated with the neurobiology of schizophrenia [23]. Taking into account the common pathway in which both genes are involved, we aimed to study whether these genes work in concert and collectively contribute to increase the risk of SSD.

2. Methods

2.1. Sample

The sample comprised 785 Caucasian subjects: 388 SSD patients (mean age (sd) = 26.94 (11.38), 74.7% males) and 397 healthy unrelated subjects (mean age (sd) = 24.38 (6.54), 44.6% males). Patients' diagnoses (DSM-IV-TR evaluated with KSADS, SCID and/or CASH) were as follows: 66.5% schizophrenia, 16.7% first episode psychotic or psychotic disorder not otherwise specified, 11.9% schizophreniform disorder and 4.9% schizoaffective disorder. This sample is partially overlapped with the ones included in previous studies [12,22].

Exclusion criteria were: major medical illnesses that could affect brain functions, substance-induced psychotic disorder, neurological conditions, and history of head trauma with loss of consciousness. All participants provided written consent after being informed about the study procedures and implications.

2.2. SNP selection and genotyping

Genotyping of all SNPs was performed using Taqman 5′ exonuclease assays. First, three *DTNBP1* SNPs were selected due to their previous association with changes in the expression of *DTNBP1* in brain [7]: SNP1: rs2619537 (5′ flanking region), SNP2: rs2743864 (intronic), SNP3: rs1047631 (3′UTR). The fact that SNPs related to changes in availability or function of the protein can have stronger penetrance on the phenotype adds relevance in these SNPs selection criteria for studying their interaction. *DTNBP1* genotypes were dichotomized according to the described allelic association linked to expression changes: rs2619537 (C carriers vs AA), rs2743864 (A carriers vs GG), rs1047631 (G carriers vs TT).

Second, two *NRN1* SNPs (rs645649 (intronic) – rs582262 (upstream)) were selected. Despite these SNPs have not been associated with changes in the gene expression, they were considered of interest due to the previous observation of the increased frequency of the corresponding risk haplotype C-C in patients with SSD compared to healthy subjects [22]. Then, analyses were conducted with the risk haplotype derived from these two SNPs, classifying participants in two groups: (i) HAP-risk C-C carriers, which included individuals with at least one risk haplotype, and (ii) HAP-risk C-C non-carriers. Although *NRN1* SNPs are located in non-coding sequences, recent data has revealed the importance of intronic and intergenic variants as regulatory elements of gene expression [24]. More specifically, these two SNPs have been described as having putative influence on biological processes by changing the binding affinity of different transcription factors and binding proteins.

2.3. Statistical analysis

Comparisons of gender between patients and controls were performed by the chi-squared test. Hardy–Weinberg equilibrium and the linkage disequilibrium (LD) were estimated using Haploview v4.1. As linkage disequilibrium (LD) between *DTNBP1* SNPs showed low pairwise D'/r^2, all analyses were conducted by using *DTNBP1* SNPs separately. The two SNPs at *NRN1* showed a strong LD ($D' = 0.85$). Then, individual *NRN1* haplotypes were estimated using Unphased-v3.1.4 and, considering only those assigned with a probability $\geq$95%, each subject was classified according to the haplotype dose (HAP-risk C-C carriers vs non-carriers).

First, an epistasis screening was conducted by using the model based multifactor dimensionality reduction (MB-MDR) by applying 'mbmdr' R-package [25]. This method merges multi-locus genotypes into a one dimension construct that reflects the risk and can be represented by 3 values: high, low and non-informative. Thus, this method overcomes the dimensionality problem and increases the power to detect gene interactions associated with disease or phenotype. It also allows applying a 10,000 permutation procedure to the analyses besides adjusting for gender.

Second, the detected interaction by MB-MDR was confirmed by using a logistic regression model (SPSS 21 software, SPSS IBM, New York, U.S.A.), similarly to other studies that have explored joint effects of two genes (i.e. [26–29]). This complementary methodology allows analysing the magnitude of the interaction effect and

Table 1
Genotypes frequencies of *DTNBP1* SNPs and *NRN1* SNPs.

	Patients $N = 388$	Controls $N = 397$
DTNBP1 SNPs		
rs2619537		
AA	0.71	0.73
AG	0.25	0.24
GG	0.04	0.03
rs2743864		
GG	0.82	0.83
GA	0.17	0.16
AA	0.01	0.01
rs1047631		
TT	0.71	0.68
TC	0.26	0.29
CC	0.03	0.03
NRN1 SNPs		
rs645649		
GG	0.40	0.44
GC	0.47	0.46
CC	0.13	0.10
rs582262		
GG	0.48	0.59
GC	0.42	0.34
CC	0.10	0.07
rs645649-rs582262: HAP-risk C-C		
C-C carriers	0.47	0.36
C-C non carriers	0.53	0.64

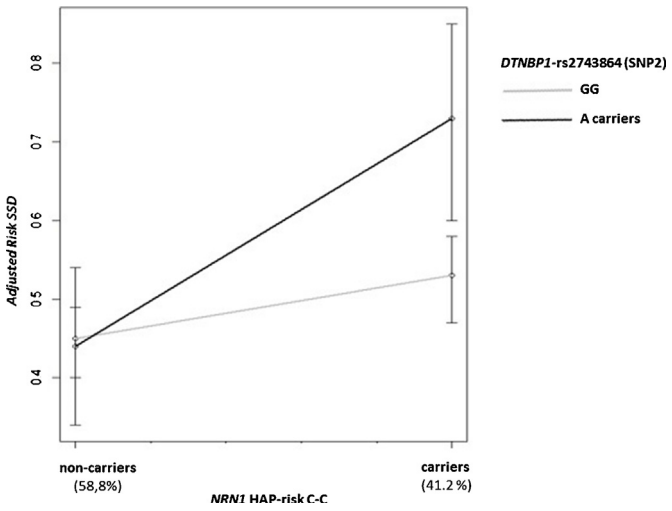

Fig. 1. Graphic representation of the interaction effect between *DTNBP1*-rs2743864 and *NRN1* HAP-risk C-C (rs645649-rs582262) on the risk probability for developing schizophrenia spectrum disorders (SSD). The concomitant presence of both A allele of *DTNBP1*-rs2743864 and HAP-risk C-C is associated with higher probability to develop a SSD. Note that risk adjusted prediction (Adjusted Risk SSD) was estimated by obtaining the predictive margins for each genotype combination group of the interaction. Subjects carrying GG genotype for *DTNBP1*-rs2743864 are shown in grey, while *DTNBP1*-rs2743864 A carriers are shown in black.

obtaining the corresponding graphical representation of the interaction.

Logistic regression was conducted between *DTNBP1* SNP and *NRN1* HAP-risk C-C (adjusted for gender). This regression analysis had two steps: first, main effects of *DTNBP1* SNP and *NRN1* HAP-risk C-C were tested on the outcome measure (risk for SSD). Second, two-way interaction effects between *DTNBP1* SNP and *NRN1* HAP-risk C-C were added to the model.

In all analyses, the significance cut-off was established at *P*-value of 0.05. Statistical power was estimated by using Quanto v1.2. For the gene–gene interaction analysis our sample had a power of 0.80 to detect an OR $\geq$ 2.1

3. Results

All SNPs were in Hardy-Weinberg equilibrium and the genotyping success rates were >96%. Table 1 depicts the genotype frequencies of *DTNBP1* and *NRN1* SNPs. As gender distribution between groups showed significant differences ($\chi^2 = 74.05$, $P < 0.001$), sex was added as a covariate in all the interaction models.

First, the MB-MDR provided evidence about the joint effect of *NRN1* HAP-risk C-C and *DTNBP1* SNP2 A allele on a higher risk for developing SSD ($P = 0.0049$ after permutation) (Table 2).

Second, this finding was confirmed with a logistic regression model. No main effect of *DTNBP1* SNP2 was detected. As expected according to our previous results [22], a main effect of the *NRN1* HAP-risk C-C on the risk for SSD was found ($\beta = 0.518$, $P = 0.001$, $R^2 = 0.155$), meaning that those subjects carrying at least one copy of the HAP-risk C-C (patients: 47%, controls: 36%) had a higher risk probability to develop SSD compared with non-carriers (Table 3). As in MB-MDR approach, this model reported a significant combined effect of HAP-risk C-C and SNP2 (rs2743864) on the risk for SDD ($\beta = 0.993$, $P = 0.033$, $R^2 = 0.162$) (Table 3). In other words, the main effect of the HAP-risk C-C was modified according to *DTNBP1* SNP2 genotype: among individuals carrying the HAP-risk C-C, the risk for SSD was higher in those who were also carriers of the A allele of *DTNBP1* SNP2 (patients: 17.64%, controls: 14.78%),

Table 2
Significant gene–gene interaction using the Model-based multifactor dimensionality reduction method (MB-MDR) for *DTNBP1***NRN1*: rs2743864*HAP-risk C-C.

Phenotype	Best multigene interaction model	Beta	Wald	*P* value	Risk category
Risk	rs2743864(A carriers)*HAP-risk C-C carriers	1.19	10.7	0.001	High
	rs2743864(GG)*HAP-risk C-C non carriers	−0.38	6.13	0.013	Low

Table 3
Main effects and interaction of *DTNBP1* SNP2 (rs2743864) and *NRN1* (rs645649-rs582262) on the risk for developing schizophrenia-spectrum disorders (logistic regression model).

	B	SE	Wald	Df	OR (95%CI)	*P*-value
Main effects						
DTNBP1 rs2743864 (A carriers vs GG)	0.32	0.21	2.22	1	1.37 (0.90–2.07)	0.136
NRN1 HAP-risk (non-carriers vs C-C carriers)	0.52	0.16	10.38	1	1.67 (1.22–2.3)	0.001
Sex	−1.4	0.16	74.82	1	0.24 (0.17–0.33)	<0.001
Interaction						
rs2743864*HAP-risk C-C	0.99	0.465	4.558	1	2.69 (1.08–6.71)	0.033

compared to GG homozygous (Fig. 1). It is noteworthy that the interaction term improved the overall fit of the model (−2log likehood $P = 0.029$). No interaction between other *DTNBP1* SNPs and *NRN1* HAP-risk haplotype was found.

4. Discussion

It is currently widely accepted that most proteins exert their biological functions in part through the interaction with other proteins [30]. Moreover, recent works have shown that neurodevelopment and plasticity processes involve an interaction between different candidate genes for schizophrenia [31,32], which is an example about the fact that the risk for developing these disorders is influenced by individual's genotype interactions.

In this study, we have explored the statistical interaction between *DTNBP1* and *NRN1* on SSD risk, considering both: (i) evidences that point towards their involvement in a common pathway, and (ii) that variation among individuals in biologically epistatic processes is likely to yield statistically epistatic effects [33].

Our data suggest that there is an interaction between *NRN1* HAP-risk C-C and *DTNBP1* SNP2 A allele modulating the risk for developing SSD, based on the analyses with the MB-MDR approach.

On the one hand, consistently with previous results, in this study we have identified a main effect of the *NRN1* risk haplotype C-C (rs645649-rs582262) on the risk for SSD. Although the effect of this haplotype is small (OR = 1.67), its significance should be evaluated under the consideration of the polygenic architecture of these disorders. Despite the fact that the two SNPs included in the risk haplotype are non-coding variants, it should be mentioned that the vast majority of the genome has gene regulatory properties [24], in which intronic and intergenic variants have a role. The impact of the non-coding variants included in the *NRN1* risk haplotype (rs645649-rs582262) can be evaluated using HaploReg (http://www.broadinstitute.org/mammals/haploreg/haploreg.php/). Accordingly, rs645649 is located in an intronic region and modifies transcription factor binding sites (ERalpha-a) where other two proteins are also bounded: SUZ12 (involved in methylation processes leading to transcriptional repression of the affected target genes) and ZNF263 (implicated in basic cellular processes as a transcriptional repressor). Moreover, rs582262 is located in the upstream region where three proteins are bounded: RAD21 (involved in repair of DNA double-strand breaks, as well as in chromatid cohesion during mitosis). In this regard, although functional studies are needed, the association of *NRN1* risk haplotype with SSD could also be linked to the final availability or functionality of the protein which, in turn, could dysregulate *NRN1* role on neurite outgrowth and arborization and/or on neuronal processes associated with plasticity.

Furthermore, despite the large number of studies supporting the role of *DTNBP1* in schizophrenia (reviewed by Williams et al. [34]) we could not detect a main effect of *DTNBP1* SNPs. This could be interpreted in light of the heterogeneity of *DTNBP1* genetic markers reported in association with SSD. Moreover, in spite of the interest of the tested SNPs due to their effect on Dysbindin-1 expression, few association studies have analyzed them in relation to the risk for SSD. However, it is remarkable that some of the *DTNBP1* haplotypes described to be associated with SSD do include these SNPs, with special mention to rs2743864, which is included in a previous identified risk haplotype involved in early-onset forms of psychosis [12].

In relation to the statistical epistasis, our results indicate that the observed main effect of *NRN1* HAP-risk is modulated by the *DTNBP1* rs2743864 (SNP2), meaning that among individuals carrying the HAP-risk C-C those also carrying the A allele of *DTNBP1* SNP2 (17.6% patients vs 14.7% healthy subjects) were more likely to develop SSD than the GG homozygous. In this sense, it should be mentioned that, although the moderate frequency of the epistatic combination, it has been reported that when the prevalence of the disease is low, as is the case of SSD (≅ 1–2%), the unmatched case-control is still the most powerful and efficient design in order to detect gene–gene interaction with less sample size [35]. Although our study does not test the biological interaction pattern directly, this statistical interaction suggests that the pair of involved genes together increases the risk for SSD according to the concept that epistasis contributes to the risk for complex diseases [1]. Research has shown that biological interactions are critical for gene regulation, signal transduction and numerous other physiological and developmental pathways, which can indicate areas for future advancement in determining the underlying aetiology of schizophrenia.

The interaction detected has to be analyzed according to the functional significance of the genetic variants. In this regard, besides the role of the *NRN1* SNPs as binding sites of different transcription factors and binding proteins, it is of mention that the A allele at rs2743864 was described to be associated with higher levels of *DTNBP1* expression in healthy subjects [7]. We have found that the concomitant presence of both A allele at rs2743864 (*DTNBP1*) and HAP-risk C-C (*NRN1*) are associated with higher risk for SSD, compared to the presence of either one of them alone. In other words, we detect a modulating effect of *DTNBP1* on the role of *NRN1*. In this connection, we could modestly infer that gene products of both genes impinge on neuronal structure and/or function, with particular emphasis in the interface with intercellular signalling at the neuron level. As other authors have suggested, plasticity genes constitute the backbone of the synaptic and cellular machinery and their regulated expression is a means to dynamically tune a correct neuronal function [20]. Then, one possible explanation for our result may be related to the crucial role that *DTNBP1* gene plays in the regulation of the synaptic homeostasis [3]. To this respect, it has been suggested that *DTNBP1* levels may be down-regulated by the action of other schizophrenia susceptibility genes [7], which could support the idea of studying genetic interactions in order to see an overall effect on a trait. In this sense, although the molecular mechanisms underlying the stabilization of neural function are broadly unknown, *DTNBP1* and *NRN1* interaction could contribute synergistically to the modification of the synaptic homeostasis. Then, on the basis of these findings, and taking into account that alterations in Dysbindin-1 have been related to abnormalities in the synaptic connectivity and glutamate signalling found in schizophrenia [6,36], our results could be of substantial value as a link to the pathophysiology of schizophrenia.

