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Analysis of the impact of synaptic plasticity genes and Human Accelerated Regions on brain function and structure: from the healthy brain to schizophrenia

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DOCTORAL THESIS

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FROM THE HEALTHY BRAIN TO SCHIZOPHRENIA

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Analysis of the impact of synaptic plasticity genes and Human Accelerated Regions on brain function and structure: from the healthy brain to schizophrenia

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"I am a brain, Watson. The rest of me is a mere appendix".

Sherlock Holmes

(The Adventure of the Mazarin Stone, by Arthur Conan Doyle)

"It is imperfection, not perfection, that is the end result of the program written into that formidably complex engine that is the human brain".

Rita Levi-Montalcini

"Nothing in biology makes sense except in the light of evolution".

Theodosius Dobzhansky

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ABSTRACT

Schizophrenia is a severe psychiatric disorder affecting around 24 million people worldwide. While we begin to disentangle the biological actors implicated in the origin of the disorder, the precise aetiological mechanisms remain largely unknown. Therefore, psychiatry research efforts still need to focus on a better understanding of the complex biological foundations of the disorder to achieve more precise diagnoses and the development of novel therapeutic strategies improving the patients' quality of life.

The prevailing etiopathological hypothesis considers that schizophrenia originates from the interplay between subtle genetic and environmental insults that disrupt the perfectly orchestrated mechanisms guiding neurodevelopment. Additionally, from an evolutionary perspective, it is suggested that schizophrenia represents a costly trade-off in the evolution of human-specific ontogenic neurodevelopmental processes sustaining the inherent complexity and variability of brain functioning, cognition, and behaviour. Along the neurodevelopmental process, the synapse formation and the organisation and maturation of neural circuits anchor the emergence of distinctive human cortical brain functions. In turn, multidisciplinary evidence indicates that synaptic alterations participate in brain dysfunctions, eventually leading to the emergence of the symptoms and cognitive deficits of schizophrenia. Accordingly, it is suggested that synaptic plasticity impairments play a critical role in the pathophysiology of the disorder.

Among genes converging in neurodevelopmental and synaptic plasticity pathways, there are genes mediating signalling pathways involved in neural homeostasis, dendritic spine development and neural excitability, such as *KCNH2*, *DISC1*, *CACNA1C* and *ZNF804A*, all of them previously associated with the risk for schizophrenia. Moreover, evolutionary approaches have identified regions that accumulated human-specific changes since the divergence from chimpanzees, like Human Accelerated Regions (HARs). These regions act as transcriptional regulatory elements that endow human neurodevelopment with unique characteristics and harbour schizophrenia genetic susceptibility variants. To facilitate the identification of the genetic and biological mechanisms involved in schizophrenia aetiology, the use of brain-based intermediate phenotypes is a valuable strategy.

Following two approaches centred on the genetic-phenotypic correlates of synaptic plasticity candidate genes and HARs sequences in the brain-based alterations in schizophrenia, this thesis includes four original articles and one systematic review. In these articles, we report the effect of common polymorphisms in *KCNH2*, *DISC1*, *CACNA1C* and *ZNF804A* genes and the polygenic load of HARs-informative sets on the differences observed between healthy brains and brains with schizophrenia.

Overall, the results validate the efficacy of neuroimaging phenotypes to identify the genetic determinants of schizophrenia and point out the complementarity of candidate genes and genome-wide approaches in the study of the genetic architecture of the disorder. First, we describe the role of *KCNH2* and *DISC1* genetic variability in modulating the attentional and working memory-related functional responses in a diagnosis-dependent manner. Furthermore, we identify that the epistasis between two schizophrenia GWAS-associated genes, *CACNAC1C* and *ZNF804A*, influence the functional ability to adapt to increased working memory difficulty euqally in healthy controls and patients with schizophrenia. Second, we present a review of how HARs underlie human neurodevelopmental signatures, brain configuration, functioning and susceptibility behind psychiatric disorders. Likewise, we report the modulatory effect of HARs polygenicity on brain cortical architectural differences in schizophrenia and provide evidence on the importance of foetal-active regulatory HARs in patients' cortical surface area variability.

Globally, the findings exposed in this thesis point towards the fact that the aetiological foundations of schizophrenia are related to the individual genetic differences altering neurodevelopment and synaptic plasticity trajectories but also to the genomic make-up that defines us as a species. This thesis provides a drop in the ocean of knowledge on disorders inherently linked to the human condition and has sought to comprehend the unique characteristics of our brain to help unravel what it means to be human.

RESUMEN

La esquizofrenia es un trastorno psiquiátrico que afecta a 24 millones de personas en todo el mundo. A pesar de que empezamos a conocer los mecanismos biológicos implicados en el origen del trastorno, los procesos etiológicos precisos continúan siendo en gran parte desconocidos. Por ello, los esfuerzos investigadores todavía necesitan dirigirse en mejorar el conocimiento de los fundamentos biológicos del trastorno, para así conseguir una mayor precisión en el diagnóstico y desarrollar nuevas estrategias terapéuticas que mejoren la calidad de vida de los pacientes.

La hipótesis etiopatogénica predominante considera que el trastorno se origina de la interacción entre factores genéticos y ambientales que modifican los mecanismos perfectamente orquestados que guían el neurodesarrollo. Además, desde una perspectiva evolutiva, se sostiene que la esquizofrenia representa "el precio a pagar" por la evolución de los procesos ontogénicos específicamente humanos que sustentan la complejidad y la variabilidad inherente al funcionamiento del cerebro, así como la cognición y comportamiento de nuestra especie. A lo largo del neurodesarrollo, la formación de sinapsis y la organización y maduración de los circuitos neurales anclan la aparición de funciones cerebrales corticales distintivamente humanas. Por su parte, evidencias multidisciplinares indican que las alteraciones sinápticas participan en disfunciones cerebrales asociadas a la aparición de los síntomas cognitivos y clínicos de la esquizofrenia. En consecuencia, se ha propuesto que las alteraciones de la plasticidad sináptica tienen un papel crítico en la fisiopatología del trastorno.

Entre los genes que confluyen en vías del neurodesarrollo y de plasticidad sináptica, hay genes que participan en vías de señalización implicadas en la homeostasis neuronal, el desarrollo de las espinas dendríticas y la excitabilidad neural, como el *KCNH2*, el *DISC1*, el *CACNA1C* y el *ZNF804A*, todos ellos previamente asociados con el riesgo para la esquizofrenia. Además, aproximaciones evolutivas han identificado regiones que han acumulado cambios específicos humanos desde la divergencia con los chimpancés, como las Regiones Humanas Aceleradas (o *Human Accelerated Regions*, HARs en inglés). Estas regiones actúan como elementos reguladores de la transcripción otorgando características únicas al neurodesarrollo humano, y albergan variantes genéticas de susceptibilidad para la esquizofrenia. Para facilitar la identificación de los mecanismo genéticos y biológicos implicados en la etiología del trastorno, el uso de fenotipos cerebrales intermedios, como medidas de neuroimagen funcional y estructural, es una herramienta de gran valor.

Siguiendo dos aproximaciones centradas en el análisis de los correlatos genético-fenotípicos entre genes candidatos relacionados con la plasticidad sináptica y secuencias HARs y las alteraciones cerebrales en la esquizofrenia, esta tesis incluye cuatro artículos originales y una revisión sistemática. En estos artículos, exponemos el

efecto de polimorfismos en los genes *KCNH2, DISC1, CACNA1C* y *ZNF804A* y la carga poligénica en conjuntos informativos de HARs sobre las diferencias observadas entre cerebros sanos y cerebros con esquizofrenia.

En su conjunto, los resultados validan la efectividad de los fenotipos de neuroimagen para identificar los mecanismos genéticos de la esquizofrenia y ponen de manifiesto la complementariedad de las aproximaciones centradas tanto en genes candidatos como en la variabilidad global del genoma para estudiar la arquitectura genética del trastorno. Primero describimos el papel de la variabilidad genética de los genes *KCNH2* y *DISC1* en la modulación de la respuesta funcional a la atención y la memoria de trabajo de manera condicional al diagnóstico. Además, identificamos que la epistasis entre dos genes asociados con la esquizofrenia a nivel de GWAS, el *CACNAC1C* y el *ZNF804A*, influye en la capacidad funcional de cerebro para adaptarse al incremento de requerimientos cognitivos en memoria de trabajo tanto en controles sanos como en pacientes con esquizofrenia. En segundo lugar, ofrecemos una revisión sobre cómo las HARs sustentan las características del neurodesarrollo humano, la configuración y el funcionamiento cerebral y la susceptibilidad para trastornos psiquiátricos. Así mismo, informamos del efecto modulador de la poligenicidad de las HARs sobre las diferencias en la arquitectura cortical en la esquizofrenia y proporcionamos evidencias sobre la especial relevancia de las HARs asociadas con elementos reguladores de la transcripción activos durante la etapa fetal.

De manera global, los resultados de esta tesis indican que los fundamentos etiológicos de la esquizofrenia están relacionados con diferencias genéticas individuales que impactan en las trayectorias del neurodesarrollo y en las vías de plasticidad sináptica, así como en la composición genética que nos define como especie. Esta tesis aporta una gota en el océano del conocimiento sobre los trastornos intrínsicamente vinculados a la condición humana y ha pretendido contribuir en la comprensión de las características únicas de nuestro cerebro para ayudar a entender qué quiere decir ser humano.

RESUM

L'esquizofrènia és un trastorn neuropsiquiàtric greu que afecta a 24 milions de persones a tot el món. Tot i que comencem a conèixer els mecanismes biològics implicats en l'origen del trastorn, els processos etiològics precisos continuen essent en gran part desconeguts. Per tant, els esforços en la recerca encara necessiten dirigir-se en millorar el coneixement dels fonaments biològics del trastorn, per tal d'aconseguir un diagnòstic més precís i el desenvolupament de noves estratègies terapèutiques que millorin la qualitat de vida dels pacients.

La hipòtesi etiopatogènica predominant considera que el trastorn s'origina a partir de la interacció entre factors genètics i ambientals que pertorben els mecanismes perfectament orquestrats que guien el neurodesenvolupament. A més, des d'una perspectiva evolutiva, s'ha suggerit que l'esquizofrènia representaria el "preu a pagar" per evolució dels processos ontogènics específicament humans que sustenten la complexitat i la variabilitat inherent al funcionament del cervell, la cognició i el comportament de la nostra espècie. Al llarg del neurodevenvolupament, la formació de sinapsis i l'organització i maduració dels circuits neurals ancoren l'aparició de funcions cerebrals corticals distintivament humanes. Al seu torn, evidències multidisciplinàries indiquen que les alteracions sinàptiques participen en disfuncions cerebrals que tenen com a resultat l'aparició dels símptomes cognitius i clínics de l'esquizofrènia. En conseqüència, s'ha proposat que les alteracions de la plasticitat sinàptica tenen un paper crític en la fisiopatologia del trastorn.

Entre els gens que conflueixen en vies del neurodesenvolupament i de plasticitat sinàptica, hi ha gens que participen en vies de senyalització implicades en l'homeòstasi neuronal, el desenvolupament de les espines dendrítiques i l'excitabilitat neuronal, com els gens *KCNH2*, el *DISC1*, el *CACNA1C* i el *ZNF804A*, tots prèviament associats amb el risc per a l'esquizofrènia. A més, aproximacions evolutives han identificat regions que han acumulat canvis específicament en humans des de la divergència amb els ximpanzés, com les Regions Humanes Accelerades (o *Human Accelerated Regions*, HARs en anglès). Aquestes regions actuen com a elements reguladors de la transcripció atorgant característiques úniques al neurodesenvolupament humà, i contenen variants genètiques de susceptibilitat per a l'esquizofrènia. Per tal de facilitar l'identificar els mecanismes genètics i biològics implicats en l'etiologia de l'esquizofrènia, la utilització de fenotips cerebrals intermedis, com mesures de neuroimatge funcional i estructural, representa una estratègia molt útil.

Seguint dues aproximacions centrades en l'anàlisi dels correlats genètics-fenotípics entre gens candidats relacionats amb la plasticitat sinàptica i regions HARs i les alteracions cerebrals de l'esquizofrènia, aquesta tesi inclou quatre articles originals i una revisió sistemàtica. En aquests articles, exposem l'efecte de polimorfismes en els gens

KCNH2, *DISC1*, *CACNA1C* i *ZNF804A* i la càrrega poligènica en conjunts informatius de HARs sobre les diferències observades entre cervells de persones sanes i persones amb esquizofrènia.

En conjunt, els resultats validen l'efectivitat dels fenotips de neuroimatge per identificar els determinants genètics de l'esquizofrènia i posen de manifest la complementarietat de les aproximacions centrades tant en gens candidats com en la variabilitat global del genoma per a l'estudi de l'arquitectura genètica del trastorn. Primer, descrivim el paper de la variabilitat genètica dels genes *KCNH2* i *DISC1* en la modulació de la resposta funcional a l'atenció i la memòria de treball de manera condicionada al diagnòstic. També, identifiquem que l'epistasi entre dos gens associats amb l'esquizofrènia a nivell de GWAS, el *CACNAC1C* i el *ZNF804A*, influeix en la capacitat funcionalde cervell per adaptar-se a l'increment de requeriments cognitius en memòria de treball en controls sans i pacients amb esquizofrènia. En segon lloc, oferim una revisió sobre com les HARs sustenten les característiques del neurodesenvolupament humà, la configuració cerebral, el funcionament i la susceptibilitat per als trastorns psiquiàtrics Així mateix, informem de l'efecte modulador de la poligenicitat de les HARs sobre les diferències en l'arquitectura cortical en l'esquizofrènia i proporcionem evidències sobre l'especial rellevància de les HARs associades amb elements reguladors de la transcripció actius durant l'etapa fetal.

De manera global, els resultats d'aquesta tesi indiquen que els fonaments etiològics de l'esquizofrènia estan relacionats amb diferències genètiques individuals que impacten en les trajectòries del neurodesenvolupament i les vies de plasticitat sinàptica, així com amb la composició genòmica que ens defineix com a espècie. Aquesta tesi aporta una gota en l'oceà del coneixement sobre els trastorns intrínsecament vinculats a la condició humana i ha pretès contribuir en la comprensió de les característiques úniques del nostre cervell per ajudar a entendre què vol dir ser humà.

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Introduction

1. The human brain developmental continuum

The human brain is the central coordinator of the nervous system and acts as the control centre for perception, behaviour, memory and thought. In humans, distinctive biological characteristics, not fully elucidated, enable the brain the ability to generate abstract thought, language, and complex cognitive processes.

All these human-specific abilities are sustained by synaptic connexions that allow the transmission of information from one neuron to another. These connexions are the basis of brain activity. Brain structure and circuitry organisation are other paramount determinants of brain function since they sustain synaptic networks and guarantee efficient communication for integrating information and generating brain functions (Jiang and Nardelli 2016; Luppi et al. 2022). The neurodevelopmental process coordinates all these complex and dynamic changes in brain architectural configuration, structural maturation, synaptic connections, and function (**Figure 1**).
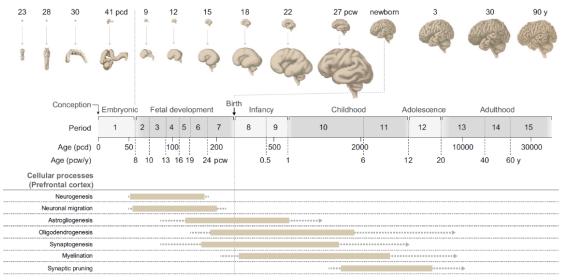
Figure 1. Timeline of the neurodevelopmental processes. At the top, the brain structural changes along the development from conception, embryonic and foetal development (in postconceptional days (pcd) and weeks (pcw)) to postnatal development with infancy, childhood, adolescence, and adulthood (in years (y)). In the middle, the timeline of human development and associated periods. At the bottom, the estimated timing and sequence of the different cellular processes. The coloured bars indicate the peak developmental period in which each process occurs, the dotted lines indicate that the process continues, although to a minor degree, and the arrows indicate that the process is present thereafter. Adapted figure (Silbereis et al. 2016).

Human neurodevelopment is a complex and protracted process that starts prenatally, with neural tube formation at approximately week 3 of gestation, and continues to early adulthood, on average, until the individual is 20 or 25 years old. This process requires extraordinary precise coordination to produce various neural and non-neural cells in proper numbers, locations, and timing. The neurogenesis and formation of the general architecture of brain regions are largely completed at birth. However, the

maturation of astrocytes and oligodendrocytes, as well as the synaptogenesis, myelination, and synapse pruning, occur during postnatal brain growth (Colver and Longwell 2013; Silbereis et al. 2016; Vasung et al. 2016; Jiang and Nardelli 2016; Teeuw et al. 2019).

Neuroimaging studies evidence continued brain development through childhood, adolescence, and early adulthood, with differential trajectories related to grey and white matter. While grey matter volume peaks in childhood and decreases through the second decade of life, white matter volume increases until mid-to-late adolescence before decelerating (Mills et al. 2016; Bethlehem et al. 2022). All these processes are genetically orchestrated, environmentally modulated, and require precise spatiotemporal transcriptional regulation (Stiles and Jernigan 2010; Silbereis et al. 2016; Jiang and Nardelli 2016; Mills et al. 2016; Hardingham et al. 2018; Teeuw et al. 2019).

Nonetheless, in comparative studies investigating the differences and commonalities between brain development in humans, macaques, and chimpanzees, it has been observed that the ontogenetic patterns, the neurogenic gene expression programs, the cytoarchitecture, and the cell-type composition are remarkably similar (Mora-Bermúdez et al. 2016; Zhu et al. 2018). Then, the differences reside in the tempo-spatial gene expression trajectories that neurodevelopmental genes exhibit in humans, especially in phases of embryonic, late mid-foetal and adolescent development (Otani et al. 2016; Zhu et al. 2018). Expression divergences in humans during embryonic development are related to neurogenesis and neuronal differentiation and are believed to underlie the increased proliferative capacity of neural progenitor cells sustaining the neocortex expansion in humans (Mora-Bermúdez et al. 2016; Otani et al. 2016; Zhu et al. 2018). In contrast, the expression differences in adolescence are associated with genes related to synaptogenesis and myelination (Zhu et al. 2018).