There were some limitations in this study, which reflect the genetic complexity of severe mental disorders. First, the sample size was limited and replication analysis in larger samples is needed to confirm these findings. To this respect, new analyses would benefit also from extending the genetic variability analyzed in both genes. Second, the variation of R^2 from the non-interaction model to the interaction was small but significant. Thus, this effect seems not of dismissible interest, since it is known that the power to detect interactions is typically lower than the power to detect main effects [37]. Third, the fact that we have analyzed the interaction by using a haplotype and SNPs could be also limiting our interpretations, adding extra complexity to the analyses. However, haplotypes can be more informative than individual SNPs, when interaction is present [38]. Finally, sex-differences found in our sample should be also mentioned. While this was corrected adjusting the analyses by gender, larger samples are needed to conduct specificity tests by gender. In summary, the interpretation of these results should be done with caution and further studies are required to determine the biological mechanisms underlying the detected epistatic effect.

To conclude, the statistical interaction observed between *NRN1* and *DTNBP1* suggests that they work collectively contributing to increase the risk of schizophrenia, which can help in the detection and characterization of the biological and biochemical pathways that underpin disease. This adds interest on investigating the interactions of genes in order to explain a substantial component of disease risk and also of more specific clinical and cognitive outcomes of SSD.

Disclosure of interest

The authors declare that they have no competing interest.

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Advisors' report on the contribution of the PhD. candidate to the article:

Dr. Lourdes Fañanás (Associate Professor) and Dr. Fatjó-Vilas (Assistant Professor) from the Department of Evolutionary Biology, Ecology and Environmental Sciences of the Faculty of Biology (University of Barcelona), both supervisors of the present doctoral thesis by Claudia Prats, hereby certify that the participation of the PhD applicant in the article "**Evidence of an epistatic effect between Dysbindin-1 and Neuritin-1 genes on the risk for Schizophrenia Spectrum Disorders"**, included the following tasks:

- Participation in the conception and design of the manuscript
- Molecular analysis design.
- Statistical analysis and interpretation of data
- First drafting of the manuscript
- Critical revision of the article for intellectual content

Signed by Dr. Lourdes Fañanás and Dr. Mar Fatjó-Vilas

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4.3

Neurotrophins role in depressive symptoms and executive function performance: Association analysis of *NRN1* gene and its interaction with *BDNF* gene in a non-clinical sample.

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Role of neurotrophins in depressive symptoms and executive function: Association analysis of *NRN1* gene and its interaction with *BDNF* gene in a non-clinical sample

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ABSTRACT

Background: Neuritin-1 is a neurotrophic factor involved in synaptic plasticity that has been associated with depressive disorders, schizophrenia and cognitive performance. The study of genotype-phenotype relationships in healthy individuals is a useful framework to investigate the etiology of brain dysfunctions. We therefore aimed to investigate in a non-clinical sample whether *NRN1* gene contributes to the psychopathological profile, with a particular focus on the clinical dimensions previously related to the *NRN1* gene (i.e. depressive and psychotic). Furthermore, we aimed to analyze: i) the role of *NRN1* on executive functions, ii) whether the association between either *NRN1*-psychopathological profile or *NRN1*-cognitive performance is moderated by the *BDNF* gene.
Methods: The sample is comprised of 410 non-clinical subjects who filled in the self-reported Brief Symptom Inventory (BSI) and were assessed for executive performance (Verbal Fluency, Wisconsin Card Sorting Test (WCST) and Letter-Number subscale (WAIS-III)). Genotyping included nine SNPs in *NRN1* and one in *BDNF*.
Results: i) GG homozygotes (rs1475157-*NRN1*) showed higher scores on BSI depressive dimension and on total scores compared to A carriers (corrected p-values: 0.0004 and 0.0003, respectively). ii) A linear trend was detected between GG genotype of rs1475157 and a worse cognitive performance in WCST total correct responses (uncorrected p-value: 0.029). iii) Interaction between rs1475157-*NRN1* and Val66Met-*BDNF* was found to modulate depressive symptoms (p=0.001, significant after correction).
Limitations: Moderate sample size; replication in a larger sample is needed.
Conclusions: *NRN1* is associated with depressive symptoms and executive function in a non-clinical sample. Our results also suggest that the role of *NRN1* seems to be modulated by *BDNF*.

1. Introduction

Brain development is an organized and dynamic process, the efficiency of which is essential for the adequate functioning of the whole brain. Both genetic and environmental inputs are involved in normal brain development, and disruption of any of them can fundamentally alter neural outcomes. Accordingly, deviances in neurodevelopmental processes are thought to contribute to the etiology of many psychiatric disorders that manifest throughout the entire lifespan. Increasing evidence suggests that neurotrophic factors (also called neurotrophins, *NTFs*) are important regulators of neural survival, growth, development, function, and plasticity (Huang and Reichardt 2001). In this sense, inadequate neurotrophic support in the brain could lead to inappropriate cortical circuitry and synaptic transmission in the developing brain, which could translate into a reduced ability to make adaptive changes (Angelucci et al., 2005). In turn, this reduction in plasticity could underlie the variability in cognitive functioning related to the development of mental disorders.

Variability in brain plasticity, from healthy to diseased states, is thought to result from complex interactions between genetic factors

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following a polygenic inheritance pattern involving multiple genes with small effects. In addition, recent molecular genetic studies have provided empirical evidence of the existence of shared genetic roots across several psychiatric disorders such as schizophrenia (SZ) and major depressive disorder (MDD) (Cross-Disorder Group of the Psychiatric Genomics Consortium, 2013), supporting the possibility of common pathophysiological mechanisms among these disorders. This notion is reinforced by the fact that both SZ and MDD show an overlapping profile of cognitive impairments, particularly in executive function (EF) (Barch and Sheffield, 2014; Chamberlain and Sahakian, 2004; Elliott, 2003).

Psychiatric research has mainly focused on subjects affected by the severe form of disorders; however, studying subjects with attenuated symptoms that are below the clinical threshold may also shed light on the etiology of mental disorders. In this sense, epidemiological studies have reported that depressive symptoms are frequent in the general population, varying between 2.1% and 7.6% (Blazer et al., 1994; Regier et al., 1988). Similarly, a recent meta-analysis reported that the median lifetime prevalence of psychotic experiences (PE) was 7.2% (Linscott and van, 2013). These data suggest that there is a continuous distribution of symptoms in the general population (Verdoux and Van, 2002). Thus, the study of the factors and mechanisms underlying psychiatric symptoms in non-clinical samples contributes to the understanding of the severe expression of these phenotypes and offers the advantage of obtaining results unbiased by treatment or the illness itself.

Due to the important role that NTFs play during neurodevelopment, Neuritin-1 (*NRN1*) and Brain-Derived Neurotrophic Factor (*BDNF*) are considered as putative candidate genes for psychiatric diseases. *NRN1*, also called candidate plasticity gene 15 (*CPG15*), encodes a small highly conserved protein that is attached to the extracellular neuronal membrane by a glycosylphosphatidylinositol link and operates as an intercellular signal between neighboring neurons (Naeve et al., 1997). As reviewed by Zhou and Zhou (2014), *NRN1* is involved in neurodevelopment and synaptic plasticity, and also in promoting processes such as dendritic and axonal growth, neurite outgrowth, neuronal migration, and the maturation of synapses. Furthermore, the expression of the *NRN1* gene has been reported to be regulated by *BDNF* (Naeve et al., 1997), which promotes the differentiation and growth of developing neurons in central and peripheral nervous systems (Buckley et al., 2007). The intracellular distribution of BDNF and its activity-dependent secretion is altered by the Met variant of a functional polymorphism in the *BDNF* gene, which consists of a valine (Val) substitution for methionine (Met) at codon 66 (Val66Met*). BDNF* gene polymorphisms have been associated not only with psychotic disorders (Krebs et al., 2000; Rybakowski et al. 2006), but also with mood disorders and a range of clinical features, including age of onset, symptoms, therapeutic responsiveness, neurocognitive function and brain morphology (Notaras et al., 2015).

On the one hand, previous studies have reported the effect of polymorphic variation of *NRN1* on the risk for developing SZ and on general cognitive performance (Chandler et al. 2010; Fatjó-Vilas et al., 2016). On the other hand, from animal model-based studies, there is evidence of a relationship between *NRN1* and depressive symptoms. First, Neuritin-1 knockdown results in depressive-like behaviors (Son et al., 2012). Second, electroconvulsive therapy, one of the most robust gene inducers among all antidepressant treatments (Segi-Nishida, 2011), induces changes in both *NRN1* and *BDNF* expression (Dyrvig et al., 2014). Third, fluoxetine increases the level of *NRN1* and *BDNF* specifically in the prefrontal cortex, hippocampus and dentate gyrus (Alme et al., 2007), which suggests that antidepressant treatment promotes gene expression responses linked to *NTF* signaling and synaptic plasticity.

Given the above, an understanding of the role of the *NRN1* gene might help explain the neuroplasticity mechanisms underlying the different patterns of psychopathological profiles. Accordingly, the main aim of our study was to investigate in a general population sample whether *NRN1* gene variability contributes to the psychopathological profile, with special interest in the clinical dimensions previously related to the *NRN1* gene (i.e. depressive and psychotic). Secondarily, we also aimed: i) to test whether executive function is an intermediate phenotype in the relationship between *NRN1* and depressive symptoms, ii) to analyze whether the association between either *NRN1*-psychopathological profile or *NRN1*-cognitive performance is moderated by the *BDNF* gene.

2. Methods

2.1. Sample description

Healthy adult Spanish individuals from the general population were recruited from the campus of Jaume I University in Castelló (Spain). Exclusion criteria were the presence of any major medical illness affecting brain function, current substance abuse (alcohol or illicit drugs), neurological conditions, history of head injury and personal history of psychiatric medical treatment. These aspects were screened by means of a short interview designed *ad hoc* for this study. In addition, participants were required to describe themselves as being of Spanish (Caucasian) ancestry to reduce the possibility of confounding by population stratification (Freedman et al., 2004).

Ethical approval was obtained from local research ethics committees. All participants provided written informed consent before inclusion in the study.

2.2. Measurements

All interviews were carried out by trained psychologists.

2.2.1. Brief Symptom Inventory

All participants filled in the Brief Symptom Inventory (BSI), which is a self-reported scale that provides information on a wide range of symptoms of psychological distress and mental disorders (Derogatis and Melisaratos, 1983) in the last 30 days. We used the validated Spanish version of the BSI, which was conceived to measure psychiatric symptoms from a dimensional perspective and designed to be used both in clinical and non-clinical populations. BSI includes 46 items grouped into six dimensions: depression, paranoid ideation, anxiety, obsession-compulsion, somatization and hostility (Ruipérez et al., 2001). Each item of the BSI is rated on a 5-point scale of distress ranging from “not at all” (1) to “extremely” (5). According to our hypothesis, depressive symptoms (measured by the *depressive dimension*) and psychotic symptoms (measured by the *paranoid ideation dimension*) were of special interest in this study. As an example, the *depression dimension* includes signs and symptoms of the clinical syndrome of depression such as dysphoric affect, loss of interest in life activities or loss of vital energy; and the *paranoid ideation dimension* includes those related to being susceptible, full of mistrust or with fear of loss of autonomy, among others.

A continuous weighted score of each symptom subscale was used in the analyses (e.g. sum of scores on the depression items divided by number of items filled in).

2.2.2. Cognitive assessment

Cognitive executive function was assessed using a battery of 3 standardized neuropsychological tests that have been shown to be sensitive to frontal/prefrontal dysfunction (Lezak et al., 2004): verbal fluency (Spreen and Benton, 1977), Wisconsin card sorting test (WCST, Heaton 1981) and the letter-number subscale of the Wechsler Adult Intelligence Scale (WAIS-III, Wechsler, 1997)). From these tests, 5 outcome variables were selected: 1) number of animals named in one minute (semantic fluency), 2) number of words starting with letter P named in one minute (phonemic fluency), 3) number of perseverative errors (WCST), 4) number of correct responses (WCST), 5) total score on Letter-Number subscale (WAIS-III).

Additionally, the Intellectual quotient (IQ) was assessed using the Block Design and Vocabulary or Information subtests of the WAIS-III, in accordance with the method suggested by Sattler (2001).

2.3. Molecular analysis

Genomic DNA was extracted from buccal mucosa using standard methods: the Real Extraction DNA Kit (Durviz S.L.U., Valencia, Spain) or the Buccal Amp DNA Extraction Kit (Epicentre® Biotechnologies, Madison, WI).

Coverage of the *NRN1* genomic sequence and ~10 kb upstream and downstream was achieved by including nine tag SNPs. The optimal set of SNPs that contained maximum information about surrounding variants was selected using SYSNPs (http://www.sysnps.org/) with a minor allele frequency (MAF) > 5%, using the pairwise option tagger (threshold of r^2=0.8). The SNPs included in Chandler et al. (2010) were also considered.

After genotyping we performed a SNP LD pruning analysis using PLINK (which allows the pruning of SNPs that are highly correlated (r^2 > 0.8)). The nine SNPs were not pruned out, supporting a single SNP approach.

The Val66Met functional SNP of the *BDNF* gene (rs6265) was also genotyped. For this polymorphism, the A allele encodes for the amino acid methionine (Met) and the G allele encodes for valine (Val). Due to the low Met allele frequency, individuals with Val/Met or Met/Met genotypes were combined (Met carriers) and compared with individuals with the Val/ Val genotype. See Table 1 for SNP details.

Genotyping was performed using a fluorescence-based allelic discrimination procedure (Applied Biosystems Taqman 5'-exonuclease assays). Standard conditions were used. The genotyping call rate for all SNPs was higher than 94.2%. After randomly re-genotyping 10% of the sample, 100% of genotyping results were confirmed. All SNPs were in Hardy-Weinberg equilibrium.

The impact of non-coding variants of the *NRN1* SNPs was examined using HaploReg (Ward and Kellis, 2012), which is a tool that uses linkage disequilibrium information from the 1000 Genomes Project to provide data on the predicted chromatin state of the queried SNPs, their sequence conservation across mammals and their effect on regulatory motifs.

2.4. Statistical analysis

Association analyses under a genotypic model were undertaken between *NRN1* SNPs and each BSI dimension using the linear regression function in PLINK (Purcell et al., 2007), including age and gender as covariates. We also explored the data under the assumptions of dominant (major homozygotes versus heterozygotes plus minor homozygotes) or recessive (major homozygotes plus heterozygotes versus minor homozygotes) models of inheritance. These analyses were corrected for multiple testing using PLINK's max (T) permutation procedure with 1000 iterations.

Based on the significant results of genotypic association analysis, the association between the *NRN1*-rs1475157 (GG vs A carriers) and cognitive performance was analyzed by means of linear regression (SPSS 21.0; IBM, New York, U.S.A). Years of education and gender were included as covariates. The relationship between cognitive performance and BSI depressive/total scores was also tested using linear regression, adjusted by years of education and gender.

Moreover, the effect of the interaction between the *NRN1*-rs1475157 (GG vs A carriers) and the *BDNF*-rs6265 (Val/Val vs Met carriers) polymorphisms on the depressive dimension and cognitive measures was explored by means of two-way interaction effects with a linear regression model (adjusted by years of education and gender). These regression analyses involved two steps: first, the main effects of *NRN1*-rs1475157 and *BDNF*-rs6265 were tested for each outcome measure: a) depressive symptoms (adjusted by sex and age), and b) cognitive measures (adjusted by sex and years of education). Second, two-way interaction effects between *NRN1*-rs1475157 and *BDNF*-*r*s6265 were added to the model. In each of these linear regression analyses, Bonferroni correction was applied.

Statistical power estimations were conducted using G*Power 3.1.7 (Faul et al., 2009). In our sample, we had sufficient power (0.80) to detect effect sizes (d) ranging from 0.32 to 0.70 between the two main genotypes of *NRN1* SNPS. Specifically, as an example, for rs1475157 (GG vs A carriers) the effect was d=0.66, which corresponds to 0.71 points on the BSI depressive dimension scale (Cohen, 1988).

3. Results

3.1. Sample description

The sample comprised 410 non-clinical subjects from the general population: 44.2% were males, mean age at interview (sd)= 22.09 (3.4). At the time of assessment, 77% of the participants were university students. In terms of education, 2.51% of individuals had only completed elementary school, 92.46% had completed high school and 5.03% had completed a university education (mean years of education (sd)= 13.5 (1.7), mean IQ (sd)=99.16 (11.64)).

In relation to the psychopathological status measured by the BSI, in the current sample between 15 and 20% of the individuals reported that they had "extremely" experienced at least one item of any BSI dimension. Weighted mean scores (sd) of each BSI dimension were as follows: depression=1.83(0.74); paranoid ideation=1.72(0.61); anxiety=1.28(0.39); obsession-compulsion=1.73(0.60); somatization=1.60(0.56); hostility=1.38(0.49) and total BSI=9.57(2.72).

Table 1
Information on *NRN1* and *BDNF* SNPs included in this study. The table includes the dbSNP number, the genomic and gene position and the alleles of each SNP (UCSC Genome Browser on Human Mar. 2006 Assembly (hg18), http://genome.ucsc.edu/cgi-bin/hgTracks). Observed allelic and genotypic frequencies are also given.

SNP	Chr	Chr Position	Gene position	Alleles[a]	MAF_{1000G}[b]	MAF_{sample}[c]	Genotype Frequency (%)		
NRN1 gene									
rs2208870	6	5992490	Intergenic	A/G	0.332	0.316	GG (9.10%)	GA (46.19%)	AA (44.71%)
rs2208870	6	5992490	Intergenic	A/G	0.332	0.316	GG (9.10%)	GA (46.19%)	AA (44.71%)
rs12333117	6	5994992	Downstream	C/T	0.347	0.423	CC (32.92%)	CT (49.26%)	TT (17.82%)
rs582186	6	6001381	Intronic	A/G	0.450	0.371	GG (38.54%)	GA (48.87%)	AA (12.59%)
rs645649	6	6004959	Intronic	C/G	0.449	0.324	GG (45.25%)	GC (44.75%)	CC (10.00%)
rs582262	6	6007991	Upstream	C/G	0.480	0.230	GG (60.36%)	GC (33.76%)	CC (5.88%)
rs10484320	6	6010437	Upstream	C/T	0.152	0.219	CC (59.61%)	CT (36.15%)	TT (4.24%)
rs4960155	6	6010539	Upstream	C/T	0.425	0.493	CC (26.10%)	CT (46.04%)	TT (27.86%)
rs9405890	6	6012721	Intergenic	T/C	0.376	0.320	TT (47.00%)	TC (41.30%)	CC (11.7%)
rs1475157	6	6017169	Intergenic	A/G	0.164	0.181	AA (68.38%)	AG (27.00%)	GG (4.62%)
BDNF gene									
rs6265 (Val66Met)	11	27598369	Exonic	A/G	0.201	0.237	Val/Val (59.10%)	Val/Met (34.22%)	Met/Met (6.68%)

[a] The less frequent allele (minor allele) is placed second.
[b] MAF refers to Minor Allele Frequency observed in the 1000 Genomes project across all populations (Abecasis et al. 2012).
[c] MAF observed in the current sample.

Table 2
Linear regression models testing the main effects and interaction of *NRN1* (rs1475157) and *BDNF* (rs6265) on the presence of depressive symptoms **(A)** and phonemic fluency **(B).**

A) BSI Depressive Dimension	**β**	**SE**	***P-value***
i) Main effects:			
NRN1 (rs1475157) (GG vs A carriers)	0.62	0.18	0.001
BDNF (rs6265) (Val/Val vs Met carriers)	0.09	0.80	0.246
Sex	0.09	0.07	0.248
Age	−0.008	0.01	0.439
ii) Interaction:			
NRN1 BDNF*	1.22	0.38	**0.001**
B) Phonemic Fluency	**β**	**SE**	***P-value***
i) Main effects:			
NRN1 (rs1475157) (GG vs A carriers)	−1.349	0.992	0.175
BDNF (rs6265) (Val/Val vs Met carriers)	−0.451	0.426	0.290
Sex	−0.211	0.423	0.618
Education years	0.119	0.058	0.042
ii) Interaction:			
NRN1 BDNF*	−4.462	2.089	**0.033**

A) i) Adj-R^2 =0.03 ii) Adj-R^2 =0.06
B) i) Adj-R^2 =0.009 ii) Adj-R^2 =0.018
β, regression coefficient; SE, standard error

With regard to executive function, the mean (sd) scores of the tests were as follows: i) phonemic fluency=16.64(4.1); semantic fluency=22.28(5.1); perseverative errors WCST=8.44(8.01); total correct response=69.98(8.88); letter-number=9.32(2.52).

Table 1 shows the genotype distribution for *NRN1* and *BDNF* polymorphisms in the sample. The observed genotypic frequencies were similar to those described by the 1000 Genomes Project.

3.2. Is the variability in the NRN1 gene associated with BSI psychopathological dimensions?

Among the six BSI psychopathological dimensions, the genetic variability in the *NRN1* gene was related to the depressive dimension and total BSI scores. In particular, the SNP rs1475157 was significantly associated with depressive symptoms (p=0.0016 – genotypic model). Specifically, GG homozygotes showed higher scores on the BSI depressive dimension than A allele carriers: 2.4 (1.08) *vs* 1.8 (0.71), respectively (β=0.62 p=0.0004 – recessive model). In addition, the total score on the BSI was also significantly higher in those individuals carrying two copies of the G allele: 10.84 (3.56) *vs* 9.51 (2.68), respectively (β=2.51 p=0.0003 – recessive model). These associations remained significant after permutation analysis.

3.3. Is the variability in the NRN1 gene (SNP rs1475157) associated with cognitive performance?

The same genotype within the rs1475157 polymorphism was also associated with cognitive performance. A linear trend was detected between the GG genotype and a worse cognitive performance in the following tests: a) phonemic fluency: GG 15.05(3.73) vs A carriers 16.72(4.11), β=-1.66 p=0.086; b) WCST total correct response: GG 65.52(5.45) vs A carriers 70.20(9.04), β=-4.5 p=0.029. However, these results did not remain significant when multiple testing corrections were applied.

No association was observed between rs1475157 polymorphism and either semantic fluency, WCST perseverative errors or letters-numbers.

According to these findings, we explored the relationship between executive function tests (those observed to have a nominal association with *NRN1),* and BSI depressive dimension and total scores (also associated with *NRN1*). A trend towards correlation was found between BSI depression dimension scores and phonemic fluency (β=-0.02 p=0.059). In addition, higher BSI total scores were correlated with lower phonemic fluency (β=-0.09 p=0.007). No relationship was found between WCST total correct responses and BSI depressive dimension or total scores. When correcting for multiple testing, only the association between phonemic fluency and BSI total scores remained significant.

3.4. Is the relationship between NRN1 *and depressive symptoms/ cognitive performance modulated by* BDNF *gene?*

We tested the effect of the interaction between *NRN1*-rs1475157 and the polymorphism Val66Met of the *BDNF* gene on: i) depressive symptoms, ii) phonemic fluency and total correct responses in WCST. First, a significant two-way interaction was found for the presence of depressive symptoms (β= 1.22 p=0.001) (Table 2, Fig. 1A). In other words, carriers of both the GG genotype of *NRN1*-rs1475157 and the *BDNF* Met allele presented significantly more depressive symptoms. Second, we found a significant two-way interaction for phonemic fluency (β=-4.462 p=0.033) (Table 2, Fig. 1B), meaning that carriers of both the GG genotype of *NRN1*-rs1475157 and Met allele of the *BDNF*-rs6265 presented significantly worse phonemic fluency. Third, there was no interaction effect for WCST or letter-number scores. After multiple testing correction, only the *NRN1xBDNF* interaction effect in depressive symptoms remained significant.

4. Discussion

Our study aimed to explore the role of the Neuritin-1 gene in the expression of psychopathological dimensions and on the performance on executive function tasks in healthy subjects from the general population. Moreover, we were also interested in investigating whether the effect of *NRN1* on these clinical and cognitive phenotypes is modulated by the *BDNF* gene (Fig. 2).

First, our results suggest that *NRN1* gene variability is associated with depressive sub-clinical symptomatology. Contrary to previous findings, in which *NRN1* was associated with SZ, in our general population-based sample, *NRN1* was not related to psychotic symptoms as measured using the paranoid ideation dimension of the BSI scale. To our knowledge, this is the first work describing a genetic association between the *NRN1* gene and depressive symptoms in a non-clinical population sample. Specifically, we found that the polymorphism upstream of *NRN1* (rs1475157) shows a significant effect on the appearance of depressive symptoms, with individuals carrying the genotype GG showing higher scores compared to A allele carriers. These results are in line with the findings from animal models in which *NRN1* has been identified as an interesting new player in depression. In this regard, it has been reported that electroconvulsive therapy and antidepressant treatment produce changes in Neuritin-1 levels in the prefrontal cortex and hippocampus (Alme et al., 2007; Dyrvig et al., 2014) and, also, that knockdown of *NRN1* produces depressive-like behavioural effects in mice models (Son et al., 2012). On the other hand, it has been reported that hippocampal expression of Neuritin-1 is down-regulated by chronic stress and that antidepressant treatment reverses this effect. In all, these results suggest the involvement of *Neuritin-1* in modulating depressive symptomatology and highlight it as a potentially interesting new target for antidepressant treatment. In addition, these studies also indicate that the relationship between Neuritin-1 and depression could be mediated by stress exposure; this is supported by i) evidence on the involvement of Neuritin-1 in the actions of BDNF (Naeve et al., 1997; Wibrand et al., 2006), and ii) the well-described effects of stress on BDNF expression (Berton & Nestler, 2006). Then, taking into account the previous associations relating BDNF and cortisol levels (Holsboer, 2000), it is plausible that stress-mediated changes in Neuritin-1 expression could also impact on stress hormones and HPA axis dysregulation; however, further studies are needed on this topic.

Second, the same genotype within the rs1475157 polymorphism showed a trend towards association with worse performance in phonemic fluency and WCST total correct responses. Although these results were not

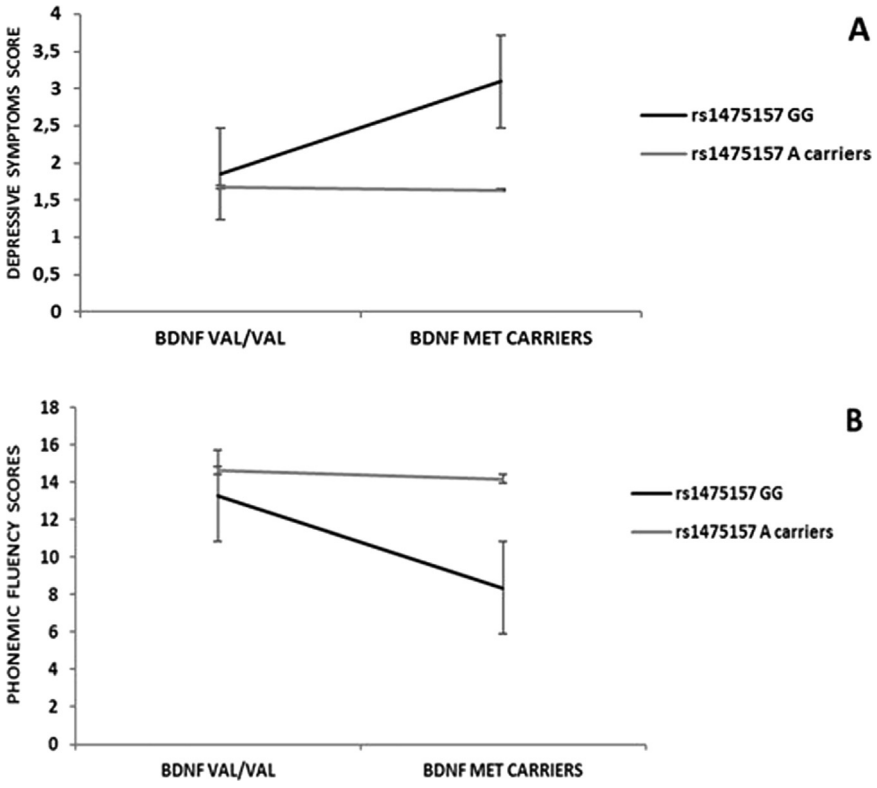

Fig. 1. A) Graphical representation of the effect of the interaction between rs1475157 of *NRN1 (GG vs A carriers)* and rs6265 of *BDNF* (Val/Val vs Met carriers) on the presence of depressive symptoms (BSI), corrected by age and sex. BSI scores $\tilde{x}$(sd): GG + Val/Val=2.02(0.9); GG + Met carriers=3.25(1.11); Met carriers + A carriers=1.82(0.69); Val/Val + A carriers=1.79(0.75). B) Graphical representation of the effect of the interaction between rs1475157 of *NRN1* and rs6265 of *BDNF* on phonemic fluency scores, corrected by years of education and sex. Phonemic fluency $\tilde{x}$ (sd): GG + Val/Val=13.27(0.9); GG + Met carriers=8.36(1.11); Met carriers + A carriers=14.17(0.69); Val/Val + A carriers= 14.62(0.75).