Despite this precision and fierce control, the neurodevelopmental map drawn by the unique individual genetic make-up is in constant dialogue with environmental forces during prenatal, early childhood and adolescent stages. These genetic and environmental factors that guide the ontogenetic process of brain formation, maturation, and in the end, structure and function are what sustain the inherent variability in cognition, behaviour and personality traits found in humans. However, these determinants, at the same time, also underlie the disorganisation and, eventually, dysfunction of the central nervous system that can be observed across the neurodevelopmental continuum in different disorders such as schizophrenia (Schmitt et al. 2014b; Owen and O'Donovan 2017).

2. Schizophrenia: from an epidemiological and clinical point of view

Epidemiological and social characteristics

According to the World Mental Health Report 2022 from the World Health Organization (WHO) (World Health Organization 2022), 24 million people worldwide suffer from schizophrenia, approximately 1 in 200 adults 20 years old and over. While this prevalence rate (the number of cases with a disease in a specific population at a particular timeframe) has relatively tiny differences across countries (Charlson et al. 2018), those with more significant income inequalities at a national level and social polarisation have been found to have a higher prevalence of the disorder (Lund et al. 2018). Also, higher rates of schizophrenia have been associated with migrant status (McGrath et al. 2008; Radua et al. 2018), but with substantial risk variation depending on the migratory context (Bourque et al., 2011).
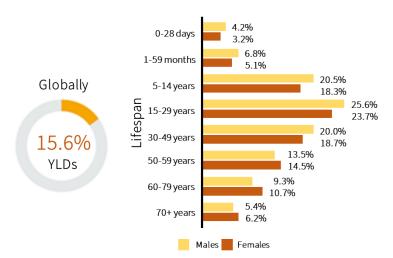
Whether the disorder is more common in men or women is constantly debated. Prevalence estimates suggest no sex differences (Charlson et al. 2018; World Health Organization 2022) but incidence statistics (the number of new cases with a disease in a specific population during a timeframe) describe a slightly higher frequency in men than in women (Jongsma et al. 2019).

As highlighted by a recent revision overviewing the disorder (Jauhar et al. 2022), schizophrenia onset occurs predominantly in early adult life. Still, it can also appear for the first time in adolescence. While the men's incidence peaks in the early twenties declining afterwards, in women, the incidence peak is less sharp as well as the decline, and from the mid-forties onwards, new cases in women exceed those in men (Kirkbride et al. 2012).

People with mental health conditions experience disproportionately higher mortality rates than the general population: affected individuals die, on average, 10 to 20 years earlier (Chesney et al. 2014; Walker et al. 2015; Erlangsen et al. 2017). Several contributing factors sustain this increased mortality rate. First, the lifetime risk of suicide in schizophrenia is estimated at a rate of around 5% (Hor and Taylor 2010), for which patients with schizophrenia are especially vulnerable, having more than ten times higher suicide mortality rates than the general population (Chesney et al. 2014). Second, the side medication effects lead to premature mortality and underlie obesity, glucose intolerance and lipid metabolism abnormalities (Correll et al. 2015). Third, unhealthy habits like smoking, alcohol use, poor dietary habits or physical inactivity are more prevalent among mental health patients (Olfson et al. 2015). Finally, side medication effects and poor lifestyle habits may contribute to premature death caused by preventable diseases such as cardiovascular diseases, cerebrovascular accidents, respiratory diseases, and

infections, which are more common in schizophrenia and other severe mental disorders (de Hert et al. 2011). Notwithstanding, evidence also suggests shared genetic signatures between schizophrenia and lifespan, cardiovascular and metabolic disorders (Andreassen et al. 2013; Muntané et al. 2021).

Moreover, individuals with severe mental illnesses like schizophrenia must deal with the self and social stigma resulting from misconceptions and stereotypes about mental disorders, which may influence the clinical outcome (Corrigan and Watson 2002; Dubreucq et al. 2021). The impact of these clinical and social characteristics on the population has been estimated by assessing the population-wide burden of living with mental illness. Burden measures include the Years of healthy Life lost due to Disability (YLDs). According to the latest WHO Mental Health report, schizophrenia, by itself, accounts for 1.8% of the global YLDs, being among the 20 leading causes contributing to the global burden. However, when combining all mental conditions, these disorders are the leading cause of years of healthy life lost due to disability, accounting for 1 in every six YLDs globally (15.6% of the global burden) (World Health Organization 2022) (**Figure 2**).

Figure 2. Mental health global and lifespan years lived with disability (YLDs). The left plot represents the proportion of all-cause YLDs attributable to mental disorders globally. The right plot represents the YLDs by sex across the lifespan. Note that YLDs attributable to people between 15 and 29 represent 24.7% of the global burden. Adapted figure (World Health Organization 2022).

In addition, schizophrenia, in its acute state, is the most impairing disorder. The WHO's Global Health Estimates, or the so-called "health state weights", represent a 0 to 1 quantitative measure used to adjust the time spent in a particular health state by its associated level of diminished health or impairment, where 0 means total health or no impairment. The estimated health state weight for acute schizophrenia is 0.78, and 0.59 after the acute episode (World Health Organization 2020, 2022). This means that a person

suffering from schizophrenia has between one and two-fifths of the health and functioning of a fully healthy person.

Clinical description

Even though schizophrenia is a biological illness, no specific biological marker, brain characteristic or symptom unequivocally identifies people with this diagnosis (Moncrieff and Middleton 2015). Its denomination as "disorder", which refers to a broader range of pathological conditions with no specific agent or group of agents identified, suits the complexity of the phenomena. Accordingly, schizophrenia is diagnosed based on diagnostic manuals. These manuals rely on clinicians' symptoms and mental state evaluations, self-reported experiences, and personal history but not on any biological and objectively assessed marker. This fact indicates that diagnostic manuals are not necessarily linked to the biological foundations, suggesting that the heterogeneous presentation and course of psychiatric disorders may not represent distinct pathogenic entities but exist on a continuum (Keshavan et al. 2011; DeRosse and Karlsgodt 2015).

Among the diagnostic classification systems, there are two on the lead: the International Classification of Diseases (World Health Organization) and the Diagnosis and Statistical Manual of Mental Disorders (DSM)(American Psychiatric Association (APA) 2013), that have reflected through their editions the evolving schizophrenia diagnosis definition. The current version of the DSM-5 defines schizophrenia by the presence of delusions, hallucinations, disorganised thinking, and behaviour and or symptoms such as apathy and social avoidance. To settle a diagnosis, two or more of these symptoms, with at least one being delusions, hallucinations, or disorganised speech, must be present for a month (see **Box 1**).

This current conception of schizophrenia as a discrete clinical entity is instrumental in medical practice. Nevertheless, there is scarce evidence indicating that the clinical categories represent discrete entities with natural boundaries (Kendell and Jablensky 2003; Clark et al. 2017). Instead, there are extensive clinical, epidemiological, and genetic indicators of the commonalities among psychiatric disorders (Anttila et al. 2018; Plana-Ripoll et al. 2019; Barr et al. 2022).

As previously mentioned, schizophrenia typically appears in adolescence and early adulthood, with a peak age between 20 and 21 years old (Solmi et al. 2022), but this is preceded by the emergence of subtler changes in belief, thought and perception that appear as attenuated symptoms (Møller and Husby 2000). This prodromal or high-risk state is characterised by non-specific signs (such as attention problems, lack of energy, and anxiety) but also by attenuated symptoms such as perplexity, unusual and overvalued beliefs, guardedness, and hearing indistinct noises (Norman et al. 2005;

Marshall et al. 2014; Cannon 2015). The line between prodromal and psychosis is based on the frequency of the symptoms, their pervasiveness and impairment (Cannon 2015).

Some of the symptoms of schizophrenia could be understood as a brain failure to offer sophisticated control of purposeful behaviour and alterations in the centralisation and integration of complex sensory inputs. Thus, the clinical presentation of schizophrenia, and its complexity in signs and symptoms, relate to the complexity of brain development and functioning. It is important to note that symptoms are not manifested in the same way in every patient or throughout the lifespan, and there is no unique pattern of signs distinguishing people diagnosed with schizophrenia from those with other mental health diagnoses or people without it (Andreasen 1999; Craddock and Owen 2010).

Attempts to characterise schizophrenia symptomatology into different categories have classically focused on a three-factor division that includes: positive symptoms, comprehending delusions (false beliefs), hallucinations (false perceptions), suspiciousness, and grandiosity; negative symptoms, such as emotional withdrawal or detachment, anhedonia, blunted affect, apathy and social avoidance; and, disorganisation, which includes formal though disorder and disorganised behaviour (Andreasen et al. 1995; Grube et al. 1998). Recently, the cognitive dimension has also been considered a core dimension, including poor sustained attention, difficulty in abstract thinking, intellectual impairment, working and long-term memory deficits and poor executive function (Jauhar et al. 2022) (**Figure 3**). Also, other symptoms which may be classified into affect and resistance categories recognize depression, anxiety, and guilt feelings; and hostility, poor impulse control, and uncooperativeness (Shafer and Dazzi 2019).

Once diagnosed with schizophrenia, individuals may be trated with a combination of medication, psychological intervention, and community-based assistance. Still, it is essential to point out that the treatment does not represent a cure in many cases, and it is common for patients not to respond to therapy and experience relapses. In this regard, along the course of the disorder, most patients will experience multiple relapses. Five years after the recovery from a first episode of psychosis, more than 80% of patients will suffer a relapse (Robinson et al. 1999), and each new episode will increase the risk of chronicity (Wiersma et al. 1998). While multiple factors influence the chance of a relapse, the most common risk factor is non-adherence to medication (Robinson et al. 1999; Alvarez-Jimenez et al. 2012; Emsley et al. 2013). In terms of pharmacological interventions, antipsychotics of first and second generation are the only drugs with proven effectiveness, especially on positive symptoms, and their action mechanism is through blockage of dopamine D2-receptors (McCutcheon et al. 2020; Jauhar et al. 2022). Most common side effects of antipsychotics physical condition such as obesity, dyslipidaemia, diabetes mellitus, thyroid disorders, cardiovascular, respiratory diseases

and movement disorders, and a higher lifetime cumulative antipsychotic dose might contribute to higher mortality (Correll et al. 2015; Yoshida and Takeuchi 2021). Apart from drug-based approaches, cognitive behavioural therapy is also recommended for patients with schizophrenia (Jauhar et al. 2014; Bighelli et al. 2018; Rodolico et al. 2022), and family interventions, psychoeducation and cognitive behavioural therapy have proven to benefit patients in reducing the risk for relapse (Bighelli et al. 2021).
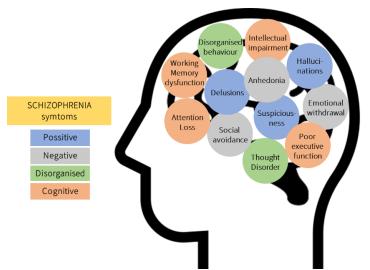
Figure 3. Classification of symptoms in schizophrenia. The clinical presentation of schizophrenia includes a wide range of symptoms that can be classified intro four major dimensions: positive symptoms (blue), negative symptoms (grey), disorganised behaviours (green) and cognitive impairments (orange).

Nonetheless, while positive symptoms are the ones that capture much of the attention and usually respond well to antipsychotic medication and psychological therapy, negative and cognitive symptoms are the ones that contribute most to the morbidity associated with schizophrenia (Keefe and Fenton 2007; McCutcheon et al. 2020). Indeed, cognitive impairments usually begin before the onset of the first psychotic episode (Fusar-Poli et al. 2012), and individuals at high risk of psychosis who later develop it present more severe prodromal negative symptoms than those who did not (Piskulic et al. 2012). Thus, negative symptoms and cognitive function alterations are considered risk factors for transition to psychosis in at-risk people (Piskulic et al. 2012; Karcher et al. 2022; Harvey et al. 2022).

Box 1. DSM-5 Criteria for the diagnosis of schizophrenia, 295.90 (F20.9)
(American Psychiatric Association (APA) 2013)

A. Two (or more) of the following, each present for a significant portion of the time **during a 1-month** period (or less if successfully treated). At least one of these must be (1, 2, or 3):

- Delusions
- Hallucinations
- **Disorganised speech** (e.g., frequent derailment or incoherence)
- Disorganised or catatonic behaviour
- Negative symptoms (i.e., diminished emotional expression or avolition).

B. For a significant portion of the time since the onset of the disturbance, **level of functioning** in one or more major areas, such as work, interpersonal relations, or self-care, **is markedly below** the level achieved **prior to the onset** (or when the onset is in childhood or adolescence, there is failure to achieve expected level of interpersonal, academic, or occupational functioning).

C. Continuous **signs of the disturbance** persist **for at least six months**. This 6-month period must include at least one month of symptoms (or less if successfully treated) that meet Criterion A (i.e., active-phase symptoms) and may include periods of prodromal or residual symptoms. During these prodromal or residual periods, the signs of the disturbance may be manifested by only negative symptoms, or two or more symptoms listed in Criterion A present in an attenuated form (e.g., odd beliefs, unusual perceptual experiences).

D. Schizoaffective disorder and depressive or bipolar disorder with psychotic features have been ruled out because either (1) no major depressive or manic episodes have occurred concurrently with the active-phase symptoms, or (2) if mood episodes have occurred during active-phase symptoms, they have been present for a minority of the total duration of the active and residual periods of the illness.

E. The **disturbance** is **not attributable to** the physiological **effects of a substance** (e.g., a drug of abuse, a medication) or another medical condition.

F. If there is a history of autism spectrum disorder or a communication disorder of childhood onset, the additional diagnosis of schizophrenia is made only if prominent delusions or hallucinations, in addition to the other required symptoms of schizophrenia, are also present for at least one month (or less if successfully treated).

(Specifications not included)

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3. The aetiological roots of schizophrenia

It is impossible to talk about the biology behind schizophrenia without talking about the brain, the organ that sustains it. Thus, the aetiological roots of the disorder are intimately related to the neurobiology underlying brain architecture and functioning.

What factors, then, underpin the neurobiology of proper brain functioning and influence the emergence of schizophrenia? Although the specific aetiological (the causes of the disorder) and pathophysiological (the biological mechanisms) actors are not fully understood, the prevailing hypothesis relies on a complex and multifactorial origin resulting from the interaction between environmental and genetic risk stimuli, which in an indissolubly way orchestrate brain development and maturation, and eventually modulate the world's perception and enable the adaptation to it (**Figure 4**) (Weinberger 1995; Owen et al. 2011; Birnbaum and Weinberger 2017).
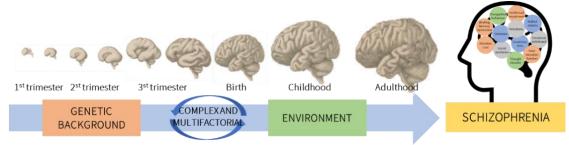
Figure 4. **The complex aetiology of schizophrenia.** Schizophrenia is a complex and multifactorial neuropsychiatric disorder that emerges from the interaction between environmental and genetic factors guiding the brain development and maturation. The figure representing the brain structural changes along the development was adapted (Silbereis et al. 2016).

Environmental factors

Several environmental influences impact on vulnerability windows along the life course and modulate an individual's likelihood towards the disorder (Stilo and Murray 2019). Notwithstanding, these determinants are not specific to schizophrenia, and there is no single or particular combination of environmental factors that inevitably causes the disorder. Instead, these same factors can also contribute to the emergence of other mental and neurodevelopmental disorders, among others (Schmitt et al. 2014a).

One of these windows of vulnerability happens even before birth. A significant proportion of schizophrenia's environmental factors occur during pregnancy and perinatal periods (Schmitt et al. 2014a, 2022). Obstetric complications arising at perinatal periods are among the most well-documented environmental risk factors for schizophrenia (Cannon et al. 2002b; Mittal et al. 2008; Belbasis et al. 2018; Radua et al. 2018; Schmitt et al. 2022) . These adverse events include foetal hypoxia, pregnancy bleeding, preeclampsia, diabetes, rhesus blood group incompatibility, and emergency

caesarean section. These events impact on child's development frequently leading to low birth weight and prematurity, which are also associated with schizophrenia risk (Cannon et al. 2002b; Abel et al. 2010; Schmitt et al. 2014a; Rubio-Abadal et al. 2015). Overall, obstetric complications are not only estimated to increase two times the odds ratio (OR) for schizophrenia-spectrum disorders (Belbasis et al. 2018) but also have been associated with cognitive deficits in patients (Amoretti et al. 2022).

Also, increased rates of childhood adversities, including physical or sexual abuse, emotional or psychological negligence during childhood, as well as stressful life events occurring in this period, such as parental death, social exclusion or bullying, have been reported in meta-analytic reviews to increase between 2.78 and 3.60 the OR for psychosis in adulthood (Varese et al. 2012; Matheson et al. 2013). Nonetheless, these factors do not solely increase the odds of psychosis; they are also linked to its severity. Severe forms of affective and positive symptomatology in adulthood, especially hallucinations, have been linked with childhood trauma (Matheson et al. 2013; Bentall et al. 2014; Longden et al. 2016).

Other events like migration, social isolation, urbanicity, and substance abuse, alone and in combination, influence an individual's likelihood towards the disorder (Stilo and Murray 2019). Through meta-analysis, migration has been strongly associated with an increased risk towards schizophrenia and other psychotic disorders, with a lasting effect transferred to generations (Bourque et al. 2011). An umbrella review (a meta-analysis of meta-analyses) assessing 170 factors for psychosis pointed out that the psychosis incidence rate among individuals of Black-Caribbean ethnicity in England was 4.87 times higher compared to individuals from the general population (Radua et al. 2018). This same study also described that belonging to an ethnic minority in a low ethnic-density area or belonging to second-generation immigrants significantly increased this risk (Radua et al. 2018). Likewise, an increasing number of results point towards the negative effect of the urban environments. For example, a meta-analysis on the impact of urbanicity reported that most dense environments has a 2.37 odds towards schizophrenia when compared to most rural areas (Vassos et al. 2012). Still, among the most consistently associated environmental risk factors for schizophrenia there is the use of cannabis. Umbrella meta-analytical evidence emphasise the robustness of the evidence and attribute the use of marihuana to increase 3.90 times the odds towards a schizophrenia-spectrum disorder (Arseneault et al. 2002; Marconi et al. 2016; Belbasis et al. 2018; di Forti et al. 2019).
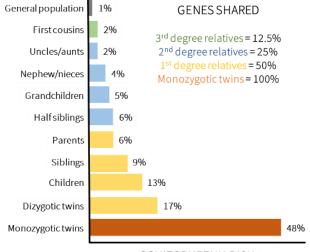
While the importance of environmental stressors should not be dismissed, their effects on the risk are also modulated by the individual's genetic background, resulting in gene and environment interactions. These interactions modulate the ontogenetic pathways, and insults from both origins bias the pre-established biological trajectories,

paving the way for the emergence of the disorder (van Os et al. 2008; Wahbeh and Avramopoulos 2021).