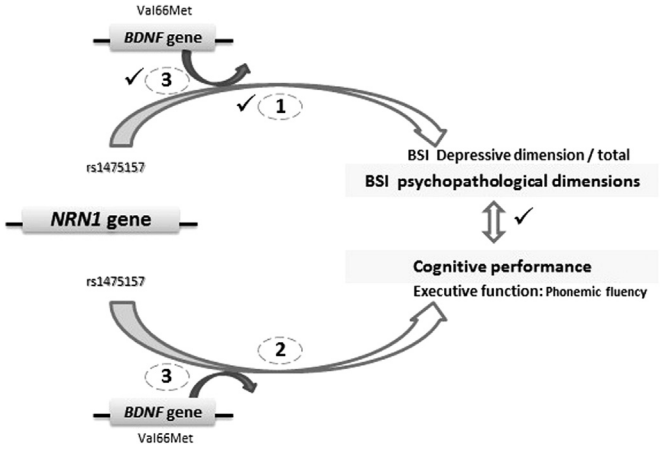

Fig. 2. Graphical representation of the different aims and results of the study. 1) To explore whether the *NRN1* gene is associated with psychopathological dimensions of the BSI. *NRN1*-rs1475157 was found to be significantly associated with depressive symptoms and BSI total score. 2) To explore whether *NRN1* is associated with executive function. An association trend was detected between *NRN1*-rs1475157 and executive cognitive performance (non-significant after correction). In addition, we detected a significant negative correlation between BSI total scores and phonemic fluency. 3) To analyze whether the association between either *NRN1*-psychopathological profile or *NRN1*-cognitive performance is moderated by the *BDNF* gene. The interaction between rs1475157-*NRN1* and Val66Met-*BDNF* was found to significantly modulate depressive symptoms, while no significant interaction was found between either polymorphism in terms of cognition. *Note that only the significant results after correction are indicated with a tick symbol.

significant after multiple testing correction, they are suggestive of an involvement of *NRN1* in executive function in the general population. The two previous studies on *NRN1* and cognition detected such an effect in SZ patients but not in healthy subjects (Chandler et al., 2010; Fatjó-Vilas et al., 2016); however, they analyzed general intelligence and not executive function. In particular, Chandler et al. found that the G allele of rs1475157 was associated with poorer performance in the abstraction component and IQ decline specifically in SZ patients; the other allele of rs1475157 (A) located within a haplotype was associated with better IQ scores and later age at onset for SZ (Fatjó-Vilas et al., 2016).

Although our findings are based on a healthy sample, it is attractive to speculate about the interest in studying genetic variability in non-clinical populations in which there is a continuum of pathophysiological dimensions (Verdoux and Van, 2002). The fact that *NRN1* plays a role in the presence of depressive symptoms and cognitive performance is also supported by evidence on that depression is associated with a number of cognitive deficits (Austin et al., 2001; Christensen et al., 1997). This is in line with studies showing that there is a negative relationship between BSI depression dimension scores and phonemic fluency. This is in line with evidence that patients with depression produce fewer words on fluency tasks (Fossati et al., 2003). From the viewpoint of an intermediate phenotypes framework, it is of note that these cognitive deficits have also been found in healthy first-degree relatives of patients with either MDD or SZ (Barrantes-Vidal et al., 2007; Christensen et al., 2006). Thus, our findings support the importance of studying the performance of executive functions as a vulnerability marker for depression in the general population.

Studying the putative effects of the analyzed polymorphic sites on regulatory mechanisms of gene expression represents a valuable way of providing additional meaning and importance for our association data. Although rs1475157 is not a functional SNP and there are no studies in animal models on whether this SNP can be associated with depressive symptoms, recent data have revealed the importance of intronic and intergenic variants as regulatory elements of gene expression (Dunham et al., 2012). Interestingly, by means of Haploreg, rs1475157 is predicted to alter various regulatory motifs such as *HP1-site-factor*, a telomere-capping protein whose function is necessary for chromosome stability (Fanti et al., 1998) and is involved in gene silencing (Jones et al., 2000).

Moreover, there is also evidence that this SNP is a binding site for the circadian rhythm-related transcription suppressor E4BP4 (Mitsui et al., 2001). This transcription factor is involved in the circadian expression of Per2, which is one of the essential components of mammalian circadian clocks (Ohno et al., 2007). Interestingly, depressive disorders have been associated with deregulation of the circadian biological clock that controls neuronal physiological processes (Landgraf et al., 2014), which gives indirect support to our findings.

Third, our data show for the first time that the effect of *NRN1*-rs1475157 on either the appearance of depressive symptoms or cognitive function is not independent of *BDNF* polymorphism. In other words, individuals with the *NRN1*-rs1475157 GG genotype that are carriers of the *BDNF-rs6265* Met allele present more depressive symptoms than individuals carrying other allelic combinations. Moreover, although not significant after correction, the same genotype combination (GG genotype of *NRN1*-rs1475157 and Met allele of *BDNF-rs6265)* was associated with poorer cognitive performance in terms of phonemic fluency. Thus, to understand this synergistic effect, beyond the above-described effects of *NRN1*, the role of *BDNF* has to be considered. Met-allele carriers show significantly lower activity-dependent expression of *BDNF* (Egan et al., 2003) and this allele has also been related to a plausible increased risk for developing depression (Buchmann et al., 2013). In addition, the Met allele has also been linked with impaired episodic memory, working memory, and reduced hippocampal volume and function in healthy populations (Dempster et al., 2005; Frodl et al., 2007; Zeni et al., 2016), all of which support our findings.

Despite the fact that evidence of a statistical interaction does not necessarily map directly onto biological interaction, our findings on *NRN1xBDNF* interaction are consistent with the previously described effect of *BDNF* on *NRN1* regulation (Naeve et al., 1997). This interaction is also supported by evidence about the positive correlation in expression between both genes (BrainCloud: http://braincloud.jhmi.edu/). Moreover, these gene–gene interaction results are in line with another study reporting the interplay between *NRN1* and *BDNF* and the risk for developing schizophrenia spectrum disorders (SSD) (Fatjó-Vilas et al., 2016).

Since both neurotrophins are essential for correct brain function and plasticity, although the molecular mechanisms underlying this interaction are unknown, we could modestly hypothesize that both genes contribute synergistically to the modification of synaptic plasticity. In turn, this interaction could have an impact on the underlying mechanisms of either the presence of depressive symptoms or changes in cognitive performance.

Our study should be interpreted in the context of various limitations. First, the moderate sample size used to detect genetic associations should be mentioned, with replication in larger samples from the general population with higher statistical power being needed to confirm these findings. Second, the characteristics of the sample need to be considered when generalizing the present findings. Although the sample was drawn from the general population, representativeness was also limited by its characteristics. Third, when multiple testing was considered, only the association between *NRN1* and BSI scores and the interaction between *NRN1xBDNF* in depressive symptoms remained significant. However, the Bonferroni correction is often considered to be overly strict and conservative (Feise, 2002; Gelman et al., 2012). Fourth, the evaluation of the psychopathological outcome could have benefited by using some other more specific scales for depressive/psychotic symptoms. Fifth, other factors not controlled in the present study such as social adjustment or quality of life may influence the mood state of participants at the time of psychopathological assessment. Sixth, it should be mentioned that as the genotype combination includes the minor alleles of both *NRN1*-rs1475157 and *BDNF-rs6265,* the interpretation of our results is hampered by the frequency of this combination in the population. Seventh, the variation in R^2 from the non-interaction models to the interaction model was small but significant; this effect cannot be ignored, since is it known that the power required to detect interactions is typically lower than the power required to detect main effects (McClelland and Judd, 1993). In summary, the results should be interpreted with caution, and further studies are required to determine the biological mechanisms underlying not only the role of *NRN1* but also the detected gene interaction effect between *NRN1* and *BDNF* in a non-clinical sample.

To conclude, our results contribute to the understanding of the genetic heterogeneity present in the general population, suggesting that *NRN1* has a role in the appearance of depressive symptoms and cognitive performance in a non-clinical sample. These findings add support to the pleiotropic effect of *NRN1* and indicate the interest in further analyses in at-risk and/or clinical samples. Moreover, although new studies are needed to better understand the role of both *NRN1* and *BDNF* genes in psychiatric symptoms, our data suggest the genetic interaction between the two neurotrophic factors, which have multiple roles in neurodevelopment and synaptic plasticity.

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Contributors

CP, LF and MFV designed the study. BA, GO, MII, JM designed and coordinated the evaluation protocol and conducted the sample recruitment. CP and MFV undertook the genetic and statistical analyses and wrote the first draft of the manuscript. All authors advised on interpretation of the results and contributed to, read and approved the final manuscript.

Conflict of interest

All authors report no biomedical financial interests or potential conflicts of interest.

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Advisors' report on the contribution of the PhD candidate to the article:

Dr. Lourdes Fañanás (Associate Professor) and Dr. Fatjó-Vilas (Assistant Professor) from the Department of Evolutionary Biology, Ecology and Environmental Sciences of the Faculty of Biology (University of Barcelona), both supervisors of the present doctoral thesis by Claudia Prats, hereby certify that the participation of the PhD applicant in the article "**Neurotrophins role in depressive symptoms and executive function performance: Association analysis of *NRN1* gene and its interaction with *BDNF* gene in a non-clinical sample"**, included the following tasks:

- Participation in the conception and design of the manuscript
- Molecular analysis design.
- Statistical analysis and interpretation of data
- First drafting of the manuscript
- Critical revision of the article for intellectual content

Signed by Dr. Lourdes Fañanás and Dr. Mar Fatjó-Vilas

Barcelona, June 1st 2017

4.4

Genetic variability of a set of white matter related genes: Association and epistatic analysis in Schizophrenia and Autism spectrum disorders

C Prats, M Fatjó-Vilas, MJ Penzol, O Kebir, L Pina, G Martinez, E Pomarol-Clotet, B Crespo-Facorro, A González-Pinto, D Demontis, M Parellada, MO Krebs, L Fañanás

(In preparation)

Genetic variability of a set of white matter related genes: Association and epistatic analysis in Schizophrenia and Autism spectrum disorders.

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ABSTRACT

Background: Schizophrenia (SZ) and Autism spectrum disorders (ASD) share a number of common genetic risk factors. Part of this genetic overlap converge on pathways that are related with connectivity and synaptic plasticity, which could be related to the white matter (WM) abnormalities also observed in both disorders. In this sense, those genes that play a role in the white matter brain and are related with myelination processes are considered as candidate genes for both SZ and ASD. In this study, we aimed to investigate the role of a set of WM related genes and its epistatic effect on the risk for SZ and ASD. This approach has been developed in three independent Caucasian population-based samples.

Methods: We examined 108 SNPs across a set of 22 WM related genes in a sample comprised of: i) 91 ASD patient-parents' trios, ii) 187 SZ patient-parents' trios, and iii) 915 SZ case-control sample (451 cases and 464 controls). The selected candidate genes include those implicated in myelin structure, oligodendrocyte development, synaptic plasticity and axonal regeneration, transcription and signaling factors and cell adhesion molecules and receptors (e.g. *MAG, CNP, MBP, QKI).* A selection of tag SNPs based on the HapMap CEU population were genotyped. Quality control assessments and association analyses were conducted with PLINK. Gene-gene interaction was explored by using the model based multifactor dimensionality reduction (MB-MDR) approach.

Results: Among our set of genes we found that some genes were specifically associated with either SZ (e.g. *NRG1*) or ASD (e.g. *QKI*) while others appear to be associated to both disorders (e.g. *MAG*, *MOG, MBP: non-corrected p-values*<0.03). Moreover, we found significant interactions between rs9966986 (*MBP*) * rs3802160 (*NRG1*) (*permutation p-value* = 0.002) in SZ trio sample and between rs2271194 (*ERBB3*) * rs7772756 (*QKI*) (*permutation p-value*<0001) in the ASD trio sample.

Discussion: Our results suggest: i) the implication of WM genes in the risk for both SZ and ASD and ii) although the gxg interaction found in each disorder is different, *ERBB3* gene seems to play a key role modulating the risk for developing both disorders, which is in line with previous findings indicating its association with both, SZ and ASD. Thus, our results support the concept of some shared pathophysiological pathways between both disorders.

Keywords: white matter, OMR genes, schizophrenia, autism spectrum disorders, genexgene interaction

INTRODUCTION

Abnormal neurodevelopment is now thought to contribute to the etiology of many psychiatric disorders that manifest throughout the entire lifespan. It is well known the importance of intact white matter for a normal brain functionality and its implication in a numerous of psychiatric diseases (e.g., (Catani and ffytche, 2005)), giving support to the notion of considering schizophrenia as a disorder of disconnectivity (Konrad and Winterer, 2007)(Konrad and Winterer, 2007). Commonly known, Schizophrenia (SZ) and autistic spectrum disorders (ASD) are severe neurodevelopmental disorders of unknown etiology affecting approximately 3% of the population disorders (DiCicco-Bloom et al., 2006; Rapoport et al., 2005). The fact that the estimated heritability (h^2) of Schizophrenia (SZ) is around 80% (Cardno and Gottesman, 2000) and 90% in ASD (Freitag, 2007), makes clear the substantial genetic contribution to both disorders. To this respect, findings from the past few years have emphasised phenotypic and genetic overlap between ASD and SZ (King and Lord, 2011; Rapoport et al., 2009) , including identification of copy number variants conferring risk of both (Levinson et al., 2011). In detail, it has been confirmed that the cross-diagnosis genetic correlations were highest correlations for SZ/BP and low but significant for SZ and ASD (of the Psychiatric Genomics Consortium et al., 2013). Although whether this overlap extends to the level of specific genetic variants is currently unclear. Interestingly, the largest molecular study of SZ reported evidence for overlap in schizophrenia GWAS regions and those with de novo non-synonymous mutations in ASD providing further support for the hypothesis that these disorders have partly overlapping pathophysiologies (Purcell et al., 2014; Sullivan et al., 2012).

These disorders share symptomatology and neurocognitive conditions and this make them to be placed in a pathophysiological *continuum*, from the more neurodevelopmentally compromised form of ASD to healthy individuals, which is influenced by genetic factors (Rapoport et al., 2009). Moreover, SZ and ASD have shown to share common deficits in connectivity and synaptic plasticity (Cheung et al., 2010; de Lacy and King, 2013), which could be related to the white matter abnormalities also observed in both disorders (Dennis and Thompson, 2013; Wheeler and Voineskos, 2014). To this respect, individuals with SZ and autism show alterations in the density of dendritic spines in cortical pyramidal cells. Considering that several genes are conferring susceptibility to both disorders, and due to the multiple genetic studies pointing to the involvement of oligodendrocyte-related genes in SZ (Karoutzou et al., 2008), those genes related with myelin structure, oligodendrocyte development, synaptic functions and axonal regeneration can be considered as putative candidate genes for both disorders (Penzes et al., 2011).

In recent years, integrative approaches combining multiple data sources have been widely used to identify susceptible genes in complex disorders such as schizophrenia (Sun et al., 2009). In this sense, the focus of *candidate gene pathway approach* is to work with different candidate genes related to multiple cellular pathways. Moreover, the interest in studying gene-gene interactions is increasing for psychiatric diseases, such as schizophrenia-spectrum disorders (SSD) and Autism spectrum disorders (ASD), where multiple genes are involved. Considering that several genes are

conferring susceptibility to both disorders, and due to the multiple genetic studies pointing to the involvement of oligodendrocyte-related genes in SZ (Karoutzou et al., 2008), more genetic studies involving white matter related genes and its genetic interactions, could provide new ways to better understand the common related pathways between both disorders.

Taking into account all mentioned above, we hypothesized that: i) the integrative effects of a set of white matter related genes will contribute to both SSD and ASD, and ii) similar epistatic interactions ill be contributing to both disorders. To test these hypotheses, we exploredthe relatio n-ship between genetic variability among our selected set of white matter related genes and the risk for developing SSD or ASD. Secondly, we aimed to analyse gene-gene interactions among our selected set of white matter genes in an already available sample of patients with schizophrenia and autistic spectrum disorders.

METHODS

Sample description

Patients were included in this study based on the presence of a diagnosis of schizophrenia-spectrum disorder or autism-spectrum disorder. When possible, healthy patients' parents were also recruited, in order to have a trio based sample. A sample of healthy subjects was also included.

The sample was drawn from three centers: i) University of Barcelona (multi-centre enrollment, Spain), ii) Hospital Universitario Gregorio Marañón, Madrid, iii) Centre Hospitallier Sainte Anne, Paris.

The global sample (1749 subjects) included: i) Sample I - ASD case-parents' trios sample -- 273 subjects in 91 nuclear families (91 cases and 182 parents), ii) Sample II - SSD case-parents' trios sample -- 561 subjects in 187 nuclear families (187 cases and 374 parents), and iii) Sample III - SSD case-control sample: 915 subjects (451 cases and 464 controls).

Probands and parents were evaluated by trained psychiatrists using a semi-structured interview: i) Autsim Diagnostic Interview (Sample I), ii) the Comprehensive Assessment of Symptoms and History (CASH) and/or the Diagnostic Interview for Genetic Studies (DIGS) (Sample II and III).

With respect to SSD-psychosis continuum Patients' DSM-IV-TR diagnoses distribution for the three independent samples were: First, for the SSD family-based sample (ii): schizophrenia (54%), schizophreniform disorder (9.9%), psychotic disorder not otherwise specified (6.8%), schizoaffective disorder (6.8%), schizotypal personality disorder (0.5%) and other psychotic disorders/NOS (0.6%) and bipolar disorder with psychosis features (20.9%). Second, for the SSD case-control sample (iii): schizophrenia (68.7%), schizophreniform disorder (11.5%), psychotic disorder not otherwise specified (10.4%), schizoaffective disorder (5.7%) and schizotypal personality disorder (0.4%) and other psychotic disorders/NOS (10.4%) and bipolar disorder with psychosis features (3.3%). Controls were recruited from university students and staff, and their acquaintances, plus independent sources in the community. They were interviewed and excluded if they reported a history of mental illness and/or treatment with psychotropic medication.

Exclusion criteria included: major medical illnesses that could affect brain function, substance-induced psychotic disorder, neurological conditions, history of head trauma

with loss of consciousness, and moderate or severe mental retardation. All participants were European-Caucasian, thereby reducing the possibility of confounding genetic differences by population stratification treatment. These aspects were screened by means of a short interview designed ad hoc for this study.

Ethical approval was obtained from local research ethics committees. All individuals underwent a clinical interview in order to evaluate their present and lifetime history of mental illness and/or treatment with psychotropic medication. All participants provided written consent after being informed of the study procedures and implications. The study was performed in accordance with the guidelines of the institutions involved and approved by the local ethics committee of each participating center.

DNA extraction and genotyping

Genomic DNA was extracted from peripheral blood cells or from buccal mucosa using standard methods. The genotyping was conducted at the Genomic Service of the Spanish National Cancer Research Center (CEGEN-CNIO) by using the *Open Array® Genotyping System of Applied Biosystems.*

The molecular genetics data_for the sample include 108 Single Nucleotide Polymorphisms (SNPs) among a set of 22 genes related with matter matter functions (Supplementary Table X). The optimal set of SNPs that contained maximum information about surrounding variants was selected using SYSNPs (http://www.sysnps.org/) with a minor allele frequency (MAF) > 5%, using the pairwise option tagger (threshold of r^2=0.8). These SNPs were also selected due to either previous associated findings or functional implications. The final set of SNPs and genes can be found in **Table 1**. The whole set of WM related genes were selected according to its functionality, which are mainly involved in: i) myelin structure (*MAG, MBP, PLP1, MOG, CNP, PTEN, AKT1* and *FYN*) ii) oligodendrocyte development (*QKI*), iii) synaptic plasticity and axonal regeneration (*OMG, CDH10, MAG*), iv) transcription and signaling factors (*OLIG2, NRG1, ErbB2, ErbB3, ErbB4*), v) cell adhesion molecules and receptors (*NRXN1*, *CNTNAP2* and *SPON1*), vi) calcium channels: *CACNA1C*, *CACNB2 and a coding for* zinc finger binding protein *(ZNF804).*

Source of molecular network data

Gene interaction graph network from STRING v.9 (Franceschini et al., 2013) was implemented. STRING integrates protein-protein interactions from literature curation, computationally predicted interactions and interactions transferred from model organisms based on orthology. As visible in **Figure 1**, most of the proteins included in our study are linked and form an interaction network. The interactive evidences in this network (from STRING network overview), support the fact of studying gene-gene interaction among our set of White matter genes.

Statistical analysis

Data was analyzed with PLINK v1.07 (Purcell et al., 2007), R and SPSS 21.0 software (SPSS IBM, New York, USA). Standard data cleaning and quality control (QC) was performed before analysis. QC of our data consisted of at least four steps, 1) identification of SNPs with an excessive missing genotype (--geno 0.1), 2) identification of SNPs demonstrating a significant deviation from Hardy-Weinberg equilibrium (HWE) (--hwe 0.000001), 3) identification of SNPs with significantly different missing genotype rates between cases and controls (--mind 0.2), and 4)

the removal of makers with a very low minor allele frequency (--maf 0.00000001).

In addition, after genotyping we performed a SNP LD pruning analysis using PLINK (which allows the pruning of SNPs that are highly correlated (r2> 0.8)).

After quality control and SNP pruning there are 91 SNPs that survived for ASD sample and 90 SNPs for the SSD family-based and SSD case-control sample. The genotyping success rates were >94%.

Association analyses

All association analysis as implemented in Plink 1.07 (Purcell et al., 2007) were used to explore the relationship between our 22 WM-related genes and the risk for developing SSD/ASD. In all analyses PLINK's max(T) permutation procedure with 10,000 iterations was performed. The specific analyses for each sample are described below.

Association analyses: *TDT testing and pseudo-controls (Sample I and II)*

In the families, cases and matched pseudo-controls were generated. For the family-based samples, PLINK software was used to perform single locus transmission disequilibrium test (TDT) association analysis, considering pedigrees. Moreover, we matched alleles transmitted to affected offspring (trio cases) with untransmitted alleles (pseudo-controls) as this corresponds to the unmatched case–control design assumed by the tests we consider (Cordell et al., 2004). Thus, pseudo-controls were created based on our trio dataset using PLINK (—tucc) software. The basic idea behind this design is to generate pseudo-controls using the parent's untransmitted alleles, thus creating a matched case-control design where the observed case is compared to all possible genotypic combinations that could have arisen from the parental mating type. For any single variant, there are three alternative genotypes for pseudo-controls that could have been transmitted to the case, thus the case: pseudo-controls ratio is 1:3 in a conditional logistic regression 10 model.

These analyses were corrected by gender and center when necessary.

Population stratification: *Cochran-Mantel-Haenszel (CMH) association (Sample II and III)*

In those samples including individuals from Spain and France (Sample II and III), Breslow-Day (BD) and Cochran-Mantel-Haenszel (CMH) tests were used for association analyses and heterogeneity testing, respectively.

The Cochran-Mantel-Haenszel (CMH) association test (2 × 2 × K, where K = nº clusters) in PLINK v1.07 correct for the potential confounding of population stratification. The CMH association test allows for comparison of cases and controls while controlling for clusters within the data, where the clusters are defined by identity-by-state (IBS) sharing among individuals (2 clusters were identified in our sample: Spanish vs French population). The CMH analysis tests each single SNP independently. Empirical *p*-values were calculated using 10.000 permutations with the adaptive permutation option in PLINK v1.07.

Cochran-Mantel-Haenszel (CMH) test (1 degree of freedom) was performed for testing differences in SNP allele frequencies between SSD cases and controls, stratified by the two different European samples (Spanish vs French Population).

Gene-gene interaction analysis: *model-based multifactor dimensionality reduction (MB-MDR)*

In order to capture second-order SNP–SNP interactions, we used the model-based

multifactor dimensionality reduction (MB-MDR) method recently developed by Calle et al (2010). It is an extension of the popular multifactor dimensionality reduction (MDR) method in which risk categories are defined using a regression model that also allows adjustments for main effects and covariates. By this approach, first, logistic regressions analyses are performed to define the nine possible genotypic combinations as high (*H*), low (*L*), or no risk (0). Then, these multilocus genotypes of the same risk category are merged and two Wald statistics (WH and WL, one for each risk) with the relevant *p* values (PH and PL) are obtained. The significance for the epistatic effect is based on the minimum between PH and PL (MIN.P). Finally, the significance of a specified model is assessed through a permutation test on the maximum Wald statistic, implemented in the function mbmdr.PermTest of MB-MDR package. It also allows adjusting for confounding effects and correcting for multiple testing.

In the present study, the MB-MDR algorithm to the stage 2 dataset was applied. Note that genexgene interactions were tested in each sample (Sample I, II and III), separately. The permutation procedure (10,000 permutations) was applied to the interaction models with a MIN.P < 0.05. Note that all analyses were adjusting either for gender or center when necessary.

RESULTS

Sample Description

Firstly, the final dataset consisted of 91 ASD trio families including pseudo-controls formed from non-transmitted alleles. Secondly, the final dataset consisted of 187 SSD trios including pseudo-controls formed from non-transmitted alleles. Thirdly, the final dataset consisted of 915 individuals (451 SSD cases and 464 controls).

Table 2 shows the final characteristics of the three independent Caucasian samples of our study. As gender distribution between groups showed significant differences, gender was added as a covariate in all the analysis.

Association analysis of *WM* SNPs – Autism Spectrum Disorders families (Sample I)

Family-based association analysis was carried out using PLINK software on a subset of ASD families, comprised of Caucasian individuals, which included 91 trio families.

The associated SNPs implicated 4 genes (MOG, QKI, MAG and MBP) (Table 3). After permutation correction analysis, no SNP remained significant.

Table 3. Results of single case/pseudo-control TDT analysis in Autism Caucasian families for 108 SNPs across a set of White matter genes. Note that only those SNPs that showed a trend association are showed.

				Single case/pseudocontrol TDT				
Chr	**Gene**	**SNP ID**	**Position (BP)**	**Allele (A1)**	**X^2**	**P-value**	**OR**	**95% CI**
6	MOG	rs9257936	29639776	A	3.98	0.045	1.97	(1.04-3.88)
6	QKI	rs6931903	163910325	T	4.91	0.026	2.56	(1.10-6.05)
19	MAG	rs2301600	35786868	T	5.56	0.018	0.51	(0.29-0.89)
18	MBP	rs12959006	74727631	T	5.65	0.017	0.50	(0.28-0.89)

After multiple testing no SNP remained significant (pval >0.05).

Association analysis of *WM* SNPs – Schizophrenia Spectrum Disorders families (Sample II)

Family-based association analysis was carried out using PLINK software on a subset of SSD families including pseudocontrols, comprised of Caucasian individuals.

The associated SNPs implicated 6 genes (*ERBB4*, *ERBB2*, *MOG*, *NRG1*, *MBP* and *MAG*) (Table 4). After permutation correction analysis, no SNP remained significant.

Association analysis of *WM* SNPs – Schizophrenia Spectrum Disorders (Sample III)

First, a Breslow–Day test was performed across all the analyzed samples for all SNPs. The Breslow-Day test found p-values of 0.01 for rs73235619 (*NRG1*) and rs2494734 (*AKT1*), and 0.005 for rs1059004 (*OLIG2*), implying heterogeneity of Odds ratio between these SNPs. However, these SNPs were not excluded due to they were not significant after multiple testing using Bonferroni correction (P > 0.05).

The associated SNPs implicated 5 genes (*CACNA1C, ERBB3, AKT1, CNP and PLP1*) (Table 5). These association were not significant after permutation correction.

Epistatic analysis effect of White matter genes on SSD and ASD

MB-MDR analysis among the 22 WM related genes provided significant evidence for an epistatic effect in: i) ASD: *ERBB3*(rs2271194) x *QKI*(rs7772756) *Perm pval*= 6e-4, ii) SSD family-based: *MBP(rs9966986) x NRG1(rs3802160) Perm pval*=0.002 and iii) SSD case-control: *AKT1 (rs2494746) x ERBB3 (rs7971751) Perm pval*=0.001 (Table 6).

Table 4. Results of single case/pseudo-control TDT analysis in Schizophrenia Caucasian families for 108 SNPs across a set of White matter genes. Note that only Trend association SNPs are showed.

					CMH test			
Chr	**Gene**	**SNP ID**	**Position (BP)**	**Allele (A1)**	**X^2**	**P-value**	**OR**	**95% CI**
2	ERBB4	rs707284	213387389	C	4.278	0.038	0.69	(0.48-0.98)
2	ERBB2	rs4252612	37863708	C	4.696	0.030	0.43	(0.19-0.93)
6	MOG	rs2535246	29636409	G	5.174	0.022	1.55	(1.06-2.25)
8	NRG1	rs73235619	31473740	A	5.335	0.020	1.75	(1.07-2.84)
8	NRG1	rs6989777	32415682	C	5.299	0.021	0.62	(0.41-0.930)
18	MBP	rs470279	74693395	T	5.828	0.015	0.56	(0.35-0.90)
18	MBP	rs523243	74714585	A	7.845	0.005	0.52	(0.33-0.82)
19	MAG	rs6510476	35784166	G	6.625	0.010	0.57	(0.37-0.87)

After multiple testing no SNP remained significant (pval >0.05).

Table 5. Unadjusted (raw) *p* -values for the White Matter Gene-SNPs from Cochran-Mantel-Haenszel (CMH) association analysis and Breslow-day test in SSD sample

					CMH test			
Chr	**Gene**	**SNP ID**	**Position (BP)**	**Allele (A1)**	**X^2**	**P-value**	**OR**	**95% CI**
12	CACNA1C	rs1024582	2402246	A	3.64	0.056	0.82	(0.48-0.98)
12	ERBB3	rs2271194	56477694	A	9.22	0.002	0.75	(0.19-0.93)
12	ERBB3	rs7971751	56478658	T	8.65	0.003	0.75	(1.06-2.25)
12	ERBB3	rs877636	56480583	G	5.64	0.017	0.79	(1.07-2.84)
12	ERBB3	rs773123	56494998	T	4.75	0.029	0.72	(0.41-0.930)
14	AKT1	rs2494743	105251720	C	6.28	0.012	1.46	(0.35-0.90)
14	AKT1	rs2494746	105257719	C	7.3	0.007	1.52	(0.33-0.82)
17	CNP	rs11296	40127060	C	4.42	0.035	0.69	
23	PLP1	rs521895	103036412	A	5.41	0.020	0.75	(0.37-0.87)

After multiple testing no SNP remained significant (pval >0.05).