Genetic factors

As identified by family-based studies, schizophrenia runs in families, and what do families share apart from a common environment? The genes. The genetic determinants of schizophrenia are a major leading factor, as the psychiatric family history is the strongest predictor of mental illness (Sandstrom et al. 2020).

The schizophrenia rate is higher among relatives of patients with a diagnosis than among the general population (Henriksen et al. 2017), and this risk increases eightfold in the case of first-degree relatives of an affected person, including twins, siblings, offspring, and parents, compared to the general population, as estimated by a recent meta-analysis (LE et al. 2020). Moreover, as the proportion of shared genes increases (as quantified by the proportion of variants shared and genetic similarity), so does the risk for schizophrenia (Gottesman 1991). For example, while half-siblings have up to 3.60 increased risk, the full siblings have up to a 9.0 risk ratio (Lichtenstein et al. 2009) (**Figure 5**). This increased risk is not only specific to schizophrenia, but it also affects other psychiatric disorders, suggesting that the genetic risk breaks down diagnostic barriers (Smoller et al. 2013; Smoller 2013; Sandstrom et al. 2020).

Figure 5. **Schizophrenia risk among relatives.** As the number of shared genes increases, the liability for schizophrenia does so. Adapted figure (Gottesman 1991).

Likewise, twin and adoption studies have highlighted the paramount importance of the genetic component. There is a higher concordance rate in monozygotic twins (sharing almost 100% of the DNA) than in dizygotic twins (sharing 50% of the DNA on average). This means that among monozygotic twins, there are more sibling pairs where both individuals are affected (concordant for the disorder) when compared with dizygotic

twins (Gottesman 1991; Cardno and Gottesman 2000). Similarly, adoption studies indicate that those adoptees with a stronger schizophrenia genetic background (with biological progenitors affected by the disorder) not only present a higher risk towards schizophrenia-spectrum disorders but also are more sensitive to environmental problems in the adoptive family (Ingraham and Kety 2000; Tienari et al. 2004).

Notwithstanding, how much weight does genetics carry? Through twin studies, it has been possible to assess the heritability, the proportion of the overall phenotypic variance between individuals attributed to genetic factors. Indeed, the heritability for schizophrenia has been estimated to be up to 80% (Sullivan et al. 2003; Hilker et al. 2018), emphasising the enormous importance of genetics.

Molecular studies have revealed that schizophrenia, like other psychiatric disorders, has a highly polygenic architecture. This means that the genetic risk results from the accumulation of thousands of common alleles of minor effects such as single nucleotide polymorphisms (SNPs) or copy number polymorphisms (CNPs), as well as from few rare mutations with larger effects like single nucleotide variants (SNVs) and copy number variants (CNVs) (Bodmer and Bonilla 2008; Geschwind and Flint 2015; Foley et al. 2017; Legge et al. 2021) (see **Box 2**).

Seeking for the identification of genes and specific variants associated with the risk for schizophrenia, hypothesis-driven investigations focus on candidate genes for association analyses. These candidate genes are selected based on their role in specific biological and pathophysiological mechanisms underlying the disorder. Given the conception of schizophrenia as a disorder related to alterations in brain developmental trajectories based on pathophysiological data (Crabtree and Gogos 2014; Sigurdsson 2016; Dienel and Lewis 2019; Jaaro-Peled and Sawa 2020), many association studies on schizophrenia have been focused on genes related to these processes (i.e., *COMT, DISC1, DAOA, DRD1, DRD2, NRG1, DTNBP1, BDNF, MIR137*), and their implication on schizophrenia has been strengthened thanks to meta-analytic results (Li et al. 2006; Watanabe et al. 2013; Pan et al. 2014; González-Castro et al. 2016a, b; Jagannath et al. 2018; Mohameda et al. 2018; Liu et al. 2019).

Box 2. A glimpse into the genetic code and population genetics.

A typical human genome

The human genome is organised in 23 pairs of homolog chromosomes and contains approximately 20,000 genes. The **genetic alphabet** consists of four letters (nucleotides): A (adenine), C (cytosine), G (guanine) and T (thymine). These nucleotides pair together (A-T and C-G) to form the DNA double helix structure. In the human genome, these base pairs are **repeated 3,200 million times**.

Human inter-individual differences

Approximately **twenty million base pairs** (0.6% of the total) are estimated to **differ between individuals**. This is where our uniqueness lies, but also where our risk or protection towards illnesses arises.

The **genetic architecture** of an individual greatly **depends on** his or her **ancestry**, and the relative allele frequencies in a population depend on the ethnic origin. Thus, population-based genetic studies must be homogenous regarding ancestry, and the findings cannot be straightforwardly translated from one population to another. Given the population rate of the less frequent variant (allele), also referred to as minor allele frequency (MAF), the **genetic variants** are classified as **common** (MAF >1%), **uncommon** (MAF 0.1-1%), **rare** (MAF <0.1%), and **ultra-rare** (MAF <0.001%).

In terms of inheritance, not every base pair is independent of the other; there are certain genomic regions where the **recombination** occurs more frequently (recombination hotspots). The genome is divided into regions with certain **linkage disequilibrium (LD)**, which define the non-random association of alleles and can be understood as the probability of joint inheritance. **Alleles located within an LD block** will be transferred together to the descendants conforming to a **haplotype**.

Common (polymorphisms) and rare variants in a population may implicate as little as one base pair, like **single nucleotide polymorphisms (SNPs)** and single nucleotide variants, or extensive segments of thousand base pairs, like copy number polymorphisms and copy number variants (CVNs), which are deletions or duplications of genomic regions.

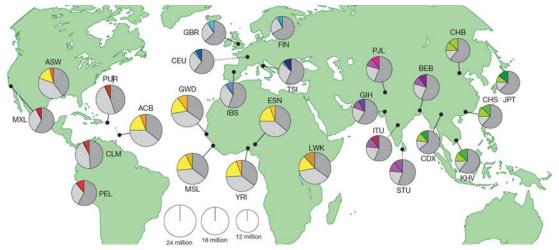Figure B2. Polymorphic variants within 1000 Genomes sampled populations. Each pie chart represents the proportion of polymorphisms within the population divided into four slices: the variants specific to the population (darker colour), specific to the continent (lighter colour), shared across the continent (light grey) and shared across all continents (dark grey). Adapted figure (Auton et al. 2015).

The association of candidate genes with a disorder is established based on the comparison of the frequency of common genetic variants across diagnostic groups through case-control or family-based approaches. Moreover, apart from assessing the modulation effect that genetic variants have on the risk, the same associations can be tested on intermediate phenotypes associated with the primary trait.

Intermediate phenotypes are heritable traits derived from quantifiable measures that appear in patients and unaffected relatives, though to a lesser extent (Gottesman and Gould 2003; Iacono 2018). In schizophrenia, there are multiple intermediate phenotypes are based on neuroanatomical measures (i.e. derived from anatomical structural imaging methods to assess grey/white matter densities, volumetric measures, cortical thickness, cortical surface area, or diffusion structural techniques), brain functional response (i.e. task associated or resting state functional imaging, functional connectivity or electroencephalography), neurocognitive performance (i.e. attention, working memory, executive function assessments), and metabolic imaging methods (i.e. positron emission tomography and magnetic resonance spectroscopy), among others (Keshavan et al. 2007; Allen et al. 2009; Kalkstein et al. 2010; Patel et al. 2010; Greenwood et al. 2011, 2016, 2019; Swerdlow et al. 2015; Cao et al. 2016; Owens et al. 2016; Birur et al. 2017). Among the intermediate phenotypes, cognitive dimensions deserve special attention. The heritability of general cognitive ability has been estimated around 70% in adulthood and the genetic effects across different cognitive and learning abilities present correlations higher than 0.60 (Tucker-Drob et al. 2013; Plomin and Deary 2015). Still, direct associations with candidate genes in these domains and cognitive alterations in schizophrenia have not always been straightforward (Rose and Donohoe 2013).

Instead, neuroimaging measures have more successfully uncovered genes and biological pathways associated with the disorder. The heritability of measures such as functional brain variability response has been reported to vary between 40 and 65%, while the heritability of cortical thickness and surface area are estimated to range between 52 and 78% (Jansen et al. 2015). Through neuroimaging techniques like functional magnetic resonance imaging (fMRI), the modulating role of genes consistently associated with schizophrenia, such as *IL1B*, *COMT*, *DISC1* and *BDNF*, has been assessed on the brain function underlying the disorder's cognitive alterations (Roffman et al. 2006; Pomarol-Clotet et al. 2010; Fatjó-Vilas et al. 2012; Duff et al. 2013; Vercammen et al. 2014; Notaras et al. 2015). Furthermore, the role of these genes in the pathophysiological roots of schizophrenia has been also strengthened by structural magnetic resonance imaging (sMRI) studies which have been associated with brain volumetric and cortical changes in the disorder (Duff et al. 2013; Notaras et al. 2015).

Therefore, while fMRI emerges as a brain-based powerful tool to assess the link between candidate genes and cognitive processes in schizophrenia (Meisenzahl and

Schlösser 2001), sMRI is also an asset for studying the genetic basis of brain architecture, which in turn sustains multiple aspects of higher-order cognitive function (Keshavan et al. 2020; McCutcheon et al. 2020; Holleran et al. 2020). Identifying the genetic basis of specific intermediate phenotypes may help classifying patients into more homogeneous subgroups, potentially improving treatment efficacy and long-term prognosis. Moreover, intermediate phenotypes may also help understanding the biology underlying schizophrenia will contribute to developing new genetically based therapeutic strategies (Gottesman and Gould 2003; Braff 2015; Greenwood et al. 2019).

Nonetheless, it has been through genome-wide association studies (GWAS) that capturing genetic variants associated with schizophrenia has turned more successful. GWAS systematically screen millions of common variants for association with a trait in the same way as candidate gene approaches but with a hypothesis-free model. However, a stringent significance threshold must be set to control for false positive findings, typically at $p<5x10^{-8}$. Thus, larger and larger sample sizes are needed to increase the study's statistical power to identify independent loci associated with the disorder. The Psychiatric Genomics Consortium has conducted four schizophrenia GWAS so far (Ripke et al. 2011, 2014; Pardiñas et al. 2018; Trubetskoy et al. 2022), with the latter identifying 287 independent loci in a sample composed of 76,755 patients and 243,649 controls.

Nevertheless, identifying specific genes associated with schizophrenia is not the unique objective of GWAS investigations. Subsequent findings on the convergence of the discovered genes in biological mechanisms and pathways point in the same direction as candidate gene studies initially did but from a hypothesis-blind starting point. The latest GWAS study reports that schizophrenia-associated genes are primarily expressed in excitatory and inhibitory neurons of the central nervous system. These genes also converge into biological processes and pathways related to development, neuronal differentiation, neuronal function, and synaptic transmission and involve cellular components like ion channels and synapses (Trubetskoy et al. 2022).

The genes identified by GWAS studies automatically become candidate genes and a starting point for further studying their implications in the underlying pathophysiological pathways. Examples of this include the *ZNF804A* and *CACNA1C* genes that, since their first association with schizophrenia through GWAS (O'Donovan et al. 2008; Ripke et al. 2013), numerous studies have advanced in their functional characterisation. The results have shown their key roles in neurodevelopmental transcriptomic regulation and synaptic plasticity mechanisms, as well as their implication in functional connectivity alterations and cognitive performance (Esslinger et al. 2011; Bhat et al. 2012; Chang et al. 2017). Therefore, from more basic approaches that allow us to identify the specific mechanisms by which these genes exert their function to approaches focused on their modulatory role in other phenotypes associated with the disorder, such as clinical, cognitive or

neuroimaging ones, candidate gene approaches allow disentangling their role in the biology of schizophrenia.

Still, is this common variability enough to explain the whole genomic picture? Although it has been suggested that the SNP-based heritability values might be considerably underestimated (Grotzinger et al. 2023), the latest GWAS sets it around 24%, which represents the proportion of variance in schizophrenia liability attributable to all measured SNPs (Trubetskoy et al. 2022). Thus, we are still left with the so-called missing heritability, which refers to the discrepancy between the amount of variation in a trait explained by common genetic variants and the amount of variation observed in the population and quantified by twin-based heritability studies. This missingness is explained by the fact that SNP-based heritability does not account for other polymorphic or rare variants not included in the GWAS, and because schizophrenia's polygenicity is furtherly complicated by the interplay between the underlying genes with each other, through epistasis, and their interactions with the environment. Thus, a much lower estimate compared to twin-based heritability is expected.

With this complex and polygenic picture, we must emphasise that most schizophrenia-associated genetic variants are common in the population. Therefore, each of us carries a certain genetic risk for the disorder. In this sense, the polygenic risk score (PRS) is a powerful tool to estimate each person's genomic burden of risk (see **Box 3**). More precisely, the PRSs quantify the individual genetic liability for a trait considering the additive effect of each genotyped variant weighted by GWAS estimated effect sizes. Thus, while PRS for schizophrenia should not be considered a diagnostic tool, it is a highly informative measure to assess the individual risk for the disorder at a research-level and has proven to be highly consistent across studies and samples (Raben et al. 2022). Also, apart from quantifying the liability for schizophrenia, a higher schizophrenia PRS has been associated with lower cognitive performance, working memory, processing speed, functional alterations and reduced synaptic plasticity in response to working-memory tasks and with cortical thickness changes in individuals at high-risk for psychosis, patients with the disorder and healthy individuals (Lencz et al. 2014; Miller et al. 2018; He et al. 2021; Cattarinussi et al. 2022; Ohi et al. 2022; Zhao et al. 2022). Another advantage of PRSs analyses is that they can be estimated based on genome-wide variability but also with subsets of SNPs defined based on their involvement in particular biological pathways of interest.

Further complicating this picture, we have epigenetic mechanisms and transcriptional and post-transcriptional regulation on the path from genotype to phenotype. From the DNA transcription to the protein translation, these processes underlie the cellular diversity found in terms of function and structure despite the homogeneity in the genetic code (Jaenisch and Bird 2003; Franks et al. 2017). These levels

of regulation include expression machinery control through DNA methylation and histone modifications that affect chromatin accessibility, but also gene expression regulatory process guiding RNA splicing, processing, and degradation (Gavin and Akbarian 2012). The importance of epigenetic and transcriptional alterations in the onset of schizophrenia has been outlined by studies describing changes in blood-based methylomic and transcriptomic signatures in individuals at high risk for psychosis between those transitioning to psychosis and those not (Kebir et al. 2017; Chaumette et al. 2019). While genetic sequence changes may also influence transcriptional and post-transcriptional regulation, beyond these, there are multiple epigenetic and post-transcriptional modifications associated with schizophrenia (Gavin and Akbarian 2012; Akbarian 2014; Smigielski et al. 2020; Srivastava et al. 2021). First, DNA methylation changes in genes from the dopaminergic, serotoninergic and glutamatergic systems have been associated with the disorder's proneness (Abdolmaleky et al. 2011, 2014; Carrard et al. 2011; Kordi-Tamandani et al. 2013; Gao et al. 2017). Also, methylome-wide association studies have been helpful for the identification of methylation marks associated with the disorder related to glutamatergic and GABAergic neurotransmission and brain development (Mill et al. 2008; Montano et al. 2016). Second, histone activity changes have been described in patients compared to controls (Sharma et al. 2008; Pang et al. 2016). Third, non-coding RNAs, which have functional roles in gene expression regulation, have been outlined in the transcriptional regulation machinery of neurogenesis and synaptic plasticity in schizophrenia (Gibbons et al. 2018; Wu et al. 2022).
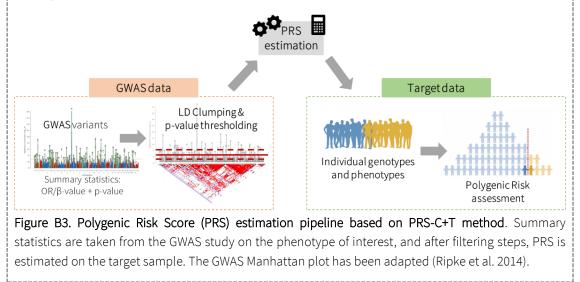
Equally to what happens with environmental risk factors, there is no genetic factor indisputably specific to schizophrenia (Lee et al. 2013; Anttila et al. 2018; Gudmundsson et al. 2019; Smeland et al. 2020a). Then, the risk of developing this disorder does not arise only based on one genetic or environmental trigger. Instead, susceptibility genes and environmental factors are considered to be associated with minor neural function deviations that are majorly harmless by themselves. It is the cumulative effect of adverse environmental events and common and rare genetic factors that, interacting together, push the individual threshold towards the expression of psychosis and towards a clinical diagnosis (McGue et al. 1983; Kelly and Murray 2000).

Box 3. Estimation of the polygenic risk score (PRS).

The Polygenic Risk Score or **PRS** is a numerical value summarising **a person's polygenic background for a particular disease, disorder, or trait**. PRS estimation is based on the combined effect of multiple and common genetic variants, usually, SNPs, pondered by their associated weights or effect sizes to a given phenotype. Schizophrenia PRS estimations have shown high consistency across different studies and samples. Thus, it is considered a highly informative measure at the research level since higher schizophrenia PRSs are associated with increased rates of schizophrenia incidence compared to the population average (Raben et al. 2022).