Table 6. Significant gene-gene interactions using Model-based multifactor dimensionality reduction method (MB-MDR) for each sample: I) ASD Risk, II) SSD Risk and II) SSD replication sample. Note that 10000 permutation analysis was applied.

Phenotype	**Best multigene interaction model**	**Beta**	**Wald**	**Perm *P* value**	**Risk category**
I) ASD Risk (family-based)	*ERBB3*_rs2271194 * *QKI*_rs7772756	1.44	14.63	6e-4	High
	*ERBB3*_rs2271194 * *QKI*_rs7772756	-0.67	2.91	6e-4	Low
Phenotype	**Best multigene interaction model**	**Beta**	**Wald**	**Perm *P* value**	**Risk category**
II) SSD Risk (family-based)	*MBP*_rs9966986 * *NRG1*_rs3802160	0.91	12.25	0.002	High
	*MBP*_rs9966986 * *NRG1*_rs3802160	-1.15	17.46	0.002	Low
Phenotype	**Best multigene interaction model**	**Beta**	**Wald**	**Perm *P* value**	**Risk category**
III) SSD Risk (case-control)	*ERBB3*_rs7971751 * *AKT1*_rs2494746	0.50	11.7	0.001	High
	*ERBB3*_rs7971751 * *AKT1*_rs2494746	-0.59	18.06	0.001	Low

DISCUSSION

The present study has been focused on the genetic variability of a group of 22 candidate genes (related with white matter pathways) and its genexgene interactions were analysed in three different samples: i) ASD case-parents' trios sample, ii) SSD case-parents' trios sample and iii) SSD case-control sample.

Our results report evidence of that some of the WM genetic risk variants seem to be shared across SZ-ASD while others seem to be disease specific. Moreover, although every combination of genexgene interaction is different for each group of samples, the *ERRB3 gene* appears to be interacting with QKI (in ASD trio sample) and with *AKT1* (in SSD case-control sample). Since *QKI* and *AKT1* genes are related with myelination pathways (Åberg et al., 2006; Flores et al., 2008), we could modestly speculate about the interplay between OMR genes and Neuregulin-ERBB signalling pathways in both disorders**.** In favour of this conception, recent studies have revealed complex Nrg/Erbb signalling networks that regulate the assembly of neural circuitry, myelination, neurotransmission, and synaptic plasticity (Mei and Nave, 2014), which are key processes involved in neurodevelopmental disorders. Moreover, evidence indicates that deviances from NRG/ERBB signalling in the brain impairs brain functions (Mei and Nave, 2014). In this sense, OMR and NRG/ERBB signalling pathways may provide therapeutic targets for neuropsychiatric diseases.

Some limitations of this study must be acknowledged. First, the polygenic nature of mental disorders and the minor effect of the common genetic variants limit the power of our sample size. Second, the antipsychotic treatment was not specified and, therefore, analyses were not adjusted by treatment type or duration. Third, although the permutation procedures have been applied, if multiple testing is addressed for the overall analyses not all the findings would remain significant. Then, the interpretation of our results should be conducted with caution and replication in larger samples are needed.

Overall, our results contribute, from a biological approach, to the understanding of the genetic mechanisms involved in SSD and ASD, suggesting a key role for the genes involved in the relationship OMR and NRG/ERBB, which modulates the risk for developing either ASD or SSD. However, new studies should be developed to further acknowledge the role of white matter related genes in the aetiology of these disorders.

Conflicts of interest: All authors report no conflict of interest

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SUPPLEMENTARY MATERIAL

Table 1. List of the genotyped 108 SNPs among the 22 white matter genes

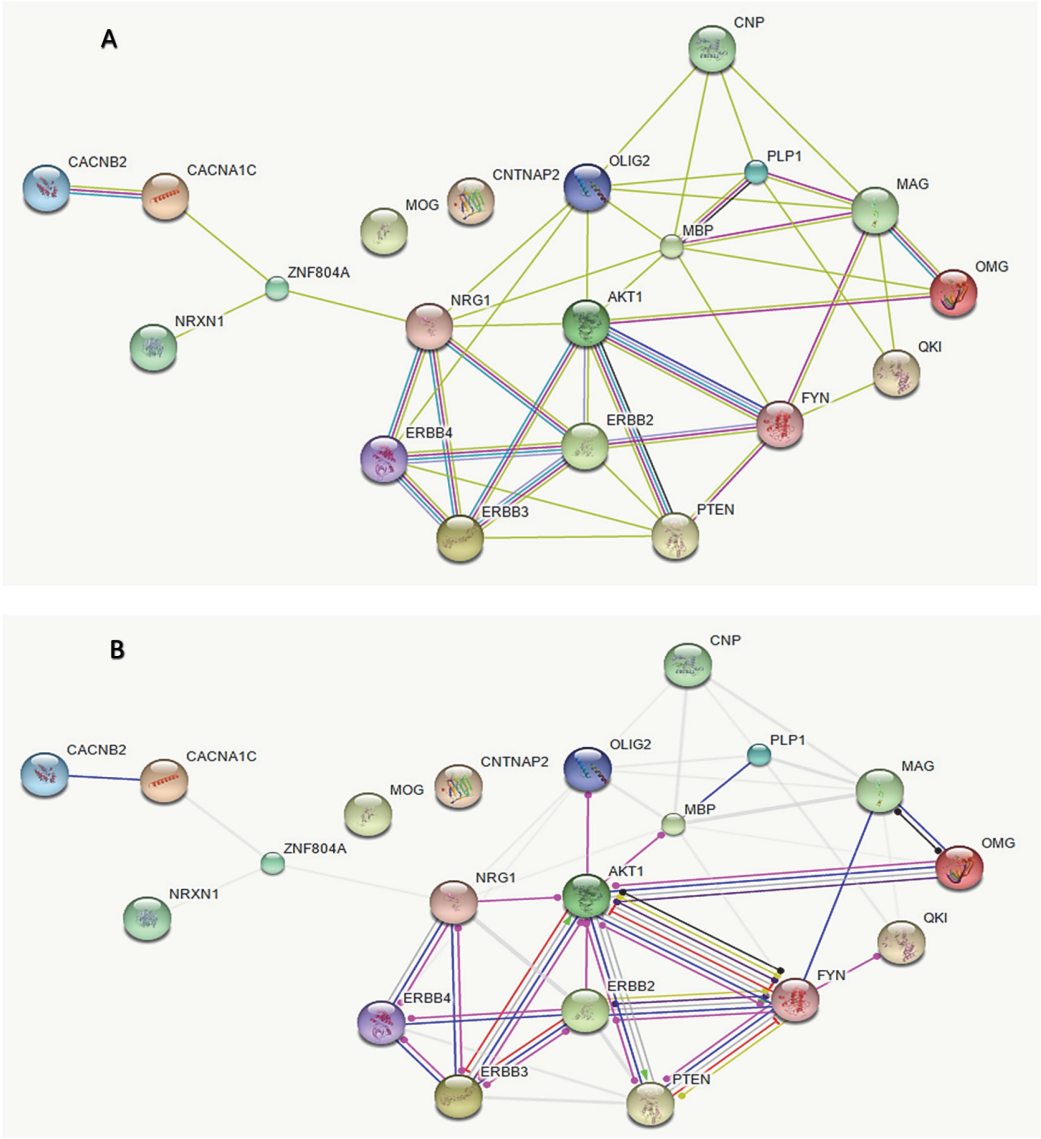
Gene symbol	Selected SNPs	Gene Description	Gene location
MAG	rs6510476, rs2301600, rs3746248, rs11669734, rs11670792, rs720309, rs720308, rs1034597, rs756796	myelin associated glycoprotein	19q13.12
MBP	rs1049004, rs470279, rs9675994, rs523243, rs12967023, rs12959006, rs11150994, rs470473, rs871673, rs1026520, rs1629089, rs9966986	myelin basic protein	18q23
PLP1	rs521895, rs2294152	proteolipid protein 1	Xq22.2
MOG	rs9468571, rs3130250, rs16895223, rs2535260, rs2857766, rs3130253, rs2071653, rs2535246, rs9257936	myelin oligodendrocyte glycoprotein	6p22.1
CNP	rs8078650, rs4258677, rs12602950, rs8077391, rs2070106, rs11079028, rs11296	2',3'-cyclic nucleotide 3' phosphodiesterase	17q21.2
PTEN	rs3781195, rs1234220, rs11202596, rs1234219, rs10490920, rs2248293, rs17562384, rs2736627, rs11202607	phosphatase and tensin homolog	10q23.31
AKT1	rs2494732, rs1130233, rs2494734, rs2494739, rs2494743, rs11847866, rs2494746, rs1130214	AKT serine/threonine kinase 1	14q32.33
FYN	rs12191154, rs2344706, rs2237257, rs4947144, rs2301465, rs6901958, rs706915	FYN proto-oncogene, Src family tyrosine kinase	6q21
QKI	rs2784867, rs7772756, rs803612, rs1744926, rs6931903, rs6931903, rs9364692, rs9458853, rs11964059, rs9456869	QKI, KH domain containing RNA binding	6q26
OMG	rs11080149, rs11655238	oligodendrocyte myelin glycoprotein	17q11.2
ZNF804A	rs1344706	zinc finger protein 804A	2q32.1
CDH10	rs4307059	cadherin 10	5p14.2-p14.1
CACNA1C	rs1024582	calcium voltage-gated channel subunit alpha1 C	12p13.33
CACNB2	rs2799573	calcium voltage-gated cannel auxiliary subunit beta 2	10p12.33-p12.31
OLIG2	rs1005573, rs1059004, rs6517137	oligodendrocyte lineage transcription factor 2	21q22.11
NRG1	rs73235619, rs62510682, rs6994992, rs3802160, rs10503929, rs6989777	neuregulin 1	8p12
ErbB2	rs4252596, rs1565923, rs2952155, rs4252612	erb-b2 receptor tyrosine kinase 2	17q12
ErbB3	rs2271194, rs7971751, rs877636, rs705708, rs10783779, rs773123	erb-b2 receptor tyrosine kinase 3	12q13.2
ErbB4	rs4673628, rs7598440, rs839541, rs839523, rs707284, rs1026882	erb-b2 receptor tyrosine kinase 4	2q34
NRXN1	rs858932, rs1045881	neurexin 1	2p16.3
CNTNAP2	rs2710102	contactin associated protein-like 2	7q35-q36.1
SPON1	rs2618516	spondin 1	11p15.2

Table 2. Descriptive data for SSD/ASD family sample and for SZ case-control sample following quality control.

	Sample II	Sample I Family-based sample			Sample III Case-only/control	
	ASD Patients (n= 91) Mean(sd)	1st degree ASD healthy relatives (n= 182) Mean(sd)	SSD Patients (n=187) Mean(sd)	1st degree SSD healthy relatives (n= 374) Mean(sd)	SSD Patients (n=451) Mean(sd)	Controls (n=464) Mean(sd)
Age at interview	*	*	23.17 (7.81)	42.50 (1.05)	31.11 (11.39)	33.72 (11.76)
Gender (% males)	84.6 %	38.5 %	59.9 %	41.78 %	71.5 %	46.9 %
Years of education	*	*	9.54 (3.71)	11.28 (0.35)	9.61 (4.33)	14.81 (3.96)
Age at onset	*	-	18.75 (5.43) [a,]	-	24.09 (7.93)	-
Illness duration in months	*	-	43.18 (52.15)	-	-	-
IQ	*	*	86.71 (15.07)[b]	94.26 (1.24)[b]	89.38 (12.83)[b]	107.22 (13.84)[b]

[a] 61.9% of the patients are early-onset
[b] Available for more than 45% of the total sample
* This data will be confirmed in the following months.
Sample I (100% Mad)
Sample II (47.9% Bcn, 32.4% Paris, 19.7% Mad)
Sample III (63.5% Bcn, 26.3% Paris, 10.2% Mad)

Figure 1. STRING human protein interaction network of the 22 White matter genes included in our study. A) Evidence for interaction between these genes is provided and is also based on information from text mining. **B)** Evidence and molecular action between these genes.



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Advisors' report on the contribution of the PhD candidate to the article:

Dr. Fañanás (Associate Professor) and Dr. Fatjó-Vilas (Assistant Professor) from the Department of Evolutionary Biology, Ecology and Environmental Sciences of the Faculty of Biology (University of Barcelona), both supervisors of the present doctoral thesis by Claudia Prats, hereby certify that the participation of the PhD applicant in the article "**Genetic variability of a set of white matter related genes: Association and epistatic analysis in Schizophrenia and Autism spectrum disorders"**, included the following tasks:

- Participation in the conception and design of the manuscript
- Statistical analysis and interpretation of data
- First drafting of the manuscript
- Critical revision of the article for intellectual content

Signed by Dr. Lourdes Fañanás and Dr. Mar Fatjó-Vilas

Barcelona, June 1st 2017

5. Global Summary of Results

Science is not only a disciple of reason
but, also, one of romance and passion.
– Stephen Hawking

The **hypotheses** were tested throughout two independent Objectives (A, B):

Specific hypothesis A [*NRN1*, candidate plasticity gene]: Genetic variability of Neuritin-1 gene (*NRN1)* will be associated with SSD and BPD, and also with some clinical and cognitive phenotypes both in patients and in healthy subjects from the general population. Moreover, *NRN1* action will be modulated by other genes such as *BDNF* and *DTNBP1*.

Main Objective A: First, to study the role of the genetic variability in a neurodevelopment and synaptic plasticity candidate gene called Neuritin-1 gene, on the risk for Schizophrenia-Spectrum Disorders and on clinical and cognitive phenotypes of interest. **S**econd, to investigate whether the role of *NRN1* is modulated by the interaction with other plasticity genes (*BDNF* and *DTNBP1*).

The articles derived from these aims are:

- **M Fatjó-Vilas & C Prats *et al.*, 2016**. *Involvement of NRN1 gene in schizophrenia-spectrum and bipolar disorders and its impact on age at onset and cognitive functioning*. World J Biol Psychiatry. 2016;17(2):129-39.
- **C Prats *et al.*, 2017a.** *Evidence of an epistatic effect between Dysbindin-1 and Neuritin-1 genes on the risk for Schizophrenia Spectrum Disorders*. Eur Psychiatry. 2017 Feb; 40:60-64.
- **C Prats *et al.*, 2017b.** *Neurotrophins role in depressive symptoms and executive function performance: Association analysis of NRN1 gene and its interaction with BDNF gene in a non-clinical sample*. J Affect Disord. 2017 Mar 15; 211:92-98.