While there are several **methods for PRS estimation** (Ni et al. 2021), all of them **require** two input data sets: i) a base data consisting of the **GWAS summary statistics** (like betas, ORs and p-values) of genotype-phenotype associations at genetic variants; and ii) a target data with the **genotypes and phenotypes in** individuals of **an independent sample** (Choi et al. 2020). A classic PRS estimation method is the PRS-C+T (Privé et al. 2019), defined as the sum of allele counts, weighted by the effect sizes obtained from the GWAS data, after two filtering steps. The first step is the LD clumping (to select the most significant SNP from any pain of SNPs in LD based on a reference population), and the second, the p-value thresholding (to establish which p-value threshold better predicts the diagnostic status in the target sample). With the resulting PRS estimates for the target individuals, it is possible to assess PRS differences across diagnostic status, but also the PRS modulatory effect on other phenotypic measures (i.e., clinical, neurocognitive or neuroimaging phenotypes). It is important to note that two individuals with the same high PRS, meaning a high genetic risk for schizophrenia, may affect different biological pathways. Interestingly, apart from whole-genome PRS estimations, biologically informative assessments can be made based on specific subsets of SNPs/genes defined by their involvement in relevant biological functions or pathways.

The **predictive** ability of **PRS depends on the** power of the **GWAS** from which PRS has been derived. Conversely to the clinically meaningful results that PRS tools represent in other medical conditions in terms of the prediction accuracy in the general population (Mavaddat et al. 2019; Mars et al. 2020; Ferrat et al. 2020; Wand et al. 2021), in psychiatric disorders, **PRS methods do not yet provide clinically actionable information** (Murray et al. 2021; Lewis et al. 2021). Nonetheless, they may be currently used for clinical populations (Perkins et al. 2020; Davies et al. 2020) and their precision will improve in the coming years (Palk et al. 2019).

Figure B3. Polygenic Risk Score (PRS) estimation pipeline based on PRS-C+T method. Summary statistics are taken from the GWAS study on the phenotype of interest, and after filtering steps, PRS is estimated on the target sample. The GWAS Manhattan plot has been adapted (Ripke et al. 2014).

4. Pathophysiological alterations in schizophrenia

Synapse and circuit maturation is thought to anchor the cortical functions (Forsyth and Lewis 2017). Among the different hypotheses aiming to explain the core pathophysiological alterations in schizophrenia, it has been suggested that the dysfunctions arise from an impaired control of synaptic plasticity (Friston 1998; Gordon Frankle et al. 2003; Lewis et al. 2005, 2012; Stephan et al. 2006; Marín 2012; Osimo et al. 2019).

Synaptic plasticity can be defined as the changes in the strength of neuronal connections at synapses that sustain neurons' communication. This process of synaptic connections remodelling is widely accepted as the biological basis of learning and memory, and it is also of paramount importance for the establishment of functional neuronal circuits during neurodevelopment (Citri and Malenka 2008; Caroni et al. 2012; Forsyth and Lewis 2017; Magee and Grienberger 2020). Synaptic plasticity mechanisms mainly rely on neuronal excitability, which is the propensity of a neuron or a neural circuit to respond to stimulus and produce an output signal. This signal is in the form of an action potential, a transient change of electrical charge of the neuronal membrane, and it requires opening voltage-gated ion channels for synapse activation (Daoudal and Debanne 2003). When the excitation/inhibition homeostasis is imbalanced, it results in the alteration of neural communication and circuitry impairments. Therefore, synaptic alterations are suggested to participate in the brain dysfunctions that lead to the development of symptoms and cognitive deficits of schizophrenia and other neurodevelopmental disorders (Insel 2010; Lewis et al. 2012; Howes and Murray 2014; Foss-Feig et al. 2017; McCutcheon et al. 2020).

Histological findings, which describe reductions in dendritic arborisation, dendritic spine density and synaptic markers in the brains of patients when compared to controls (Glantz and Lewis 2000; Black et al. 2004; Law et al. 2004), support the implication of synaptic abnormalities. Synaptic defects can result from excessive spine pruning during late childhood and adolescence (Feinberg 1982; Laskaris et al. 2016; Sakai 2020). While the molecular and cellular basis of this excessive pruning is still under study, *in vivo* and *in vitro* evidence suggests the involvement of inflammation and alterations in microglia, the immune cells in the central nervous system. Different events such as immune activation, traumatic stimuli or psychosocial stress have been associated with microglia activation, which in turn has been implicated in synaptic pruning (Paolicelli et al. 2011). Thus, microglia dysfunction in schizophrenia would contribute to pruning alterations and reduced dendritic spine densities associated with the disorder (Howes and McCutcheon 2017; Wolf et al. 2017; Selvaraj et al. 2018; Sellgren et al. 2019; Marques et al. 2019).

The synaptic plasticity dysfunction can be understood from the point of view of neurotransmission imbalances, neuroanatomical and neurofunctional alterations, and neurocognitive deficits which have been extensively reported in the disorder.

Alterations in synaptic plasticity would be closely related to pathophysiological mechanisms associated with neurotransmission dysregulations. On the one hand, many lines of evidence indicate that dopamine dysfunction is a common pathway to psychosis. The primary evidence is related to the fact that all current pharmacological treatments impact the same biological mechanism, i.e. the blockage of dopamine D2 receptors (Johnstone et al. 1978; McCutcheon et al. 2020). Convergently, other dopamine-releasing drugs, such as amphetamines, induce psychotic symptoms in healthy volunteers and worsen symptoms in patients with schizophrenia (Abi-Dargham et al. 1998; Li et al. 2014; Harro 2015). Based on this evidence, it has been proposed that multiple insults interact in the brain to alter dopaminergic pathways and that dopamine dysregulation may be a common denominator in the disorder (Howes and Kapur 2009; McCutcheon et al. 2018). Molecular brain imaging studies (i.e. using positron emission and single photon emission computed tomography) have described higher dopamine synthesis capacity in individuals at ultra-high risk for psychosis and those with an established diagnosis (Lindström et al. 1999; Howes et al. 2009; Egerton et al. 2013; McCutcheon et al. 2018). In line, post-mortem studies described higher dopamine levels and associated receptors in patients with the disorder (Lee and Seeman 1980; Seeman and Kapur 2000). Additionally, several lines of evidence suggest that genetic and environmental factors impact the dopaminergic system rendering it it vulnerable to stress. This dopaminergic dysfunction will eventually lead to an altered cortical function, and to the disorder's emergence, which may be associated with striatal dopamine hyperactivity (Howes et al. 2017).

On the other hand, apart from dopaminergic alterations, dysfunctions in the glutamatergic system have been also described. Glutamate is the most abundant excitatory neurotransmitter in the brain and was related to schizophrenia through the effect that ketamine and phencyclidine, initially used as anaesthetics, had on the development of psychotic, negative and cognitive symptoms in healthy subjects. The effects of ketamine and phencyclidine on the brain were mediated by their antagonistic role in NMDA glutamate receptors (Javitt and Zukin 1991; Krystal et al. 1994; Moghaddam and Krystal 2012; Uno and Coyle 2019). Later, many other sources of evidence from clinical, neuroimaging and post-mortem studies have provided evidence on NMDA hypofunction and glutamatergic neurotransmission abnormalities in the pathophysiology of schizophrenia (Egerton et al. 2020). Even though these are the neurotransmitter systems with the strongest evidence in schizophrenia, other neurotransmitter types, including endocannabinoid and serotonin systems, have been associated with the disorder (Stahl 2018; Jauhar et al. 2022; Durieux et al. 2022).

Apart from neurotransmission alterations, structural imaging studies, which have implicated alterations in white matter tracts, have furtherly supported synaptic abnormalities (Ellison-Wright and Bullmore 2009; Xie et al. 2022). Meta-analyses also point to global grey matter reductions in the hippocampus, prefrontal and temporal cortices, as well as in the insula, thalamic and striatal areas (Wright et al. 2000; Heckers 2001; Davidson and Heinrichs 2003; Fornito et al. 2009; Sheffield and Barch 2016). Additionally, brain volume decreases, and widespread cortical thinning and surface area reductions have been also reported in patients, with the most significant effect sizes in frontal and temporal regions (Haijma et al. 2013; van Erp et al. 2016, 2018).

These grey and white matter alterations, in turn, partially sustain impairments in neural communication and function (Keshavan et al. 2007, 2020; McCutcheon et al. 2020). Thus, synaptic plasticity deviations are also consistent with evidence from electrophysiology and magnetic resonance imaging pointing to the role of altered neuronal excitability in some of the clinical and cognitive features of the disorder (Stephan et al. 2006; Uhlhaas and Singer 2010). Electrophysiological and fMRI studies have consistently described disrupted neural activity synchronisation in first episode and chronic patients (Uhlhaas and Singer 2010; Sheffield and Barch 2016). Functional alterations frequently reported include connectivity dysfunctions within brain networks such as the default-mode, the ventral attentional, the frontoparietal and the somatomotor networks (Stephan et al. 2006; Dong et al. 2018), which play essential roles in higher-order brain functions (Perlstein et al. 2001; Buckner et al. 2008; Raichle 2015).

Synaptic changes and neurofunctional abnormalities likely underlie the cognitive impairments that patients with schizophrenia experience, which in fact seem to be mediated by neural excitability imbalances. Indeed, the cognitive alterations in schizophrenia involve a wide range of domains, such as executive function, attention, learning, and memory, which in turn are related to the loss of excitation and inhibition homeostasis (Keefe and Fenton 2007; Uhlhaas and Singer 2010; Lisman 2012; Kahn and Keefe 2013; Lepage et al. 2014; Sutcliffe et al. 2016; Mould et al. 2021) (see **Box 4**).

Further data from genetic association studies, focused on genes implicated in synaptic plasticity and neural excitability (Berger and Bartsch 2014; Bauer and Schwarz 2018; Zhou et al. 2018; Tropea et al. 2018) have highlighted the involvement of genes such as *ZNF804A, CACNA1C*, *KCNH2* or *DISC1* in the susceptibility for schizophrenia (Hashimoto et al. 2013; Zhou et al. 2018; Ma et al. 2018; Liu et al. 2020) (see **Box 5**).

Box 4. Assessing working-memory through functional magnetic resonance imaging

Working memory is the ability to **hold and manipulate information** to guide **goal-directed** behaviours (Baddeley 1992). Impairments in this domain are considered central (Lett et al. 2014), and its impairment has been shown to significantly limit the global every-day functioning of patients (Lepage et al. 2014). Moreover, it is significantly determined by genetic factors. Its heritability is estimated to range between 43 and 49%, patients with schizophrenia and affected siblings present impairments relative to healthy individuals, and several genomic loci have been associated with this trait at the genome-wide level (Pirkola et al. 2005; Greenwood et al. 2007, 2019).

Brain functional magnetic resonance imaging (**fMRI**) **allows**, in a non-invasive way, to **study** the **functional characteristics of the brain** and is been widely **used to assess** brain **activity changes in response to cognitive tasks**. In fMRI, the physiological response of neurons is measured through the blood oxygen-dependent (BOLD) signal, which is a proxy of neuronal activity.

By means of **working memory fMRI tasks** several **functional alterations**, latter replicated though other functional paradigms, **have been described in schizophrenia**. For example, it has been extensively documented activation increases in frontal and fronto-lateral regions and also failures in deactivation in the medial prefrontal cortex in patients when compared to controls (Meyer-Lindenberg et al. 2001; Callicott et al. 2003; Pomarol-Clotet et al. 2008; Minzenberg et al. 2009; Whitfield-Gabrieli et al. 2009; Mannell et al. 2010; Schneider et al. 2011; Landin-Romero et al. 2015; Haatveit et al. 2016).

Among the typical **working memory** fMRI **paradigms**, there is the **N-back task**. This task engages storage and executive process related to attention and working memory and can be structured in **different difficulty levels** requiring increases in memory load. Depending on the difficulty and the specific cognitive requirements, different fronto-parietal regions may be relevant for task performance. It has been reported that at lower difficulty levels **frontally-centered networks** are **relevant for the attention-dependant task** performance, while at higher memory load requirements, **frontal areas together with parietally-centred networks** are engaged for the **working memory** performance (Owen et al. 2005; Egli et al. 2018).
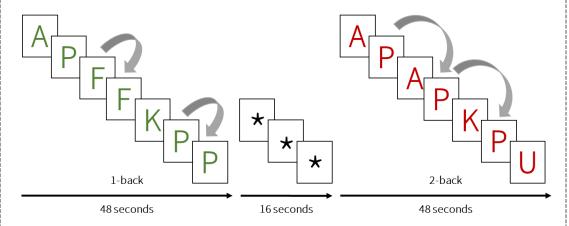
Figure B4. Representation of the sequential-letter version of the N-back task used in the fMRI paradigm of this thesis. The objective of the task is to indicate the letter repetitions. There are two memory load levels: the 1-back (most accessible level, in green) and the 2-back (most challenging level, in red), presented in a blocked design manner. The whole task consists of four blocks for each level presented in an interleaved way and separated by a baseline stimulus (asterisk), each containing five letter repetitions randomly located.

Moreover, the combination of the outputs from large-scale genomic studies on common and rare variants associated with schizophrenia has consistently reported an enrichment in processes related to synaptic organization and function (Pardiñas et al. 2018; Hall and Bray 2022; Singh et al. 2022; Trubetskoy et al. 2022). Similarly, PRS approaches focused on specific gene sets related to biological pathways of interest also outlined the importance of neural function. Several studies have described that schizophrenia polygenic load on axon and synapse-related genes, as well as dopaminergic- and glutamatergic-related gene sets influenced brain structure, working memory, attention and the associated brain functional responses in healthy controls (Rampino et al. 2017; Wang et al. 2018; Barbu et al. 2022; Pergola et al. 2022).

Box 5. List of candidate genes studied in this thesis.

Potassium voltage-gated channel subfamily H member 2 gene or *KCNH2* (7q36.1) encodes for the α pore-subunit of the human ether-à-go-go (hERG) voltage-gated potassium channel. It is expressed in excitatory cells and modulates neuronal firing patterns (Bauer and Schwarz 2018) *KCNH2* genetic variability has been associated with increased mRNA levels of the primate-specific KCNH2-3.1 isoform, with specific electrophysiological properties, in the brain (Huffaker et al. 2009). Furthermore, increases in the KCNH2-3.1 isoform expression cause alterations in dendritic spines, cognitive impairments, and changes in neuronal function in mice models (Carr et al. 2016).

Disrupted in schizophrenia 1 gene or *DISC1* (1q42.2) encodes for DISC1 protein that participates in the neurodevelopment, neurosigniling, and functional regulation of synaptic plasticity (Jaaro-Peled et al. 2009; Thomson et al. 2013; Tomoda et al. 2017). The protein directly interacts with the dopamine D2 receptor; thus, functional changes in the gene and/or protein can interfere with the dopaminergic signalling pathway (Su et al. 2014). It was first associated with schizophrenia through linkage studies and identified when a balanced chromosomal translocation was found to segregate with major mental disorders (St Clair et al. 1990). Its genetic variability underpins the variance in cognitive domains such as attention and working memory (Vázquez-Bourgon et al. 2015; Teng et al. 2018).

Zinc finger protein 804A gene or *ZNF804A* (2q32.1) possibly encodes for a transcription factor expressed in the foetal and adult brain that participates in the gene-expression regulatory machinery of synaptic plasticity genes and is implicated in mRNA processing and RNA translation (Hill and Bray 2012; Deans et al. 2017; Zhou et al. 2018). Genetic changes at *ZNF804A* seem related to expression changes in the prenatal and adult brain (Hill and Bray 2012). In addition to its association with schizophrenia risk by GWAS studies (O'Donovan et al. 2008), this gene has been associated with memory, higher schizotypy scores, a schizophrenia risk factor, in the general population (Hashimoto et al. 2010; Soler et al. 2019; Meller et al. 2019).

Calcium voltage-gated channel subunit α-1C gene or *CACNA1C* (12p13.33) encodes for the α subunit of the L-type calcium channel. This channel regulates the calcium influx into the cell and represents the main calcium channel in the brain with critical roles in maintaining synaptic plasticity homeostasis (Striessnig et al. 2014). Besides its association with schizophrenia through GWAS (Ripke et al. 2013), *CACNA1C* genetic variability modulates the channel expression and density in the brain, modulating neural excitability mechanisms (Yoshimizu et al. 2015), which may underly its association with cognitive performance variability (Hori et al. 2012; Cosgrove et al. 2017).

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5. Neurodevelopmental and evolutionary origin of schizophrenia

Overall, considering evidence from environmental, genetic, and pathophysiological roots of schizophrenia demarcate a pathway intimately linked to how our brain develops. The ontogenetic plan guides neurodevelopment to ensure that the brain appropriately responds to stimuli. Consequently, alterations in this plan cause neurodevelopmental deviations, neuronal dysfunctions and neurotransmission alterations that lead to the emergence of the disorder later in life (Eastwood 2004; Forsyth and Lewis 2017; Birnbaum and Weinberger 2017; Jaaro-Peled and Sawa 2020; Hall and Bray 2022).

The early neurodevelopmental roots

The neurodevelopmental hypothesis of schizophrenia states that the emergence of the disorder during adolescence or early adulthood results from deviations of the neurodevelopmental homeostasis, which cause altered neuronal architecture and neurotransmission and synaptic dysfunctions later in life. These deviations would be caused by genetic and environmental risk factors impacting during vulnerability windows along the course of the neurodevelopment and even occurring in very early prenatal periods (Weinberger 1995; Rapoport et al. 2005; Birnbaum and Weinberger 2017; Jaaro-Peled and Sawa 2020; Schmitt et al. 2022) (**Figure 6**).
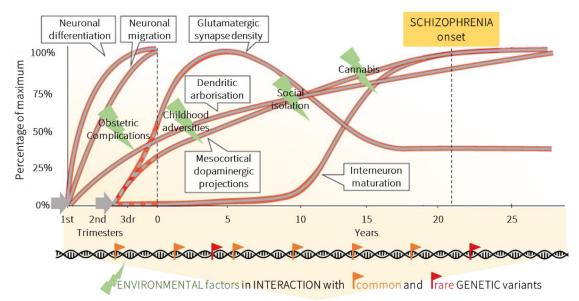

Figure 6. **The neurodevelopmental hypothesis in schizophrenia.** The neurodevelopmental trajectories tightly regulated are disrupted and deviated by the interaction of environmental and genetic risk factors and underlie the emergence of the disorder later in life. Adapted figure (Birnbaum and Weinberger 2017).

This hypothesis is widely supported by multidisciplinary evidence. On the one hand, considering the previously mentioned environmental risk factors, results from population-based studies point to stressful events and obstetric complications during prenatal and perinatal periods as more prevalent among people who later develop schizophrenia (Rapoport et al. 2005). Moreover, those individuals suffering obstetric adversities and having a delay in the achievement of developmental milestones present an increased risk for schizophrenia-spectrum disorders in adulthood (Clarke et al. 2011). In line, children who later develop schizophrenia have early developmental and educational and social impairments, signs that, in addition, contribute to the prediction of psychotic symptoms in childhood and adulthood (Cannon et al. 2002a; Woodberry et al. 2008; Sørensen et al. 2010).

Furthermore, there is higher co-occurrence of non-specific neurologic soft signs and minor physical anomalies in patients with schizophrenia (Bramon et al. 2005; Mittal et al. 2008; Chan et al. 2018). Also, structural and functional neuroimaging alterations observed in high risk individuals before the onset of the disorder were higher in subjects that later developed psychosis. Such alterations include grey matter reductions, disrupted white matter integrity, cortical surface area decreases, widespread cortical thinning, ventricular enlargement and functional impairments (Pantelis et al. 2003; Harrison et al. 2003; Mechelli et al. 2011; Haijma et al. 2013; Dietsche et al. 2017; Niendam et al. 2018; Fortea et al. 2021, 2023; Jalbrzikowski et al. 2021).

On the other hand, from the molecular point of view, many genes associated with the disorder from linkage, candidate and genome-wide association studies converge into neurodevelopmental pathways and processes (Fatemi and Folsom 2009; Rees et al. 2020; Singh et al. 2022; Trubetskoy et al. 2022). In addition, among the biological markers that reflect the occurrence of disrupted prenatal neurodevelopmental trajectories in schizophrenia, there are also expression changes in proteins related to the early migration of neurons and glia, cell proliferation, axonal outgrowth, and synaptogenesis (Fatemi and Folsom 2009). Likewise, transcriptomic and epigenetic analyses in post-mortem brain tissues have provided evidence that specific mechanisms during early brain development are also implicated in schizophrenia risk, further supporting the neurodevelopmental theory (Tebbenkamp et al. 2014).

The evolutionary roots

As previously highlighted, the onset in late adolescence or early adulthood and the impairments associated with the condition lead schizophrenia to represent a handicap to reproductive success (fitness), especially for males. Such statement is based on studies assessing the fertility of individuals with psychosis, showing that patients have fewer offspring than unaffected siblings and the general population. Interestingly, when inspecting the differences in offspring numbers separately in males and females, different tendencies were reported. Uniquely among affected males the number of descendants was significantly lower as compared to male siblings, who in turn also had fewer offspring compared to the general population. (McGrath et al. 1999; Haukka et al. 2003; Power et al. 2013). These results are consistent with findings based on schizophrenia PRSs in the general population, describing that higher a schizophrenia liability is associated with being childless in males (Escott-Price et al. 2019).

Considering the evolutionary principles: genetic changes or alleles are introduced in the genetic pool by mutation, persist due to random genetic drift or due to positive selection when advantageous (conferring higher fitness) and are negatively selected and eventually eliminated when not (are associated with low fitness) (Pritchard and Cox 2002; Kondrashov et al. 2004; Kryukov et al. 2007; Blekhman et al. 2008). The same applies to genetic diseases. Still, schizophrenia is relatively common and ubiquitous across countries, indicating that its genetic load persists in our genetic pool.

Different mechanisms, not mutually exclusive, have been proposed to explain why schizophrenia risk variants remain at common frequencies. Mechanisms proposed include processes such as balancing selection (where schizophrenia alleles would be maintained because of different selection pressures such as antagonistic pleiotropy, where the effect of a genetic variant is associated with advantageous and disadvantageous traits); fitness trade-offs (where schizophrenia would be the extreme of normal variation in cognitive abilities); sexual selection (where schizophrenia genetic load would be maintained in the population by non-affected individuals that compensate the fitness reduction from affected patients); mutation-selection balance (when schizophrenia damaging mutations would be removed but novel mutations would occur all the time); and background selection (where negative selection against highly deleterious variants would produce a diversity reduction and the appearance by drift of alleles with small deleterious effect) (Keller and Miller 2006; van Dongen and Boomsma 2013; Keller 2018; Pardiñas et al. 2018). Still, the common denominator in many of these theories is that the disorder emerged as a costly by-product of the evolution of the ontogenetic mechanisms underlying human-specific neurodevelopment able to sustain complex cognitive abilities that distinguish humans, such as cognition, language, social functioning, and creativity (Crow 2000, 2008; Brüne 2004; Burns 2004, 2006b, a; Wynn and Coolidge 2011).

There are multiple sources of evidence supporting this view. First, schizophrenia has not been identified in non-human species. Second, many of the features of the disorder involve higher-order cognitive processes, such as cognitive impairment, delusions and hallucinations that converge with characteristic human traits (Polimeni and Reiss 2003; Burns 2006b, a; Preuss 2017). Third, the neural circuits sustaining some of the previously cognitive domains, such as the fronto-parietal and the default-mode networks, are among the networks that suffered the largest expansion in humans as compared to our closest relatives, the chimpanzees (Crespi et al. 2007; Wei et al. 2019). Additionally, these networks are among the neural circuits more affected by functional and structural changes in schizophrenia (Buckner et al. 2008; Rotarska-Jagiela et al. 2010; Chang et al. 2014; Fox et al. 2017). Lastly, several genes with essential roles in brain development and associated with schizophrenia show signs of positive selection in humans, including *DISC1*, dystrobrevin binding protein 1 (*DTNBP1*) and neuregulin 1 (*NRG1*) (Crespi et al. 2007), and polygenic data seem to indicate that language, creativity and psychopathology have a shared genetic background (Power et al. 2015; Rajagopal et al. 2023).

However, when in the evolution has schizophrenia's genetic basis emerged. Genetic association studies in African, Asian, and European populations point towards the same schizophrenia susceptibility genes and affected pathways across different ethnic groups (Lam et al. 2019; Gulsuner et al. 2020; Trubetskoy et al. 2022). These commonalities imply that the genetic variability underlying the disorder must be shared in populations that diverged between 50,000 and 100,000 years, suggesting that schizophrenia's susceptibility should have preceded the split of these human populations and could be associated with changes that determine the human condition.

While the evolutionary signatures of schizophrenia are hard to decipher, studying human-specific genomic changes may lead to a better comprehension of human-specific phenotypic traits (O'Bleness et al. 2012). Furthermore, as more reference genomes are available, evolutionary, and comparative genomics fields can advance and identify genomic regions under different evolutionary forces and regions harbouring highly divergent genomic loci across closely related species. In this sense, regions under positive selection in humans after chimpanzee divergence, like Human Accelerated Regions (HARs) (see **Box 6**) (Pollard et al. 2005; Prabhakar et al. 2006; Bird et al. 2007; Bush and Lahn 2008; Lindblad-Toh et al. 2011; Gittelman et al. 2015), or after neanderthal divergence, like the Neanderthal Selective Sweep (NSS regions) (Burbano et al. 2010; Green et al. 2010b) have been described. Accordingly, in the last ten years, multiple studies have taken advantage of the GWAS data to inspect the evolutionary signatures of schizophrenia to indicate that the susceptibility variants for the disorder would be enriched in these NSS regions and HARs, emphasising the associations between human-

specific genomic changes after neanderthal and chimpanzee divergence with schizophrenia polygenicity (Xu et al. 2015; Srinivasan et al. 2017).

Box 6. Human Accelerated Regions in neurodevelopment and schizophrenia

Human Accelerated Regions (**HARs**) are **evolutionarily conserved genomic elements** that have **evolved rapidly in humans** since the divergence from the human-chimpanzee ancestor. The accelerated divergence of HARs between humans is suggested to reflect their role in human evolution and their **association with some human-specific traits**, such as neurodevelopment mechanisms and outcomes (Pollard et al. 2006; McLean et al. 2011; Somel et al. 2013).

Throughout human evolution, these regions have accumulated human-specific variability, notwithstanding currently HARs are under a strong genetic constraint and are depleted of rare variants suggesting that after the accelerated evolution along human evolution, they have suffered a switch back to negative selection (Burbano et al. 2012; Doan et al. 2016).

From a functional point of view, HARs do not code for proteins and are predominately located in intergenic regions or within introns near protein-coding genes, transcription factors and DNA-binding proteins (Pollard et al. 2006; Capra et al. 2013; Hubisz and Pollard 2014; Doan et al. 2016; Won et al. 2019). Studies characterising their **function** point towards their role **as developmental enhancers**, some **with human-specific activity** (Doan et al. 2016; Won et al. 2019; Uebbing et al. 2021; Girskis et al. 2021).

Additionally, there is converging data showing the relationship between HARs cortical expression trajectories and **human-specific cortical expansion**, human-specific **brain functional connectivity** and the brain's **neural information processing** (van den Heuvel et al. 2019; Wei et al. 2019; Li et al. 2021; Luppi et al. 2022). As well these evidences are accompanied by results showing that HARs' genetic variability influences the **risk for schizophrenia** (Xu et al. 2015; Srinivasan et al. 2017; Bhattacharyya et al. 2021; Erady et al. 2022).
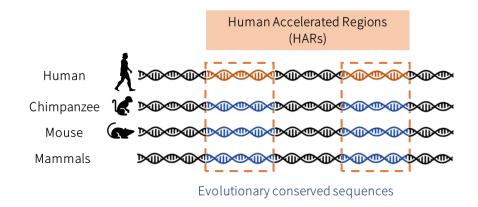

Figure B5. Schematic representations of Human Accelerated Regions. HARs are genomic sequences highly conserved along mammal evolution (represented in blue), that accumulated a higher number of human-specific substitutions after human and chimpanzee divergence (represented in orange).

Hypotheses & Objectives

The varying forms of brain functioning, cognition and behaviour observed in human populations, as well as the heterogenous presentation of psychotic disorders such as schizophrenia, reflect the complex underlying genetic and biological architecture of the human brain. The biological foundations of the disorder involve hundreds of genetic variants interacting with each other and with environmental influences to modify neurodevelopmental trajectories. Then, early neurodevelopment deviances affecting synaptic function led to eventual brain functional and organisational disruptions that sustain the emergence of schizophrenia symptomatology later in life.

The characterisation of these genetic influences and the dissection of the underlying biological causative determinants of schizophrenia are challenges still to be reached. Pursuing these goals may pave the way towards the development of better diagnostic strategies, designs and implementation of novel therapeutic interventions, thereby improving the lives of individuals and families affected by schizophrenia.

This thesis pursues a better understanding of the complex aetiology of schizophrenia by the identification of the biological and evolutionary mechanisms that impact the neural configuration and synaptic plasticity and contribute to the differences in brain function and anatomy observed between healthy subjects and patients with the disorder. Considering the affected neurodevelopmental and synaptic plasticity homeostasis identified in schizophrenia, which in turn influences the risk for the disorder and its specific manifestations, the strategies followed in this thesis have been:

i) the implementation of **neuroimaging association analyses** with a dual approach on **candidate genes** and **whole-genome variability on informative gene sets** to characterise the brain neurofunctional, cognitive and neuroanatomical alterations underlying schizophrenia

ii) the use of **intermediate phenotypes** interrelated with schizophrenia, such **as brain function, cognition, and brain anatomy,** to potentiate the identification of the underlying genetic factors.

Hypotheses

Therefore, we hypothesised that the genetic variability in genes and regions involved in neurodevelopmental and synaptic plasticity mechanisms would underlie the brain functional and anatomical differences observed in affected individuals. From this general hypothesis, two specific hypotheses have been derived:

1. The **genetic variability** at synaptic plasticity-related genes **will sustain**, to some extent, the differences between patients with **schizophrenia** and healthy individuals at the **brain functional** level in **response** to **higher-order cognitive processes**.

2. The **recently evolved genomic regions** will underly the unique neurodevelopmental features and brain traits and **harbour genetic variants associated with schizophrenia**, therefore the genetic variability within these regions will **contribute** to the **brain's cortical architecture differences** between healthy individuals and patients with schizophrenia.

Objectives

The two aforementioned hypotheses of the present thesis have been tested through the following aims:

AIM 1. To test the modulation effects of common variability at synaptic plasticity-related genes on brain activity response in schizophrenia through case-control functional neuroimaging genetic association studies. **Three specific objectives** have been established to achieve this aim:

1.1 To study whether the common genetic variability at *KCNH2* gene underlies the **brain functional differences** observed between patients with schizophrenia and healthy controls while performing a **working-memory** task.

1.2 To assess whether the **haplotypic architecture** of *DISC1* is associated with **schizophrenia liability** and whether these haplotypes modulate the **working-memory brain functional response** conditional to the health/disease status.

1.3 To investigate the **epistatic effect of** two schizophrenia genome-wide associated genes, *CACNA1C* and *ZNF804A*, on **brain function** associated with a **working-memory** task in patients with schizophrenia and healthy controls.

AIM 2. To investigate whether Human Accelerated Regions (HARs), as recent human-specific evolutionary markers, contribute to the comprehension of the aetiological and pathophysiological roots of schizophrenia. **Two specific objectives** have been determined to achieve this aim:

2.1 To **review** the HARs' implication in the human-specific **neurodevelopment**, **brain configuration**, and **development of psychiatric disorders**, to assess whether further HAR-based studies could help to **shed light on** the understanding of the biological determinants of **schizophrenia**.

2.2 To study the **correlates** between the polygenic **variability in HARs' informative gene sets** and the **architectural differences** of the **cerebral cortex** between patients with **schizophrenia** and healthy subjects.

Supervisors' Report

The doctoral thesis "**Analysis of the impact of synaptic plasticity genes and Human Accelerated Regions on brain function and structure: from the healthy brain to schizophrenia**" presented by **Maria Guardiola Ripoll** is based on the original results obtained by the doctoral candidate.

These results are centred on the assessment of the genetic influence of synaptic plasticity genes and Human Accelerated Regions (HARs) on brain functional and structural differences in healthy subjects and patients with schizophrenia. Additionally, this doctoral thesis also includes a review of the role of HARs in neurodevelopment and psychiatric disorders.

Five original studies are presented: three research articles and one systematic review have been published in international peer-reviewed journals, and one research article is currently submitted.

Study 1.

New insights of the role of the KCNH2 gene in schizophrenia: an fMRI case-control study.

Guardiola-Ripoll M, Almodóvar-Payá C, Lubeiro A, Salvador R, Salgado-Pineda P, Gomar JJ, Guerrero-Pedraza A, Sarró S, Maristany T, Fernández-Lisenbarth I, Hernández-García M, Papiol S, Molina V, Pomarol-Clotet E, Fatjó-Vilas M. *European Neuropsychopharmacology,* 2022 Jul;60:38-47. doi: 10.1016/j.euroneuro.2022.04.012. PMID: 35635995.

The **European Neuropsychopharmacology** is an international peer-reviewed journal focused on clinical and basic science contributions that advance our understanding of brain function and human behaviour. The journal promotes findings that are expected to have a major impact on both our understanding of the biological bases of mental disorders and the development and improvement of treatments, ideally paving the way for prevention and recovery.

According to the Journal Citation Reports (Science Edition, 2021), the impact factor of the journal is **5.415**, classified in the first quartile (**Q1**) of the area of Clinical Neurology (47/212).

The doctoral candidate has participated in the study conception, DNA extraction, and genotyping. Also, the candidate has led the data curation, the statistical analysis, the interpretation of the results and the visual presentation of the findings. As well, the doctoral student has written the first version of the draft and has participated in the revision and edition of the subsequent draft versions. This article is original and none of the co-authors has used, implicitly or explicitly, this work for the elaboration of another doctoral thesis.

Study 2.

Combining fMRI and DISC1 gene haplotypes to understand working memory-related brain activity in schizophrenia.

Maria Guardiola-Ripoll, Alejandro Sotero-Moreno, Carmen Almodóvar-Payá, Noemí Hostalet, Amalia Guerrero-Pedraza, Núria Ramiro, Jordi Ortiz-Gil, Bárbara Arias, Mercè Madre, Joan Soler-Vidal, Raymond Salvador, Peter J McKenna, Edith Pomarol-Clotet, Mar Fatjó-Vilas. *Scientific Reports*, 2022 May 5;12(1):7351. doi: [10.1038/s41598-022-10660-8](10.1038/s41598-022-10660-8). PMID: 35513527.

The **Scientific Reports** belongs to Nature publishing group and is an international peer-review journal focused on all areas of the natural sciences, psychology, medicine, and engineering. The health sciences subject encompasses articles examining health, disease, and healthcare. This field of study aims to develop knowledge, interventions, and technology for use in healthcare to improve the treatment of patients.

According to the Journal Citation Reports (Science Edition, 2021), the impact factor of the journal is **4.997**, classified in the second quartile (**Q2**) of the area of Multidisciplinary Sciences (19/74).

The doctoral candidate has participated in the study conception, DNA extraction, and genotyping. Also, the candidate has led the data curation, the statistical analysis, the interpretation of the results and the visual presentation of the findings. As well, the doctoral student has written the first version of the draft and has participated in the revision and edition of the subsequent draft versions. This article is original and none of the co-authors has used, implicitly or explicitly, this work for the elaboration of another doctoral thesis.

Study 3.

A functional neuroimaging association study on the interplay between two schizophrenia genome-wide associated genes (CACNA1C and ZNF804A).

Guardiola-Ripoll M, Almodóvar-Payá C, Lubeiro A, Sotero A, Salvador R, Fuentes-Claramonte P, Salgado-Pineda P, Papiol S, Ortiz-Gil J, Gomar JJ, Guerrero-Pedraza A, Sarró S, Maristany T, Molina V, Pomarol-Clotet E, Fatjó-Vilas M. *European Archives of Psychiatry and Clinical Neuroscience*, 2022 Jul 7. doi: [10.1007/s00406-022-01447-z](10.1007/s00406-022-01447-z). PMID: 35796825.