Referring to hypothesis/Objective A, the following results were obtained:

I. Objective A1: The manuscript (Fatjó-Vilas & Prats et al., 2016) reported evidence on that genetic variability of *NRN1* is associated with the risk for developing SSD and BPD. In detail, we found that the frequency of the haplotype C-C (rs645649-rs582262=*NRN1* HAP-risk) was significantly increased in patients compared to controls (p=0.0043, OR (CI 95%)=1.28 (1.08-1.51)), while the haplotype T-C-C-T-C-A (rs3763180-rs10484320-rs4960155-rs9379002-rs9405890-rs1475157) was more frequent in controls ($p=3.1x10^{-5}$; OR (CI 95%)=0.09 (0.02-0.37)). Moreover, the variability at *NRN1* was also related to changes in age at onset and to differences

in intelligence quotient in the group of SSD patients. Finally, we found that the effect of *NRN1* on the risk for SSD-BPD was modulated by $BDNF_{Val66Met}$ (p=0.005). Specifically, the effect of $BDNF_{Val/Val}$ in combination with *NRN1* variants (SNP1 (GG: β=0.654 p=0.001), SNP3 (AA: β=0.514 p=0.003) and SNP9 (TG: β=0.457 p=0.0004)) revealed an increased risk for developing both SSD and BPD ($BDNFxNRN1_{SNP9}$ $p_{permutation}=0.005$).

II. Objective A2: The results of the second manuscript (Prats *et al*, 2017a) supported a join effect of *NRN1 and DTNBP1 gene* on the risk for SSD. Based on the previous findings, we explored whether the effect of *NRN1* HAP-risk was modulated by 3 *DTNBP1* SNPs (rs2619537, rs2743864, rs1047631) related to changes in *DTNBP1* expression. An interaction between *DTNBP1* rs2743864 and *NRN1* HAP-risk was detected by using the model based multifactor dimensionality reduction (MB-MDR) approach (p=0.0049, after permutation procedure), meaning that the risk for SSD is significantly higher in those subjects carrying both the A allele of rs2743864 *(DTNBP1)* and the HAP-risk C-C *(NRN1)*. This finding was confirmed with a logistic regression model. Particularly, first a main effect of the *NRN1* HAP-risk C-C on the risk for SSD was found (β=0.518 p=0.001 R^2=0.155) and second, this model revealed a significant combined effect of HAP-risk C-C and SNP2 (rs2743864) on the risk for SSD (p=0.033, OR (95%CI) = 2.699 (1.08–6.71), R^2 = 0.162). That is, among individuals carrying the HAP-risk C-C, the risk for SSD was higher in those who were also carriers of the A allele of *DTNBP1* SNP2 (patients: 17.64%, controls: 14.78%), compared to GG homozygous.

III. Objective A3: In the manuscript published at the J Aff Disorders (C Prats *et al*., 2017b), we investigated in a non-clinical sample whether *NRN1* gene contributes to the psychopathological profile, with a particular focus on the clinical dimensions previously related to the *NRN1* gene (i.e. depressive and psychotic) and the cognitive performance. We found that i) GG homozygotes (rs1475157-*NRN1*) showed higher scores on BSI depressive dimension and on total scores compared to A carriers (corrected p-values: 0.0004 and 0.0003, respectively), ii) a linear trend was detected between GG genotype of rs1475157 and a worse cognitive performance in Wisconsin Card Sorting Test (WCST) total correct responses (uncorrected p-value: 0.029) and, iii) interaction between rs1475157-*NRN1* and $BDNF_{Val66Met}$ was found to modulate depressive symptoms (p=0.001,

significant after correction).

In summary, the findings reported in the three latter manuscripts suggest that genetic variability of *NRN1* gene has an impact on the risk for developing SSD/BPD and also on the presence of depressive symptoms in the general population. Moreover, this pleiotropic effect of Neuritin 1 is also evidenced by its effect on different phenotypes: such as cognitive performance and age at onset. Regarding the notion of the importance of considering gxg interaction, our results suggest that *NRN1* action is modulated by the *BDNF* and *DTNBP1* genes. Overall, these results add support to an aetiological perspective considering SZ as a neurodevelopmental disorder influenced by multiple synaptic plasticity genes.

Specific hypothesis B [*White matter related genes*]: Integrative effects of a set of white matter related genes will contribute to both SSD and ASD. Moreover, we hypothesize that similar epistatic interactions will be contributing to both disorders.

Main Objective B: To study the involvement of the epistatic effects of a selected set of white matter related genes on the risk for neurodevelopmental disorders, including SSD and ASD.

- **C Prats et al., 2017.** *Genetic variability of a set of white matter related genes: Association and epistatic analysis in Schizophrenia and Autism spectrum disorders.* Currently in preparation.

Referring to hypothesis/Objective B, the following results were found:

Among our set of WM related genes, in a single SNP approach we found that some genes were specifically associated with SZ (e.g. *NRG1*) or ASD (e.g. *QKI*) while others appear to be associated to both disorders (e.g. *MAG*, *MOG*, *MBP*: non-corrected p-values<0.03). Moreover, we found significant interactions between rs9966986 (*MBP*) * rs3802160 (*NRG1*) (permutation p value = 0.001) in SZ trio sample and between rs2271194 (*ERBB3*) * rs7772756 (*QKI*) (permutation p value<0001) in the ASD trio sample. Moreover, in the SSD case-control sample, an interaction between rs7971751 (*ERBB3*) * rs2494746 (*AKT1*) was found (permutation p value=0.001).

The findings reported in the last manuscript of the present thesis suggest that some of the WM genetic risk variants seem to be shared across SZ-ASD continuum. The fact that some OMR genes are marginally associated with both disorders and also, due to their involvement in the detected epistatic effects, support the notion that dysregulation in myelination processes may underlie susceptibility to develop ASD or SSD. Although the epistatic interactions found in each disorder are different, *ERBB3* gene seems to play a key role. Furthermore, the gxg interactions found, seems to indicate the interplay between OMR genes and Neuregulin-ERBB signalling pathways. Despite there is no clear evidence of the role of NRG1-ERB signalling in ASD, previous findings indicate its importance in Neuropsychiatric Diseases such as SZ and BPD. Thus, our results support the concept of some shared pathophysiological pathways between SSD and ASD.

6. Discussion and Conclusions

Reserve your right to think,
for even to think wrongly is better
than not to think at all.
– Hypatia

The present thesis, which can be framed in the field of psychiatric genetics, was aimed at studying how genetic variability at genes related with either synaptic plasticity and/or white matter related pathways can explain part of the risk for developing neurodevelopmental psychiatric disorders. Specifically, the two main objectives have been achieved with the final result of four articles. Next, a brief discussion of the research findings of this work is presented.

***A. NRN1*, candidate plasticity gene**. Neurotrophic factors are considered powerful molecular mediators of central synaptic plasticity. They are important regulators of neural survival, growth, development, function and plasticity (Huang and Reichardt, 2001). Considering that brain is a highly dynamic and sensitive system to environment challenges, immediate early gene (IEG) play a key role in maintaining the homeostatic plasticity which is very important for the correct brain functioning. In this sense, *NRN1* is considered an IEG and its expression is activated by a specific pathway that responds very quickly to regulatory signals. Particularly, this gene is expressed in early and adult development and itsexpression can be induced in response to neuronal activity and by neurotrophins such as *NGF*, *BDNF* and *NT3*. Due to the important role that NTFs and plasticity genes have in SZ and neurodevelopmental related disorders, there is an increasing interest in conducting further analyses involving plasticity genes. In this case, *NRN1* gene has been considered as an interesting candidate gene to be studied due to both, its function and implication in synaptic plasticity processes and also, because of previous association of *NRN1* with cognitive performance in SZ (Chandler *et al*, 2010).

[Manuscript A1]: In view of the role that neurotrophic factors and Neuritin-1 have in synaptic plasticity processes, a genetic design was implemented on *NRN1* in order to explore the relationship between its genetic variability with the risk for developing SSD and BPD. Based on the bases of this study (Fatjó-Vilas *et al*, 2016), it was concluded that *NRN1* has an involvement in the risk for developing SSD and BPD. Specifically, within the SSD patients, genetic variability of *NRN1* was also related to age at onset and cognitive performance. As discussed in the article, we supported the notion that specific genetic variability plays a role in defining illness subgroups. Moreover, considering the gxg interaction found between *NRN1* and *BDNF,* it could be hypothesised that the reported functional effects of the $BDNF_{Val66Met}$ polymorphism

could impact on NRN1 availability or function, explaining therefore their contribution to the risk for developing SSD and BPD. Overall, these findings suggest the role of Neuritin 1 gene as a mixed susceptibility/modifier gene (Fanous and Kendler, 2008), which increases the susceptibility to develop either SSD or BPD and modifies certain presentations of the disorder.

[Manuscript A2]: Findings of the second article (Prats *et al*, 2017a) support an interaction between *NRN1 HAP-risk C-C* (including rs645649-rs582262, previously associated with SSD and BPD in the first article) and *DTNBP1 SNPs* related with changes in expression, suggesting that they can work collectively to increase the risk for SSD. In detail, the concomitant presence of both A allele at rs2743864 (*DTNBP1*) and HAP-risk C-C (*NRN1*) are associated with higher risk for SSD, compared to the presence of either one of them alone. Although no previous association between these genes has been reported before, the modulating effect of *DTNBP1* on the role of *NRN1* could be related with the fact that gene products of both genes impinge on neuronal structure and/or function, with emphasis in the interface with intercellular signalling at the neuron level. These findings open a line of work in the detection of new biological and biochemical pathways between both genes that could underpin disease.

[Manuscript A3]: A third work reported that variability of *NRN1* is associated with depressive symptoms and executive function in a non-clinical sample (Prats *et al*, 2017b). In addition, we found that these effects were modulated by $BDNF_{Val66Met}$, indicating again, a gxg interaction between both neurotrophic factors. In this case, contrary to previous findings, in which *NRN1* was associated with SZ, *NRN1* was not related to psychotic symptoms as measured using the paranoid ideation dimension of the BSI scale. To our knowledge, this is the first work describing a genetic association between the *NRN1* gene and depressive symptoms in a non-clinical population sample. Specifically, we found that the polymorphism upstream of *NRN1* (rs1475157) shows a significant effect on the appearance of depressive symptoms, with individuals carrying the genotype GG showing higher scores compared to A allele carriers. Although this study was conducted in a non-clinical sample, these are in line with previous findings from animal models identifying *NRN1* as an interesting new player in depression. In this

sense, it has been reported that electroconvulsive therapy and antidepressant treatment produce changes in Neuritin-1 levels in the prefrontal cortex and hippocampus (Alme *et al*, 2007; Dyrvig *et al*, 2014) and, also, that knockdown of *NRN1* produces depressive-like behavioural effects in mice models (Son *et al*, 2012b).

B. White matter related genes: The importance of intact white matter for normal brain function and its implication in numerous psychiatric diseases (e.g., Catani and ffytche, 2005) give support to the notion of considering SZ as a disorder of disconnectivity (Konrad and Winterer, 2007). SZ and ASD have found to share common deficits in connectivity and synaptic plasticity (Cheung *et al*, 2010; de Lacy and King, 2013), moreover, there is also evidence indicating white matter abnormalities in both disorders (Dennis and Thompson, 2013; Wheeler and Voineskos, 2014). Considering that several genes are conferring susceptibility to both disorders, and due to the multiple genetic studies pointing to the involvement of oligodendrocyte-related genes in SZ (Karoutzou *et al*, 2008b), more genetic studies involving white matter related genes and its genetic interactions, could provide new ways to better understand the common related pathways between both disorders.

Considering this point, the following research article was developed:

[Manuscript B1] (In preparation)**:** To complement and genetically extend all previous work, where we have mainly focused on the genetic variability of one candidate gene, a group of 22 candidate genes (related with white matter pathways) and its genexgene interactions were analysed in three different samples: i) ASD case-parents' trios sample, ii) SSD case-parents' trios sample and iii) SSD case-control sample. Briefly, our results indicated that some of the WM genetic risk variants (e.g. *MAG*, *MOG*, *MBP)* seem to be shared across the SZ-ASD continuum while others seem to be disorder specific. Moreover, although every combination of genexgene interaction is different for each group of samples, the *ERRB3 gene* appears to be interacting with QKI (in ASD trio sample) and with *AKT1* (in SSD case-control sample). Since *QKI* and *AKT1* genes are related with myelination pathways (Åberg *et al*, 2006; Flores *et al*, 2008), we could modestly speculate about the interplay between OMR genes and Neuregulin-ERBB signalling pathways in both disorders**.** In favour of this conception, recent studies have revealed complex Nrg/Erbb signalling networks that regulate the assembly of neural

circuitry, myelination, neurotransmission, and synaptic plasticity (Mei and Nave, 2014) which are key processes involved in neurodevelopmental disorders. Moreover, evidence indicates that deviances from NRG/ERBB signalling in the brain impairs brain functions (Mei and Nave, 2014). In this sense, OMR and NRG/ERBB signalling pathways may provide therapeutic targets for neuropsychiatric diseases.

Finally, as stated in all the manuscripts, the **limitations** of these reports should be considered. Single-marker genetic effects in complex psychiatric disorders are likely to be very small; therefore, the relatively small sample sizes included in the present thesis and the associated low power to detect associations may be one of the strongest limitation. If we consider genexgene interaction analysis, this limitation is even more pronounced. Thus, the findings require replications in larger samples. Also, we should mention that the environmental background, including genexenvironment interactions, may also play an important role for SSD susceptibility and should be considered in potential studies. An important other issue which we have not touched upon is the use of matched cases and controls. In this thesis, we did not use matched data, but we have had many considerations in order to consider certain characteristics of cases and controls (e.g. gender and age), and correct for it when appropriate. In favour, the unmatched case-control is still the most powerful and efficient design in order to detect gene-gene interaction with less sample size (Wang and Zhao, 2003). Finally, other specific limitations have been further discussed within each article.

Global Discussion

Evidence from the first three manuscripts would be in favour of a pleiotropy effect of a plasticity gene called *NRN1,* affecting different phenotypes. More precisely, these results allow hypothesizing that some of the genetic factors conferring a pleiotropic load for psychiatric disorders may also modulate synaptic plasticity and neurodevelopmental trajectories. In genetics, the notion of pleiotropy has been extensively reviewed (Paaby and Rockman, 2013; Solovieff *et al*, 2013; Stearns, 2010), but is still not well understood in terms of its extent, mechanisms, and consequences.

Given the complex nature of mental disorders, it is important to try to understand the underlying mechanisms of pleiotropy and to identify specific genes and pathways driving such pleiotropy. Thus, a simple and reductionist way to start dissecting this potential pleiotropic mechanism is to select those genes related with the disease and study their possible impact into different phenotypes (candidate gene study). In this sense, the observation of a tendency of the same effect direction for *NRN1* SNPs associated with either SZ or BD, gives some insight concerning the underlying mechanism of their shared genetic factors. Along these lines, candidate gene approaches to date have yielded a number of associations between the polymorphism of a given gene and a clinical phenotype. However, new approaches such as GWAS have been appeared opening a new door in the investigation of the importance of genetic factors in the variability for developing psychiatric disorders. In this GWAS era, pleiotropy could may explain correlations among disorders, and may also enhance statistical power to detect genetic associations (Cross-Disorder Group of the Psychiatric Genomics Consortium, 2013; Lee *et al*, 2012, 2013; Li *et al*, 2014; Vattikuti *et al*, 2012). To date, there is strong evidence for sharing of common genetic variation (pleiotropy) between psychiatric disorders (Gratten *et al*, 2014; Lee *et al*, 2013), particularly SCZ, BPD and MDD[71]. In detail, it has been confirmed that the cross-diagnosis genetic correlations were highest correlations for SZ/BP; moderate for SZ/MDD and BP/MDD, and low but significant for SZ and ASD (of the Psychiatric Genomics Consortium *et al*, 2013). Although whether this overlap extends to the level of specific genetic variants is currently unclear.