The **European Archives of Psychiatry and Clinical Neuroscience** is an international peer-review journal focused on all aspects of psychiatry and related clinical neuroscience. Clinical psychiatry, psychopathology, epidemiology as well as brain

imaging, neuropathological, neurophysiological, neurochemical, and molecular genetic studies of psychiatric disorders are among the topics covered.

According to the Journal Citation Reports (Science Edition, 2021), the impact factor of the journal is **5.760**, classified in the first quartile (**Q1**) of the area of Clinical Neurology (41/212).

The doctoral candidate has participated in the study conception, DNA extraction, and genotyping. Also, the candidate has led the data curation, the statistical analysis, the interpretation of the results and the visual presentation of the findings. As well, the doctoral student has written the first version of the draft and has participated in the revision and edition of the subsequent draft versions. This article is original and none of the co-authors has used, implicitly or explicitly, this work for the elaboration of another doctoral thesis.

Study 4.

A systematic review of the Human Accelerated Regions in schizophrenia and related disorders: where the evolutionary and neurodevelopmental hypotheses converge.

Guardiola-Ripoll M, Fatjó-Vilas M. *International Journal of Molecular Sciences*, 2023 Feb; 24(4):3597. doi: 10.3390/ijms24043597. PMID: 36835010

The **International Journal of Molecular Sciences** is an international peer-reviewed journal providing an advanced forum for molecular and cell biology, molecular medicine, and all aspects of the molecular research. The special issue where the manuscript has been published is focused on reviews that describe general genetic studies uncovering various human diseases and is centred on genetic mechanisms and neurobiology behind schizophrenia and autism spectrum disorders.

According to the Journal Citation Reports (Science Edition, 2021), the impact factor of the journal is **6.208**, classified in the first quartile (**Q1**) of the area of Biochemistry & Molecular Biology (69/297).

The doctoral candidate has participated in the study conception, the literature search and the revision of the articles. As well, the doctoral student has written the first version of the draft and has participated in the revision and edition of the subsequent draft versions. This article is original and none of the co-authors has used, implicitly or explicitly, this work for the elaboration of another doctoral thesis.

Study 5.

Human-specific evolutionary markers linked to foetal neurodevelopment modulate brain surface area in schizophrenia.

Guardiola-Ripoll M, Almodóvar-Payá C, Arias-Magnasco A, Latorre-Guardia M, Papiol S, Canales-Rodríguez EJ, García-León MA, Fuentes-Claramonte P, Salavert J, Tristany J, Torres L, Rodríguez-Cano E, Salvador R, Pomarol-Clotet E, Fatjó-Vilas M. Submitted for peer review in an indexed journal and available as a preprint at medRxiv doi: 10.1101/2023.03.01.23286609.

The doctoral candidate has participated in the study conception, DNA extraction, and genotyping. Also, the candidate has led the data curation, the statistical analysis, the interpretation of the results and the visual presentation of the findings. As well, the doctoral student has written the first version of the draft and has participated in the revision and edition of the subsequent draft versions. This article is original and none of the co-authors has used, implicitly or explicitly, this work for the elaboration of another doctoral thesis.

As the director and co-director of this thesis, we confirm the quality of the articles published and that no co-author of the studies here presented has implicitly or explicitly used the results to elaborate another doctoral thesis.

Mar Fatjó-Vilas Mestre
Director
March 3rd 2023

Raymond Salvador Civil
Codirector
March 3rd 2023

Publications

Study 1.

New insights of the role of the KCNH2 gene in schizophrenia: an fMRI case-control study.

Guardiola-Ripoll M, Almodóvar-Payá C, Lubeiro A, Salvador R, Salgado-Pineda P, Gomar JJ, Guerrero-Pedraza A, Sarró S, Maristany T, Fernández-Lisenbarth I, Hernández-García M, Papiol S, Molina V, Pomarol-Clotet E, Fatjó-Vilas M.

New insights of the role of the *KCNH2* gene in schizophrenia: An fMRI case-control study

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KEYWORDS

Abstract

The *KCNH2* gene, encoding for a subunit of a voltage-gated potassium channel, has been identified as a key element of neuronal excitability and a promising novel therapeutic target for schizophrenia (SZ). Nonetheless, evidence highlighting the role of *KCNH2* on cognitive and brain activity phenotypes comes mainly from studies based on healthy controls (HC). Therefore, we aimed to study the role of *KCNH2* on the brain functional differences between patients with SZ and HC. The fMRI sample comprised 78 HC and 79 patients with SZ (matched for age, sex and premorbid IQ). We studied the effect of the polymorphism *KCNH2*-rs3800779 on attention and working memory-related brain activity, evaluated through the N-back task, in regions with detected diagnostic differences (regression model, controlled for age, sex and premorbid IQ, FEAT-FSL). We report a significant diagnosis x *KCNH2* interaction on brain activity (1-back *vs* baseline contrast) at the medial superior prefrontal cortex (Zmax=3.55, $p = 0.00861$). In this region, patients with SZ carrying the risk genotype (AA) show a deactivation failure, while HC depict the opposite pattern towards deactivation. The brain region with significant diagnosis x *KCNH2* interaction has been previously associated with SZ. The results of this study, in which the role of *KCNH2* on fMRI response is analysed for the first time in patients, suggest that *KCNH2* variability contributes to inefficient brain activity modulation during the N-back task in affected subjects. These data may pave the way to further understand how *KCNH2* genetic variability is related to the pathophysiological mechanisms underlying schizophrenia.

1. Introduction

Despite that the aetiological and pathophysiological underpinnings of schizophrenia (SZ) remain largely undetermined, the prevailing etiological hypotheses maintain that the interaction of multiple genetic and environmental risk factors results in brain developmental and functional alterations predisposing to the disorder later in life (Birnbaum and Weinberger, 2017; Kahn et al., 2015). In fact, from GWAS data, it is currently accepted that SZ has a polygenic architecture, and it results from the additive effect of thousands of gene variants (Purcell et al., 2009; Sullivan et al., 2003). Resulting from the convergence of GWAS associated genes in signaling and biological pathways, synaptic plasticity has been identified as a presumed mechanism involved in SZ (Pardiñas et al., 2018; Ripke et al., 2014). Neuronal excitability is an important determinant of synaptic plasticity and requires the opening of voltage-gated ion channels for synapse activation (Daoudal and Debanne, 2003). Imbalanced excitation/inhibition homeostasis results in the alteration of neural circuits, and it is suggested that such alterations may participate in the brain dysfunctions observed in neurodevelopmental disorders (Foss-Feig et al., 2017). Indeed, pieces of evidence from electrophysiology, magnetic resonance imaging, and animal models converge on the role of altered neuronal excitability in some clinical and cognitive features of SZ (Foss-Feig et al., 2017; Marín, 2012; Uhlhaas and Singer, 2010).

Among the genes responsible for neuronal excitability modulation, there is the *KCNH2*, which encodes for the α subunit of a human ether-a-go-go (hERG)-family voltage-gated potassium channel (Bauer and Schwarz, 2018; Trudeau et al., 1995). It has been repeatedly associated with SZ in different studies (Atalar et al., 2010; Hashimoto et al., 2013; Huffaker et al., 2009), and different polymorphic variants have been related to changes in the mRNA levels of the primate-specific isoform KCNH2-3.1, which is enriched in the human brain. This isoform has unique electrophysiological kinetics, characterised by rapid deactivation rates, and the increased expression of its transcript has been associated with immature neuronal architecture, impaired cognition and altered neuronal function in mice models, which also showed analogous cognitive deficits to those found in SZ (Carr et al., 2016). Besides, evidence reveals that transgenic mice with KCNH2-3.1 isoform overexpression had impaired working memory due to abnormal synchrony and neural transmission between the hippocampal formation and the prefrontal cortex (Ren et al., 2020), a mechanism that has already been suggested to be involved in SZ's etiology (Meyer-Lindenberg et al., 2005). Higher levels of KCNH2-3.1 channels may contribute to the uncoordinated neuronal firing patterns observed in patients affected by this disorder (Heide et al., 2012). Furthermore, it has been demonstrated that the expression and cell trafficking of KCNH2-3.1 isoform can be specifically modified pharmacologically without affecting its kinetic properties (Calcaterra et al., 2016). This, together with the evidence from different studies demonstrating that KCNH2 is a binding site for several antipsychotic drugs (Kongsamut et al., 2002) and that its genetic variability is associated with antipsychotic response (Apud et al., 2012; Heide et al., 2016), highlight the role of KCNH2 as a possible key element of neuronal excitability mechanisms and as a promising novel therapeutic target for the treatment of SZ.

In the attempt to establish bridges between the biological pathways identified by genetic approaches and the neurobiological basis of SZ, the use of intermediate quantifiable biomarkers may contribute to getting closer to the polygenic architecture of the disorder (Braff, 2015). Cognitive symptoms represent a core dimension in the clini-

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cal presentation of SZ (Lepage et al., 2014), and cognitive dysfunction becomes evident in different domains such as executive function, attention, learning and working memory (Kahn and Keefe, 2013). Indeed, these cognitive alterations have also been related to changes in the excitation/inhibition balance in the prefrontal cortex (reviewed in Lisman, 2012). Considering that, albeit to a lesser extent, SZ's healthy relatives exhibit cognitive affectations (Swerdlow et al., 2015), and that GWAS have informed about several genomic regions related to working memory (Greenwood et al., 2019), this cognitive dimension has been recognised as a valuable intermediate phenotype to study SZ's neurobiological basis. This phenotype can be studied at a behavioural level by the application of different cognitive tests, or at a brain level through other methodologies such as functional magnetic resonance imaging (fMRI) while conducting a cognitive task. Then, fMRI emerges as a brain-based powerful tool to assess the link between candidate genes and mechanisms underlying cognitive processes (Meisenzahl and Schlösser, 2001). Accordingly, studying the correlates between genetic variability and brain functional phenotypes related to cognitive tasks, such as working memory, in healthy controls and patients with SZ would help in the comprehension of this disorder.

Most of the data on the role of *KCNH2* on different SZ's cognitive and brain intermediate phenotypes come mainly from studies exclusively focused on healthy subjects. It has been observed an association between *KCNH2* variability and cognitive impairment (IQ, processing speed, attention or working memory) (Hashimoto et al., 2013; Henningsson et al., 2015; Huffaker et al., 2009). Likewise, different MRI studies have associated the polymorphism rs3800779 at *KCNH2* with decreased gray matter volume in the hippocampal formation and inefficient activation and connectivity of the hippocampus and the dorsolateral prefrontal cortex in healthy controls (Huffaker et al., 2009; Ren et al., 2020). Additionally, one study, including patients, highlighted the *KCNH2* modulation effect on electrophysiological brain activity measures (Lubeiro et al., 2019).

Considering the above-mentioned data, the goal of the current study was to extend to SZ patients the current knowledge on the fMRI modulation role of *KCNH2* by developing a neuroimaging genetic association study including both healthy subjects and patients diagnosed with SZ. We hypothesised that the *KCNH2* genetic variability would be associated with the differences observed at a brain functional and behavioural level in response to the N-back task in patients with SZ and healthy controls.

2. Experimental procedures

2.1. Sample

The patients' sample was composed of individuals with a confirmed DSM-IV-TR diagnosis of SZ (according to two psychiatrists). The healthy controls (HC) had no personal or familiar history of psychotic disorder or treatment. All participants met the following inclusion criteria: European origin, age between 18 and 65 years, IQ $\geq$ 70 (WAIS-III) (Wechsler, 2001) and right-handed. The exclusion criteria included: major medical illness affecting brain function, neurological conditions, history of head trauma with loss of consciousness

and present or history of drug abuse or dependence. All patients were in medical discharge and were evaluated with the Positive and Negative Symptoms Scale (PANSS) (Kay et al., 1987; Peralta and Cuesta, 1994). The Premorbid IQ of all these subjects was assessed using the Word Accentuation Test (Gomar et al., 2011).

According to these inclusion and exclusion criteria, the recruited sample initially consisted of 109 HC and 152 patients. After excluding data due to non-valid N-back performance or MRI movement, the sample comprised 108 HC and 133 patients. Then, to control for diagnostic differences, the individuals were matched for age, sex and premorbid IQ, and the case-control neuroimaging comparisons were conducted in a sample conformed of 78 HC and 79 patients. The description of the sample is given in Table 1.

Ethical approval was obtained from the Germanes Hospitalàries Research Ethics Committee, and all participants provided written consent after being informed about the study procedures and implications. All procedures were carried out according to the Declaration of Helsinki.

2.2. Molecular analysis

Genomic DNA was extracted from peripheral blood or buccal mucosa through standard methods. A single nucleotide polymorphism (SNP) in the Intron 2 of *KCNH2* (7q36.1), rs3800779-A/C (minor/major alleles in GRCh38 position: 150,974,126), was genotyped using Applied Biosystems Taqman 5′-exonuclease assays under standard conditions. The genotyping call rate was >0.99 and the accuracy of the method was tested by re-genotyping 10% of the samples and confirming all the repeated genotypes. The minor allele (rs3800779-A allele) frequency in our sample was 0.34, similar to the one described for the European population by the 1,000 Genome Project. The genotype frequencies in our sample were in Hardy-Weinberg equilibrium in both diagnostic groups.

2.3. fMRI task description and acquisition parameters

2.3.1. N-back task
Functional images were acquired while participants performed a sequential-letter version of the N-back task (Gevins and Cutillo, 1993), which engages storage and executive processes related to attention and working memory. The task had two levels of memory load: 1-back, the easiest level, and 2-back, the most difficult level, presented in a blocked design manner. Each block consisted of 24 letters that were shown every 2 seconds (1 second on, 1 second off) and all blocks contained 5 letter repetitions located randomly within the blocks. Individuals were told to indicate repetitions by pressing a button. Four 1-back and four 2-back blocks were presented in an interleaved way, and between them, a baseline stimulus (an asterisk flashing with the same frequency as the letters) was presented for 16 seconds. To identify the level to be performed, characters were shown in green and red for 1-back and 2-back blocks, respectively. The same day and before the scanning session, all participants underwent a training session outside the scanner.

2.3.2. N-back performance data
The behavioural measure used was the signal detection theory index sensitivity, d' (Green and Swets, 1996). Higher values of d' indicate better ability to discriminate between targets and distractors, while negative values indicate that subjects were not performing the task. Individuals with d' negative scores (three patients) were excluded prior to the sample matching, and therefore all individuals included in the case-control neuroimaging comparisons had valid d' values.

Table 1   Sample description. The table shows the demographic characteristics of the sample, the clinical description of patients with schizophrenia (SZ), and the genotypic data. All the quantitative variables include the mean value and standard deviation (sd). The observed allelic and genotypic counts (frequency) of the polymorphism rs3800779 are given separately for healthy controls (HC) and patients with SZ.

		Healthy Controls ($n = 78$)	Patients with SZ ($n = 79$)
Demographic data	Male:Female (male frequency)	53:25 (0.68)	54:25 (0.68)
	Age	37.20 (10.13)	37.47 (9.77)
	Premorbid IQ	104.10 (7.22)	102.14 (8.08)
Clinical data	Illness duration [a]	-	13.87 (9.92)
	PANSS Total [b]	-	72.85 (21.23)
	PANSS Positive [b]	-	17.20 (6.33)
	PANSS Negative [b]	-	21.11 (8.19)
	PANSS general psychopathology [b]	-	34.54 (10.05)
	Antipsychotic type (frequency) [c]		5(0.07):53(0.72):16(0.22)
	CPZ equivalents [c]	-	568.35 (444,20)
Genetic data	KCNH2 A allele [d]	52 (0.33)	56 (0.35)
	KCNH2 C allele [d]	104 (0.67)	102 (0.65)
	KCNH2 AA genotype [d]	8 (0.10)	10 (0.12)
	KCNH2 AC genotype [d]	36 (0.46)	36 (0.46)
	KCNH2 CC genotype [d]	34 (0.44)	33 (0.42)

[a] Data of Illness duration was available for 74 patients. There was no KCNH2 genotypic effect on Illness duration ($F = 0.01$, $p > 0.1$).

[b] Data of PANSS scores were available for 74 patients. There was no KCNH2 genotypic effect on any PANSS domain (PANSS Total $F = 0.61$, $p > 0.1$; PANSS Positive $F = 0.97$, $p > 0.1$; PANSS Negative $F = 0.26$, $p > 0.1$; PANSS general psychopathology $F = 0.45$, $p > 0.1$).

[c] Data of antipsychotic medication was available for 77 patients. There was no KCNH2 genotypic effect on antipsychotic type (typical:atypical:both) ($\chi^2 = 1.86$, $p > 0.1$) or on chlorpromazine (CPZ) equivalents ($F = 0.76$, $p > 0.1$).

[d] There were no differences in the allelic or genotypic distribution between HC and SZ patients (allelic $\chi^2 = 0.16$, $p > 0.1$; genotypic $\chi^2 = 0.21$, $p > 0.1$).

2.3.3.   Acquisition parameters

In each scanning session, 266 volumes were acquired from a 1.5-T GE Sigma scanner. A gradient echo-planar imaging (EPI) sequence depicting the blood oxygenation level-dependent (BOLD) contrast was used. Each volume contained 16 axial planes acquired with the following parameters: TR=2000 ms, TE=20 ms, flip angle=70°, section thickness=7 mm, section skip=0.7 mm, in-plane resolution=3 × 3 mm. To improve the signal strength in 1.5-T scanners, the signal-to-noise ratio was improved by reducing the number of slices and increasing the voxel volume. The first 10 volumes were discarded to avoid T1 saturation effects.

2.4.   Statistical analyses

The demographic, clinical and genetic data were described using SPSS 23.0 software (IBM SPSS Statistics for Windows, version 23.0, released 2015, IBM Corporation, Armonk, New York). Clinical data (illness duration, PANSS scores, antipsychotic type (typical, atypical or both), and chlorpromazine (CPZ) equivalent dose) were compared among patients' genotypes using ANOVA or χ^2 tests when appropriate (Table 1).

2.4.1.   Brain imaging association analyses

fMRI image analysis was performed using FEAT tool included in FSL Software (FMRIB Software, University of Oxford, Oxford, UK). Images were corrected for movement and co-registered to a common stereotaxic space (Montreal Neurologic Institute [MNI] template). The exclusion movement threshold was set at an estimated maximum absolute movement >3.0 mm or an average absolute movement >0.3 mm, in order to minimize unwanted movement-related effects. Individuals not meeting these movement criteria (1 HC and 17 patients) were excluded before the sample matching. Normalised volumes were spatially smoothed using Gaussian filter with

a full-width at half-maximum of 5 mm, and general linear models (GLMs) were fitted to generate individual activation maps for two different contrasts: 1-back vs baseline and 2-back vs baseline. Additionally, to control for the movement parameters, the movement variables were added to the model as nuisance variables. The statistical tests were performed at the cluster level with a corrected p-value of 0.05 and a Z-threshold of 2.3 (equivalent to a p-value <0.01) to define the initial set of clusters, using the Standard Field Theory correction implemented in FSL.

At the group level, we first studied in the matched sample of 78 HC and 79 subjects with SZ the effect of the disease. The analyses were performed in the two contrasts of the task (1-back vs baseline and 2-back vs baseline) using an ANOVA model (whole-brain corrected) that compared brain activity between HC and patients with SZ (adjusted for age, sex, and premorbid-IQ). For each contrast, those clusters showing significant brain activity differences between HC and patients were defined as brain masks (the results of the diagnosis main effect are described in Supplementary Material). Thus, for each N-back contrast, two brain masks were used: one mapping the clusters with higher activation in HC as compared to patients and another of the clusters with higher activation in patients as compared to HC. Within these four masks, we tested the diagnosis x genotype interaction through a regression model, with the diagnosis in two levels (HC and patients with SZ) and the genotype in three levels (AA, AC and CC) as independent variables, adjusting for age, sex and premorbid IQ. To interpret the direction of the interaction results, we subsequently estimated and plotted the individual mean activity scores from the areas where a significant interaction was detected (FSLSTATS tool in FSL and SPSS).

Several re-analyses were conducted to ensure the strength of the interaction. On the one hand, considering the limited number of individuals homozygous for the risk allele (AA), we also repeated the diagnosis x genotype interaction considering the genotype as a dichotomous variable (CC vs AC/AA) following the same procedure and statistical conditions as previously explained. Also, to rule out

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the possibility of bias due to multiple testing, we reconducted all the analyses using a more restrictive threshold to define the initial set of clusters: a Z-threshold of 3.1 (equivalent to a p-value <0.001).

2.4.2. N-back performance analyses

We studied the effect of diagnosis (HC *vs* patients with SZ) and the diagnosis x *KCNH2* genotype interaction on the N-back behavioural scores for both difficulty levels (d'1 and d'2), by means of ANOVA tests (adjusted for age, sex and premorbid IQ, SPSS). The analyses were carried out considering the diagnosis as a two-level variable and the genotype as a three-level variable.

Following the same criteria as for the fMRI data, the diagnosis x *KCNH2* genotype interaction analyses on N-back behavioural scores were also reconducted with the genotype as a dichotomous variable.

3. Results

3.1. Brain imaging association analyses

As regards the brain activity association analysis, we were interested in studying whether *KCNH2* variability modulated the functional differences observed between HC and patients with SZ. Therefore, we previously extracted those regions where there were diagnostic differences in functional activity. As expected, according to previous data of our group performed in an overlapping sample (Pomarol-Clotet et al., 2008) and in line with other studies (Kim et al., 2009; Whitfield-Gabrieli et al., 2009), patients with SZ presented a pattern of reduced activation compared to HC in several regions located at the dorsolateral prefrontal cortex, occipital cortex, thalamus and cerebellum and, on the other hand, a deactivation failure in several regions along the prefrontal cortex and the occipital lobe. For a detailed description of these results (1-back *vs* baseline and 2-back *vs* baseline), see Supplementary Material and Supplementary Figures S1 and S2.

Then, the following analyses were focused on identifying whether the *KCNH2* genotype modulates the brain activity differences between HC and patients with SZ. Accordingly, the diagnosis x group interaction assessment was limited to the regions with group differences observed in our sample. We detected a significant diagnosis x *KCNH2* interaction in the 1-back *vs* baseline contrast located at one cluster in the medial superior frontal cortex (322 voxels, peak activation at MNI $[-4,62,2]$, Zmax=3.55, $p = 0.00861$). This cluster represented a region where patients with SZ showed higher activation than HC (Figure 1.A). With the mean activity score estimation, it was observed that all HC deactivated these areas (as seen by the negative values), with higher deactivation within the AA homozygous group. On the other side, while patients with CC or AC genotypes also presented a deactivation, AA homozygotes showed an activation pattern (with positive scores). In perspective, HC deactivated this region with an A allele dose-effect while patients showed the opposite pattern towards activation (Figure 1.B). The analysis considering 2-back *vs* baseline contrast did not reveal any significant interaction.

As regards the re-analyses, on the one hand, the results on the diagnosis x genotype interaction considering the dichotomised genotype confirmed the interaction at the me-

dial superior frontal cortex (367 voxels, peak activation at MNI $[-4,64,2]$, Zmax=3.56, $p = 0.00532$). Also, the diagnosis x genotype interaction repeated with the strengthened Z-threshold of 3.1 validated these results but with a significant cluster size reduction (33 voxels, peak activation at MNI $[-4,62,2]$, Zmax=3.55, $p = 0.0133$). For a detailed description of these results after re-analyses, see Supplementary Material and Supplementary Figures S3, S4, S5 and S6.

3.2. N-back performance analyses

Concerning the N-back performance analyses, subjects with SZ obtained lower d'1 and d'2 scores, evidencing a poorer performance as compared to HC in both difficulty levels of the task. However, there was no significant diagnosis x *KCNH2* genotype interaction (Table 2). Using the genotype as a dichotomous variable, the results remained unchanged (data not shown).

4. Discussion

Our study aimed to extend to schizophrenia the already reported association of *KCNH2* polymorphisms with poorer cognitive performance (Hashimoto et al., 2013; Huffaker et al., 2009) and with inefficient activation of different brain areas in healthy subjects (Huffaker et al., 2009; Ren et al., 2020). For this purpose, we investigated the involvement of *KCNH2* genetic variability on fMRI brain activity phenotypes in HC and, for the first time, in patients with SZ. As a result, we report a differential modulating role conditional to the disease-health status in N-back task-related brain activity.

While the *KCNH2* gene has not been associated with SZ through GWAS, different studies have succeeded in identifying its role in various cognitive phenotypes such as intelligence, attention, memory and brain function, which are, in turn, also related to SZ (Hashimoto et al., 2013; Heide et al., 2016; Henningsson et al., 2015; Lubeiro et al., 2019; Ren et al., 2020). In this regard, we aimed to analyze the role of *KCNH2* on brain activity in those regions where differences between HC and subjects with SZ were detected. The brain area where we find the significant diagnosis x genotype interaction, located at the medial superior frontal cortex, can be considered a region within the default mode network (Hu et al., 2017; Raichle, 2011). Certainly, this region has been previously described in studies assessing this network throughout N-back and other working memory tasks (Kim et al., 2009; Pomarol-Clotet et al., 2008; Whitfield-Gabrieli et al., 2009), so it could be relevant for an adequate cognitive response. Brain regions located within this network are highly active during the resting state and are deactivated in response to a cognitive-demanding task, such N-back. Indeed, alterations in the default mode network have been previously described in SZ (Mingoia et al., 2012; Öngür et al., 2010), and some studies have highlighted it as a promising SZ endophenotype since it is highly heritable and mild alterations are observed in first-degree relatives of subjects with SZ (Landin-Romero et al., 2015; Meda et al., 2014).
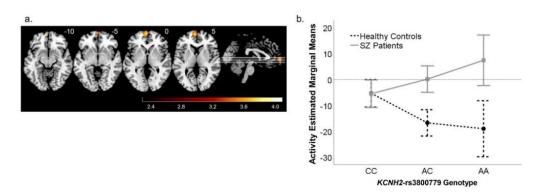
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Figure 1 Diagnosis x KCNH2 genotype interaction brain activation map and mean activity plot (Z-threshold 2.3). a) Axial view of the brain regions showing the cluster with a significant diagnosis x KCNH2 genotype interaction in 1-back vs baseline contrast. The right side of the image represents the right side of the brain. The MNI coordinates are given for the shown slices. Units of the bar are the corresponding values from the regression standardised to Z scores. b) Plot with activity estimated marginal mean and ± 2 standard errors (se) for this cluster regarding the KCNH2-rs3800779 genotype separately for healthy controls (HC) (black dashed line) and patients with schizophrenia (SZ) (grey solid line). Activity estimated marginal means (se) for HC (CC; AC; AA) = −5.30 (2.74); −16.70 (2.63); −20.15 (5.58); and for patients with SZ (CC;AC;AA) = −5.29 (2.75); −1.26 (2.64); 8.59 (5.04).

Table 2   N-back performance data. Behavioural scores for Healthy Controls (HC) and subjects with schizophrenia (SZ) are firstly shown (TOTAL). Subsequently, scores by *KCNH2* genotype within each group are also presented. Estimated marginal means and standard error (se) are reported in each case. The F scores and the corresponding p-values are given for the comparison between both groups and the diagnosis x genotype interaction analysis (adjusted for age, sex and premorbid-IQ in both cases). Significant p-values are in bold.

		Healthy Controls	Patients with SZ	
d'1 (1-back)	TOTAL	4.26 (0.10)	3.61 (0.10)	F^a=22.08, *p*<**0.001**
	AA	4.67 (0.31)	3.44 (0.28)	F^b=1.09, *p*>0.05
	AC	4.27 (0.15)	3.70 (0.16)	
	CC	4.14 (0.15)	3.56 (0.15)	
d'2 (2-back)	TOTAL	3.31 (0.10)	2.55 (0.1)	F^a=32.39, *p*<**0.001**
	AA	3.61 (0.30)	2.62 (0.27)	F^b=0.24, *p*>0.05
	AC	3.26 (0.14)	2.45 (0.14)	
	CC	3.31 (0.15)	2.62 (0.15)	

[a] The F score corresponding to the diagnosis comparison (adjusted for age, sex and premorbid IQ).
[b] The F score corresponding to the diagnosis x genotype interaction (adjusted for age, sex and premorbid IQ).

We would have also expected a significant diagnosis x genotype interaction in the most difficult level of the task, the 2-back level. Nonetheless, the detection of the interaction only in 1-back contrast could be due to the differences in the cognitive and attentional requirements between task levels (Egli et al., 2018). The fact that the interaction results remained significant and slightly strengthened after grouping the genotype in two-levels highlights the impact of the *KCNH2*-A allele at the brain functional level. While there is a need for replication, our result would represent a hint of evidence regarding the impact of *KCNH2* variability on cognitive functional response.

Since there are no *KCNH2*-fMRI association approaches including both HC and patients with SZ, our data can be only compared with healthy subjects-based studies. Within HC, we report that individuals carrying the genotype AA are the ones with higher deactivation of the medial superior frontal cortex. This is in accordance with a previous study in which the A allele was also associated with an incremented activation in the dorsolateral prefrontal cortex

in controls, an area that is expected to be activated during the performance of the N-back task (Huffaker et al., 2009). These authors interpreted this result as an inefficient response since this increased activity was not accompanied by a better performance. We could do so since we do not find genotypic differences associated with the task performance neither on HC nor on SZ patients. Nonetheless, as the relationship between brain activation and performance is much more complex, it would be expected that in the coordinated response that the brain gives to different cognitive-demanding stimuli, other brain regions frontally-cantered relevant for attention-dependent task performance (Egli et al., 2018), would coordinate and compensate the so-called neural dysfunction, and a much broader genetic background would explain the behavioural results (Tan et al., 2006). Also, concerning the behavioural results, one study reported the *KCNH2* effect on another cognitive test involving attention and working memory dimensions (the digit span subtest from the Wechsler Memory Scale-Revised) (Hashimoto et al., 2013). The differences be-

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tween our results and theirs could be accounted by differences in the task, the sample size, and the allelic distribution of the Japanese origin sample. Notwithstanding, our lack of effect found at the performance level should not diminish the significant results at the functional level since behavioural phenotypes are further from the genetic background and the genetic variability at this level is considered to be less penetrant (Rose and Donohoe, 2013).

As regards the activity pattern described within patients with SZ, our fMRI-based results are in line with the limited evidence reporting effects of *KCNH2* in affected individuals using electrophysiological data, which also quantifies global functional brain activity. Lubeiro et al. (2019) evidenced an inefficient functional activity modulation in patients carrying the A allele during another working memory task (oddball task) measured through electrophysiological activity. Their data may be coherent with ours, which also show a diminished deactivation within the medial prefrontal cortex in patients with an A allele dose-effect. Although electroencephalography measures reflect global network connectivity, taking all the data together, one might think that the genetic modulation effect captured locally through fMRI could be translated into a much wider effect since the medial prefrontal cortex is a highly relevant hub in global cognitive networks (Fox et al., 2005; Raichle, 2011).

Concerning the neurobiological effects of *KCNH2* variability, it is remarkable that the A allele is also associated with increased mRNA expression of the KCNH2-3.1 brain isoform (Huffaker et al., 2009). In turn, this overexpression has been associated with altered neuronal structure and function, and with decreased dendritic spine density in mice, which might be due to the specific electrical properties that this isoform confers to the neuron, like higher firing rate and faster deactivation (Carr et al., 2016). These particular kinetic properties might affect the spread of action potentials directly and, consequently, the coordination and communication between distant groups of neurons (Heide et al., 2012), fundamental properties to perform higher mental functions such as cognitive tasks (Varela et al., 2001). This accumulating knowledge, despite not being extensive, offers a new vision to understand how this risk variant affects the biology of the gene in a way that converges on key aspects of the biology of the disorder, such as alterations in neuron excitability homeostasis.

Some limitations of our study should be acknowledged. Our sample could be considered relatively small; however, as previous studies included only HC, a matched sample of 78 HC and 79 patients with SZ is not trivial. To ensure the statistical consistency of the results, we first used a cluster-defining Z-score of 2.3 (equivalent to a p-value <0.01) and the FLAME1 algorithm implemented in FSL to run our analyses, considered one of the best for performing cluster-wise inference (Carter et al., 2016; Eklund et al., 2016). Additionally, we also applied a stricter Z-threshold of 3.1 (equivalent to p-value <0.001) and verified that our interaction results remained significant. However, replication analyses are needed. Finally, we must consider that with our analyses comparing patients and HC, variables related exclusively to the illness status could not be included. With this in mind, we checked the possible effect of illness duration as well as medication type and dose on: i) the mean brain activity (illness duration ($r = 0.05$, $p = 0.68$); medication type

($F = 0.23$, $p = 0.80$); medication dose ($r = 0.02$, $p = 0.87$)), and ii) N-back performance scores (d'1: illness duration ($r=-0.20$, $p = 0.08$), medication type ($F = 1.56$, $p = 0.22$), medication dose ($r=-0.23$, $p = 0.05$); and d'2: illness duration ($r=-0.19$, $p = 0.12$), medication type ($F = 0.34$, $p = 0.71$), medication dose ($r=-0.14$, $p = 0.23$)); with none of them reaching significance .

In conclusion, this study adds evidence to the effect of the *KCNH2* gene on brain functioning, showing that HC and subjects with SZ have opposite activation patterns at the medial superior frontal cortex conditional to the rs3800779 A allele dose. In perspective, this same allele not only has been associated with the susceptibility for SZ (Atalar et al., 2010; Hashimoto et al., 2013; Huffaker et al., 2009) but also with cognitive and brain functional phenotypes (Hashimoto et al., 2013; Henningsson et al., 2015; Huffaker et al., 2009; Lubeiro et al., 2019; Ren et al., 2020). In conclusion, all these data highlight the putative role of the *KCNH2* gene in the etiology of schizophrenia and pave the way to further understand how *KCNH2* variability is related to gene expression differences, neuronal repolarization, cortical physiology, and cognitive performance.

Role of the funding sources

Declaration of Competing Interest

All the authors declare that they have no conflicts of interest.

CRediT authorship contribution statement

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tigation, Writing – review & editing. **Salvador Sarró:** Investigation, Writing – review & editing. **Teresa Maristany:** Investigation, Resources, Writing – review & editing. **Inés Fernández-Linsenbarth:** Investigation, Writing – review & editing. **Marta Hernández-García:** Investigation, Writing – review & editing. **Sergi Papiol:** Methodology, Writing – review & editing. **Vicente Molina:** Conceptualization, Funding acquisition, Writing – review & editing. **Edith Pomarol-Clotet:** Funding acquisition, Investigation, Resources, Writing – review & editing. **Mar Fatjó-Vilas:** Conceptualization, Funding acquisition, Methodology, Resources, Supervision, Writing – original draft, Writing – review & editing.

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Guardiola-Ripoll et al., 2022 – New insights of the role of the *KCNH2* gene in schizophrenia: An fMRI case-control study

Supplementary Material
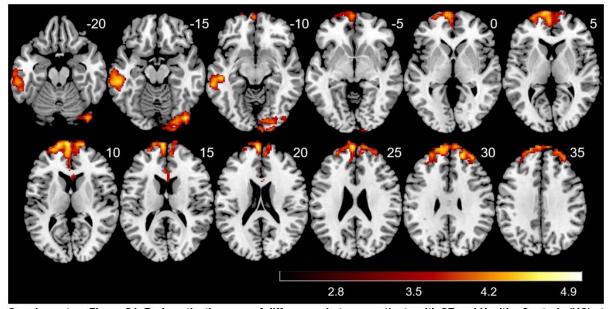
S1. Supplementary Results

S1.1 Brain activity association analyses with Z-threshold of 2.3: case-control effect
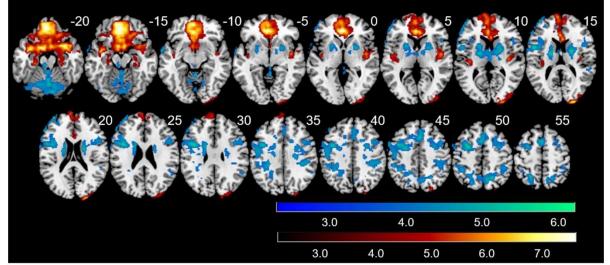
The main effect of diagnosis on brain activity was studied in the sample of 78 HC and 79 patients with SZ matched for age, sex, and premorbid-IQ, in order to first obtain brain functional masks of group differences, and second to test the effect of the *KCNH2* gene on these differences through a diagnosis x *KCNH2* genotype interaction analysis.