Regrettably, the vast majority of the genetic variants previously reported by top candidate gene studies have not been replicated in GWAS, which it can be attributable to different methodological points: i) the consistency of the phenotype definition, ii)

clinical heterogeneity of the sample, which can reduce the statistical power to detect associations and this may lead to the lack of replication between studies, iii) the complexity of the phenotype, as is the case of mental disorders, where multiple factors should be considered (i.e. age, gender, disease course, treatments) and also, iv) the lack of power due to small size of samples and its consequent insufficiency of statistical power to detect small to moderate genetic effects.

Considering both approaches, genetic association studies based on single nucleotide polymorphisms (SNPs) are used to gain more insight into primary genetic processes that are potentially disease causing. Despite of that, sometimes single SNP associations will not necessarily lead to the definitive underlying molecular or cellular mechanisms (Lips *et al*, 2012; Ramanan *et al*, 2012).Alternatively, other methods are becoming increasingly considered such as pathway analysis, genexgene interaction (epistasis) or functional gene set analysis, involving the combined effect of multiple SNPs in functionally related genes that individually have small effect sizes that do not reach significance. Thus, these approaches take into account genetic contributions that may only be observed if the appropriate combination of genes is tested (Lips *et al*, 2012; Ramanan *et al*, 2012; Ruano *et al*, 2010; Torkamani *et al*, 2008), and can be helpful to understand the nature of genetic pleiotropy.

Beyond the evidence of pleiotropy effects of Neuritin-1 gene and highlighting the importance of studying gxg interactions, this thesis also support somehow psychiatric disorders are involving more than one plasticity gene, which are modulating one each other. In this sense, we observed that the effect of *NRN1* can be modulated by other genes related with plasticity such as *BDNF* and *DTNBP1*. In this regard, although the interactions detected throughout this thesis do not test the biological interaction pattern directly, these statistical interactions found suggest that the pair of involved genes together increases the risk for SSD according to the concept that epistasis contributes to the risk for complex diseases (Cordell, 2009b). Research has shown that biological interactions are critical for gene regulation, signal transduction and numerous other physiological and developmental pathways, which can indicate areas for future advancement in determining the underlying aetiology of SZ. For these reasons, considerations of protein-protein interactions and gxg interactions seem unavoidable in this endeavour for elucidating the mechanisms behind psychiatric disorders.

Furthermore, the term plasticity is chosen as a general concept for neuronal dynamics explaining neuropathology of psychiatric disorders. It is known that normal mental functions require optimal balance among all the plasticity timescales. Then, mental disorders arise when such balance is disturbed, thus mental disorders could be reformulated as deficiencies of the different plasticity processes. In general terms, this thesis also gives support to the fact that dysregulation of neuronal activity and synaptic functions are behind disorders such as SZ and BPD, as these diseases are related to alterations mainly in genes associated with activity-dependent gene expression and synaptic maturation (Purcell *et al*, 2014; West and Greenberg, 2011).

From a biological perspective, our results involving *NRN1* gene contribute to the understanding of the genetic mechanisms involved in SSD and BPD and also, of the relationship between genetic variability and the clinical heterogeneity of these disorders. *NRN1* gene role can be suggest as a mixed susceptibility/modifier gene (Fanous and Kendler, 2008), which increases the susceptibility to these disorders and modifies certain presentations.

Despite *NRN1* has not been identified by GWAS approaches in SZ, recent evidence from whole genome approaches adds support to its involvement in SZ. On the one hand, a recent whole methylome comparison between postmorten brain tissue in SZ patients and healthy subjects (cortex and cerebellum), reported that 29 adjacent CpG sites across *NRN1* were consistently hypomethylated in SZ cortex (Pidsley *et al*, 2014b). On the other hand, the importance of proposing *NRN1* as a candidate gene to be involved in SZ can also rely upon a recent study in animal model showing that Neuritin enhances synaptic transmission in medial prefrontal cortex by increasing CaV3 surface expression, meaning the involvement of the Ca^{2+}-dependent mechanism (Lu *et al*, 2017). Since intracellular Ca^{2+} is so important for cellular signalling processes, and its intracellular levels are so tightly regulated in neurons, it is likely that dysregulation of these calcium channels in either direction could cause disruption of neural developmental pathways as is the case of SZ. This is also consistent with the fact that genetic variation in calcium channel genes, particularly those found in *CACNA1C,* have been linked to the risk with a spectrum of psychiatric disorders (Cross-Disorder Group of the Psychiatric Genomics Consortium, 2013; Ripke *et al*, 2014). Although new studies should be developed to further explore the role of *NRN1* in the aetiology of mental disorders, overall, our results try to reasonably add a grain of sand to the multiple functions that Neuritin 1 gene is

involved, proposing this gene as an interesting potential therapeutic target for future studies.

Last but not least, our study considering white matter related genes bring light to the fact that genes related with white matter pathways are involved in both SZ and ASD. Results of neuroimaging studies suggest that SZ and ASD patients also share neural vulnerability, most notably in the frontal lobe and in frontal lobe circuitry (Minshew and Keller, 2010; Pettersson-Yeo *et al*, 2011). Therefore, researchers support the idea that genes that confer susceptibility to both SZ and ASD might contribute to altered brain structure and/or function common to both disorders. In general terms, findings from the past few years have emphasised phenotypic and genetic overlap between ASD and SZ (King and Lord, 2011; Rapoport *et al*, 2009), including identification of copy number variants conferring risk of both (Levinson *et al*, 2011). Interestingly, the largest molecular study of SZ reported evidence for overlap in schizophrenia GWAS regions and those with de novo non-synonymous mutations in ASD providing further support for the hypothesis that these disorders have partly overlapping pathophysiologies (Purcell *et al*, 2014; Sullivan *et al*, 2012a). In this sense, although there are no clear previous evidences suggesting a shared involvement of white matter related genes in both disorders, SZ risk variants have been related by a large GWAS, on total brain and white matter volumes in SZ patients (Terwisscha Van Scheltinga *et al*, 2013) pointing to the importance that white matter exert for the correct brain functioning (Catani and ffytche, 2005).Moreover, taking into account that WM maturation continues in the late development of the human brain (2–18 years of age), alterations on genes having a role in white matter, could be affecting the WM changes across development, which plays a key role in establishing interregional processing and neuronal synchrony in the brain (Hagmann *et al*, 2010).In short, we could refer to white matter as the subway of the brain – connecting different regions of grey matter to one another. In this sense, abnormalities in the correct functioning of this white matter related genes could lead to affect not only white matter functioning but also could influence the white-grey matter dialogue or interconnection affecting the whole brain functionality.

To conclude, considering all mentioned above, we would like to remark that the obvious progresses that psychiatric genetics era has facilitated to researchers has led to the recognition that genetics, alongside environmental factors, play an important role in developing psychiatric disorders; however, the function of individual genes is still

largely unknown. In this sense, international and multi-disciplinary teams/studies are needed, aiming to uncover and examine those genes involved in mental disorders, with the hope of improving knowledge for the efficient prevention, diagnosis and treatments. We mainly believe that genetic studies will help infer what is going on biologically, and that can ultimately lead to better treatment, which is one of the most important goals, in order to increase the quality life of the patients who suffer from mental disorders.

General Conclusions

Our results, focused in the analyses of genetic variability at *NRN1* gene and at white matter-related genes, have allowed the detection of some minor and moderate effects of specific genetic variants and also, some specific *genexgene* interactions that could explain at least, part of the genetic basis underlying the development of SSD and other neurodevelopmental disorders. For this reason, the main conclusions suggested by the main results are the following:

[*NRN1*, candidate plasticity gene]

1) *NRN1* variability is a shared risk factor for both SSD and BPD. This supports the alteration of neurite outgrowth and arborization and/or on neuronal processes associated with synaptic plasticity in these disorders. Interestingly, we have identified a risk haplotype (rs645649-rs582262: C-C) that is associated with the risk for developing these disorders. As expected, due to the polygenic architecture of the studied disorders, the effect of this haplotype is small although significant (OR (CI 95%)=1.28 (1.08-1.51)).

2) Allelic variants in the haplotype Block 3 (rs3763180-rs10484320-rs4960155-rs9379002-rs9405890-rs1475157: T-C-C-T-C-A) of *NRN1* represent a protective factor, not only due to their association to a reduction of the risk for SSD and BPD, but also because within patients, these variants are related to a later of age at onset and a better cognitive performance.

3) The selective impact of *NRN1* on general intelligence in SSD, and not in BPD and healthy subjects, may suggest its involvement in illness specific cognitive alterations rather than in general cognitive processes. In addition, *NRN1* (rs1475157) shows a*lso* a significant effect on the appearance of depressive symptoms in a non-clinical sample, with individuals carrying the genotype GG showing higher *scores*on BSI depressive dimension and on total scorescompared to A allele carriers. The same genotype within the rs1475157 polymorphism

showed a trend towards association with worse performance in phonemic fluency and WCST total correct responses. This suggest also, an involvement of *NRN1* in executive function in the general population.

4) Our data suggest an interaction between *NRN1xBDNF* on the risk for both SSD and BPD, and also on the appearance of depressive symptoms in a non-clinical sample. In addition, the detected interaction between *NRN1xDTNBP1* on the risk for SSD, also suggests that these genes work in concert and collectively contribute to increase the risk of SSD. Although new studies are needed to better understand the precise mechanisms underlying the joint effect between *NRN1xBDNF* and *NRN1xDTNBP1* interactions, it is interesting to note that these three genes are involved in neurodevelopment and synaptic plasticity, which are key processes in the pathophysiology of schizophrenia.

5) In summary, our findings support the pleiotropic effect of *NRN1* influencing multiple phenotypic traits. In this regard, the molecular genetic overlap between schizophrenia and bipolar disorder, and between schizophrenia and autism are consistent with pleiotropy concept.

[White matter related genes]

6) Our findings suggest a marginally significant effect for the involvement of white matter related genes (e.g. *MAG*, *MOG*, *MBP)* in both SZ and ASD, suggesting common pathophysiological pathways between both disorders. However, more genetic studies involving white matter related genes and its genetic interactions are needed to provide new ways to better understand the common related pathways between both disorders.

7) In our study, the relationship between OMR and NRG/ERBB related genes, seems to play a key role in modulating the risk for developing either ASD or SSD. In this sense, OMR and NRG/ERBB signalling pathways may provide therapeutic targets for neuropsychiatric diseases.

7. References

The Only Thing That Is Constant Is Change.
— Heraclitus

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8. Appendix

She's mad, but she's magic. There's no lie in her fire.
(Ella está loca, pero es mágica. No hay mentira en su fuego).
— Charles Bukowski

Breve reflexión: Enfermedad Mental y sociedad actual

Para concluir y como breve reflexión, me gustaría mencionar la definición sobre enfermedad mental que en 1948 el psiquiatra y psicoanalista Henry Ey definió como *la pérdida de la libertad*. En este sentido, creo que los principales problemas de las personas diagnosticadas con una enfermedad mental tienen que ver principalmente con la dificultad de llevar una vida "normal", aprendiendo a convivir con los síntomas y a sentirse aceptados y vinculados dentro de ciudadanía y sociedad en la que vivimos.

Si algo es evidente, es que una de las principales barreras para las personas que padecen de un trastorno mental es la estigmatización de ésta dentro de la sociedad, amigos y familia. No obstante, a pesar del existente avance y conocimiento sobre estas enfermedades, el desconocimiento, los prejuicios e incluso la discriminación siguen siendo problemas que solo refuerzan dicha barrera de diferenciación y "anormalidad". Las presiones sociales que vivimos a todos los niveles y en los distintos tipos de sociedades, no hacen más que reforzar el aislamiento de grupos cuyo comportamiento se aleja de unos patrones y expectativas del grupo mayoritario o también conocido como "normal". Bajo mi punto de vista, todo tipo de discriminación no es más que un maltrato contra los derechos de las personas, que imposibilita su desarrollo humano volviendo a las personas vulnerables. En concreto, las distorsiones de la percepción, pensamientos y de las emociones que una persona con enfermedad mental puede sufrir, son muchas veces agravadas por el rechazo que su conducta produce en nuestra sociedad.

Además, es bien conocido cómo las personas que sufren de enfermedades mentales se enfrentan a condiciones de vida adversas, que reducen sus oportunidades para optar a unos niveles de educación mínima, trabajo, viviendas, etc; lo cual dificulta todavía más su óptima evolución. A este respecto, me gustaría también destacar que a pesar de la creciente evolución en las políticas públicas u otros planes nacionales/internacionales de salud mental necesarios para incluir la protección de los derechos de estas personas tanto a nivel de la salud pública como a nivel extra-hospitalario, todavía faltan mejoras y nuevos enfoques que permitan potenciar la inclusión del conjunto de enfermos mentales en la población.

Si bien es cierto el hecho que en las últimas décadas los avances en el campo de neurociencia han revolucionado la manera de entender nuestro cerebro y la conducta humana, permitiendo, además, destacar el concepto de mente cuya base biológica emerge en el cerebro. Todavía queda un largo camino para comprender las bases

neurológicas de estas enfermedades, destacando el hecho de que es un fenómeno ampliamente complejo del que no se pueden extrapolar conclusiones deterministas o minimalistas, y en todo caso deben complementarse gracias a la colaboración e integración de múltiples disciplinas.

En este sentido, cabe resaltar la importancia del trabajo conjunto y coordinado que debe haber entre investigadores, profesionales clínicos y pacientes para poder avanzar juntos en la misma dirección de conocimiento. Por ejemplo, creo que la investigación a nivel biológico ha podido ser de gran ayuda frente al sentimiento de culpa que muchas veces los pacientes tienen por el hecho de ser diagnosticados con una enfermedad mental. Por ello, la investigación a nivel biológico es clave para ayudar a entender que, aunque no debemos obviar el papel que el ambiente tiene (complicaciones obstétricas, maltrato infantil, negligencia, etc), hay una parte intrínseca biológica y genética asociada a ello, lo cual ayuda a minimizar la culpa que muchos pacientes sienten, con la finalidad de aceptar, entender y normalizar su enfermedad. Esto es importante porqué a mi modo de ver, da sentido a que la investigación en el campo de las neurociencias siga su curso con la finalidad de ayudar a la comprensión y mejora de la vida del enfermo. Una comprensión que es útil tanto para los pacientes y como para la evolución del conocimiento neurocientífico.

- *Claudia Prats*

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