Group differences were revealed in both N-back contrasts. In the 1-back *vs* baseline contrast, the significant differences between HC and patients were found in three clusters. All three represented deactivation difficulties in patients (Supplementary Figure S1) compared to HC. These clusters were located: i) at the superior frontal cortex bilaterally (3606 voxels, peak activation at MNI coordinates [-4,66,22], Zmax=4.57, p=3.34e-10); ii) at the right lingual gyrus (834 voxels, peak activation at MNI coordinates [42,-84,-16], Zmax=2.44, p=0.00364); and iii) at the left middle and inferior temporal cortex (810 voxels, peak activation at MNI coordinates [-60,-32,-12], Zmax=2.36, p=0.00439).

Supplementary Figure S1. Brain activation map of differences between patients with SZ and Healthy Controls (HC) at the 1-back *vs* baseline contrast (Z-threshold of 2.3). Three clusters show significantly higher activation in SZ patients than in HC. The right side of the image represents the right side of the brain. MNI coordinates are given for the shown slices. Units of the bar are F values from the ANOVA standardised to Z scores.

Supplementary Information 1

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In the 2-back *vs* baseline contrast, we observed significant differences in five clusters that were more activated in HC than in patients with SZ and two other clusters that represented a deactivation failure in patients compared to HC (Supplementary Figure S2). The five clusters more activated in HC were located at: i) the middle frontal cortex, the supplementary motor area, the medial superior frontal cortex, the inferior frontal cortex, the thalamus, the putamen and the caudate, all bilaterally (9444 voxels, peak activation at MNI coordinates [-40,6,26], Zmax=5.21, p=2.59e-18); ii) the cerebellum bilaterally (2451 voxels, peak activation at MNI coordinates [12,-66,-22], Zmax=4.78, p=7.75e-18); iii) the superior and inferior parietal cortex, the precuneus and the supramarginal gyrus, all in the right hemisphere (1690 voxels, peak activation at MNI coordinates [18,-58,50], Zmax=4.39, p=4.07e-05); iv) the left superior and inferior parietal cortex (1456 voxels, peak activation at MNI coordinates [-22,-58,56], Zmax=3.81, p=0.000155); and v) the left middle frontal cortex and inferior frontal cortex (671 voxels, peak activation at MNI coordinates [-40,60,20], Zmax=1.57, p=0.0267). The two clusters more activated in patients affected: i) the superior and inferior orbital frontal cortex, the rectus, the superior medial frontal cortex, the anterior cingulate, the superior and middle temporal pole and the parahippocampal region, all bilaterally (14502 voxels, peak activation at MNI coordinates [30,10,-26], Zmax=7.17, p=1.03e-24); and ii) the right superior, inferior and middle occipital lobe (923 voxels, peak activation at MNI coordinates [26,-100,16], Zmax=5.07, p=0.00442).

Supplementary Figure S2. Brain activation map of differences between patients with SZ and Healthy Controls (HC) at the 2-back *vs* baseline contrast (Z-threshold of 2.3). The five clusters that HC significantly activate more than patients are shown in blue, and the two clusters that patients activate more than HC are shown in red. The right side of the image represents the right side of the brain. MNI coordinates are given for the shown slices. Units of the bars are the corresponding F values from the ANOVA standardised to Z scores.
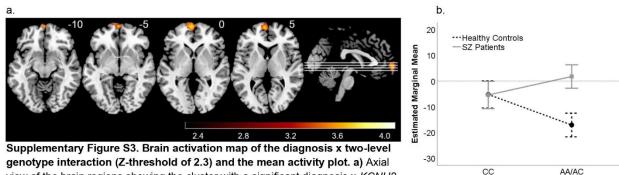
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S2. Brain activity association analyses with Z-threshold of 2.3: diagnosis x KCNH2 interaction (dichotomised genotype)

We reconducted the diagnosis x genotype interaction considering the three-level *KCNH2* genotype as a dichotomous variable (CC *vs* AC/AA genotypes). All the results remained unchanged and the interaction effect detected at the medial superior frontal cortex in the 1-back *vs* baseline contrast remained significant (367 voxels, peak activation at MNI [-4,64,2], Zmax=3.56, p=0.00532) (Supplementary Figure S3.A). The directionality of the results was in line with the diagnosis x genotype interaction results with the *KCNH2* genotype in three levels (Supplementary Figure S3.B): while HC with CC genotype present scores below zero, indicative of deactivation, patients with AC/AA genotypes present positive activity scores, indicative of activation.
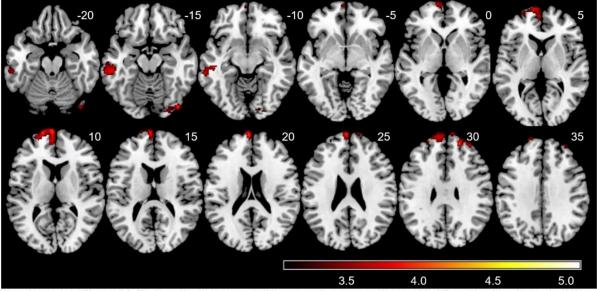
Supplementary Figure S3. Brain activation map of the diagnosis x two-level genotype interaction (Z-threshold of 2.3) and the mean activity plot. a) Axial view of the brain regions showing the cluster with a significant diagnosis x *KCNH2* genotype (in two levels) interaction in the 1-back *vs* baseline contrast. The cluster is located at the medial superior frontal cortex. The right side of the image represents the right side of the brain. MNI coordinates are given for the shown slices. Units of the bar are the corresponding β values from the regression standardised to Z scores. **b)** Plot with estimated marginal means and ±2 standard errors (se) for this cluster regarding *KCNH2*-rs3800779 genotype (dichotomised) separately for HC (black dashed line) and patients with SZ (grey solid line). Estimated marginal mean (se) for HC (CC; AC/AA) = -5.24 (2.65); -17.08 (2.30); and for patients with SZ (CC; AC/AA) = -5.44 (2.66); 1.74 (2.28).

S3. Brain activity association analyses with Z-threshold of 3.1: case-control main effect

The main effect of diagnosis on brain activity was re-tested in the same sample with a strengthened Z-threshold of 3.1 to define the initial set of clusters. These results confirmed the previously reported results in both N-back contrasts but with several clusters divided and narrower. The 1-back *vs* baseline contrast evidenced significant differences in four clusters representing deactivation difficulties in patients (Supplementary Figure S4) compared to controls located at: i) the superior, medial superior frontal cortex, and the orbitofrontal cortex (944 voxels, peak activation at MNI coordinates [-4,66,22], Zmax=4.57, p=5.96e-8); ii) in the left middle and inferior temporal cortex (307 voxels, peak activation at MNI coordinates [-60,-32,-12], Zmax=4.33, p=0.00122); iii) the right superior and medial superior frontal cortex (221 voxels, peak activation at MNI coordinates [20,52,30], Zmax=4.49, p=0.00777);
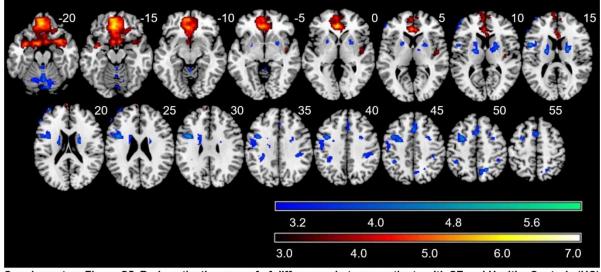
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and iv) the right lingual gyrus and the right inferior occipital cortex (166 voxels, peak activation at MNI coordinates [42,-84,-16], Zmax=4.28, p=0.0286).

Supplementary Figure S4. Brain activation map of differences between patients with SZ and healthy Controls (HC) at the 1-back *vs* baseline contrast (Z-threshold of 3.1). Patients showed significantly higher activation than HC in four clusters. The right side of the image represents the right side of the brain. MNI coordinates are given for the shown slices. Units of the bar are F values from the ANOVA standardised to Z scores.

The 2-back *vs* baseline contrast showed differences in 10 clusters that were more activated in HC than in patients with SZ and two clusters that represented a deactivation failure in patients compared to HC (Supplementary Figure S5). The ten clusters more activated in HC were located at: i) the right hemisphere in the Rolandic operculum, the opercular part of the inferior frontal gyrus, the precentral gyrus reaching the middle frontal cortex (1033 voxels, peak activation at MNI coordinates [-40,6,26], Zmax=5.21, p=5.96e-8); ii) the cerebellum and the vermis (812 voxels, peak activation at MNI coordinates [2,-66,-22], Zmax=4.78, p=1.13e-6); iii) the right hemisphere in the putamen and caudate and reaching the middle frontal cortex (599 voxels, peak activation at MNI [20,-1,14], Zmax=5.06, p=2.25e-5); iv) the left hemisphere in the putamen and the caudate (502 voxels, peak activation at MNI [-20,-6,14], Zmax=5.09, p=9.8e-5); v) medial superior frontal cortex, the supplementary motor area and the middle cingulate cortex (442 voxels, peak activation at MNI [0,18,46], Zmax=4.71, p=0.000256); vi) the right hemisphere within the precuneus, the superior parietal cortex and the superior occipital cortex (217 voxels, peak activation at MNI [18,-58,50], Zmax=4.71, p=0.0152); vii) the left hemisphere at the postcentral and precentral gyri (212 voxels, peak activation at MNI [-48,-12,42], Zmax=3.82, p=0.0169); viii) in the right hemisphere in the

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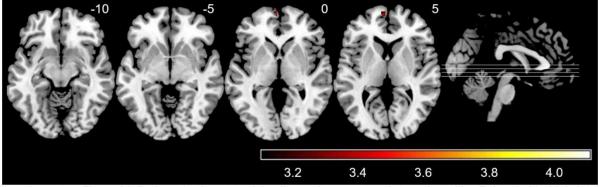
postcentral and the supramarginal gyri and the inferior parietal cortex (202 voxels, peak activation at MNI [34,-32,38], Zmax=3.90, p=0.0208); ix) the left middle frontal cortex and the inferior frontal gyrus (186 voxels, peak activation at MNI [-40,60,20], Zmax=5.98, p=0.0292); and x) the left inferior parietal cortex (174 voxels, peak activation at MNI [-38,-44,48], Zmax=4.14, p=0.0378). The two clusters showing a deactivation failure in patients compared to controls were located at: i) bilaterally at the superior and medial orbital frontal cortex, reaching the right inferior orbitofrontal cortex, the rectus, the anterior cingulate, the superior and middle temporal pole and the parahippocampal region and amygdala, (7500 voxels, peak activation at MNI [30,10,-26], Zmax=7.17, p=1.1e-30); and ii) the right superior temporal cortex and the right insula (164 voxels, peak activation at MNI [44,-6,0], Zmax=4.19, p=0.047).

Supplementary Figure S5. Brain activation map of of differences between patients with SZ and Healthy Controls (HC) at the 2-back *vs* baseline contrast (Z-threshold of 3.1). The ten clusters that HC significantly activate more than patients are shown in blue and the two clusters that patients activate more than HC are shown in red. The right side of the image represents the right side of the brain. MNI coordinates are given for the shown slices. Units of the bars are the corresponding F values from the ANOVA standardised to Z scores.

S4. Brain activity association analyses with Z-threshold of 3.1: diagnosis x KCNH2 interaction

The diagnosis x genotype interaction repeated with the strengthened threshold confirmed the interaction results found at 1-back *vs* baseline contrast but with a significant cluster size reduction (33 voxels, peak activation at MNI [-4,62,2], Zmax=3.55, p=0.0133) (Supplementary Figure S6).

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Supplementary Figure S6. Brain activation map of the diagnosis x genotype interaction with a Z-threshold of 3.1. Axial view of the brain regions showing the cluster with a significant diagnosis x *KCNH2* genotype interaction in 1-back *vs* baseline contrast at the medial superior frontal cortex after strengthening the significance threshold. The right side of the image represents the right side of the brain. MNI coordinates are given for the shown slices. Units of the bar are the corresponding β values from the regression standardised to Z scores.

Study 2.

Combining fMRI and DISC1 gene haplotypes to understand working memory-related brain activity in schizophrenia.

Maria Guardiola-Ripoll, Alejandro Sotero-Moreno, Carmen Almodóvar-Payá, Noemí Hostalet, Amalia Guerrero-Pedraza, Núria Ramiro, Jordi Ortiz-Gil, Bárbara Arias, Mercè Madre, Joan Soler-Vidal, Raymond Salvador, Peter J McKenna, Edith Pomarol-Clotet, Mar Fatjó-Vilas.

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Combining fMRI and *DISC1* gene haplotypes to understand working memory-related brain activity in schizophrenia

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The *DISC1* gene is one of the most relevant susceptibility genes for psychosis. However, the complex genetic landscape of this locus, which includes protective and risk variants in interaction, may have hindered consistent conclusions on how *DISC1* contributes to schizophrenia (SZ) liability. Analysis from haplotype approaches and brain-based phenotypes can contribute to understanding *DISC1* role in the neurobiology of this disorder. We assessed the brain correlates of *DISC1* haplotypes associated with SZ through a functional neuroimaging genetics approach. First, we tested the association of two *DISC1* haplotypes, the HEP1 (rs6675281-1000731-rs999710) and the HEP3 (rs151229-rs3738401), with the risk for SZ in a sample of 138 healthy subjects (HS) and 238 patients. This approach allowed the identification of three haplotypes associated with SZ (HEP1-CTG, HEP3-GA and HEP3-AA). Second, we explored whether these haplotypes exerted differential effects on n-back associated brain activity in a subsample of 70 HS compared to 70 patients (diagnosis × haplotype interaction effect). These analyses evidenced that HEP3-GA and HEP3-AA modulated working memory functional response conditional to the health/disease status in the cuneus, precuneus, middle cingulate cortex and the ventrolateral and dorsolateral prefrontal cortices. Our results are the first to show a diagnosis-based effect of *DISC1* haplotypes on working memory-related brain activity, emphasising its role in SZ.

The *Disrupted in Schizophrenia 1* gene (*DISC1*) was first recognised in the context of psychiatric illness when a balanced chromosomal translocation (1;11)(q42.1;q14.3) was found to segregate with major mental disorders, including schizophrenia (SZ)[1]. Since then, molecular investigations have highlighted that the liability of the *DISC1* gene towards psychosis is mediated by the protein role in processes associated with the pathophysiology of SZ, such as neurodevelopment and neurosignalling[2,3]. In neurodevelopment, the DISC1 protein acts as a central coordinator of neuronal trafficking, enabling the proper delivery of a range of neuronal cargoes with spatial and temporal precision, thereby ensuring normal neuronal development and functional homeostasis[4]. More specifically, DISC1 is involved in many stages of neurogenesis, such as neural precursor proliferation, neuronal migration, and neuronal integration/maturation[5]. Also, the synaptic location of DISC1 in adult dendritic spines and its enrichment in the post-synaptic density have suggested a role in the functional regulation of synaptic plasticity[4], which is supported by several studies that show synaptic plasticity impairments in a variety of different DISC1 mouse mutant models[6]. Finally, it is worth mentioning that DISC1 protein interacts directly with the dopamine D2 receptor[7], the main target of antipsychotic medications, suggesting that functional changes in the *DISC1* sequence could interfere with dopamine signalling and antipsychotic drug response. Overall, these

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	Healthy Subjects	Patients with SZ	
HEP1-CTG	15.15%	9.94%	$W = 6.04$, $p = 0.013$, OR (95% CI) = 0.63 (0.41–0.98)
HEP3-GA	30.91%	22.13%	$W = 6.56$, $p = 0.010$, OR (95% CI) = 0.63 (0.45–0.89)
HEP3-AA	6.68%	12.52%	$W = 6.17$, $p = 0.011$, OR (95% CI) = 2.03 (1.17–3.53)

Table 1. Haplotypic association results. Only those haplotypes showing significant frequency differences between healthy subjects (n = 138) and patients with SZ (n = 238) are reported in this table. The haplotype allelic combinations and the corresponding frequencies are shown for each group, as well as the logistic regression statistic (Wald, W), the p value (obtained after applying 10,000 permutations procedure) and the odds ratio (OR) and its 95% confidence interval (95% CI).

data indicate that any factor that compromises normal DISC1 function will likely impact brain development and create neurosignalling deficits[5].

After identifying the *DISC1* translocation, numerous genetic association studies and meta-analyses have also provided support for the role of Single Nucleotide Polymorphisms (SNPs) and mutations at this gene in the risk for SZ[8-11], as well as in other mental disorders and psychosis-related traits[12-14]. Additionally, recent data has shown lower *DISC1* expression levels in patients with SZ, which, in turn, were associated with increased severity of symptoms[15]. Therefore, the *DISC1* gene is currently considered one of the most relevant susceptibility genes for psychosis[16]. However, while many genes identified through Genome-Wide Association Studies (GWAS) in SZ form part of the *DISC1* regulome and interactome[17,18], this gene has never been identified through genome-wide approaches by itself[19-21]. This lack of direct GWAS significance may be due to the *DISC1* genetic structure, which is complex and includes protective and risk single-SNP variants[11,14]. Efforts to characterise such complexity have identified epistatic effects among *DISC1* polymorphisms on the susceptibility towards SZ[14,22], bipolar disorder, psychosis-related traits, and emotional liability[12,14,22,23]. As well, from haplotype-based approaches, the combined effect of different alleles has been related to risk and protective effects towards SZ and schizoaffective disorders[13,24-28], confirming the interest in analysing the *DISC1* variability considering its haplotypic structure. In this sense, among the haplotypes more robustly associated with psychosis are the so-called HEP3 haplotype (spanning at intron 1/exon 2), which includes the rs751229 and the rs3738401, and the HEP1 haplotype (spanning at exon 9/intron 9), which includes SNPs such as the rs6675281, the rs1000731 and the rs999710[13,26-28].

To better comprehend this complex genetic landscape and how *DISC1* contributes to SZ, strategies based on quantifiable brain-derived phenotypes have been proposed[29,30]. On the one hand, many studies have reported associations between different *DISC1* SNPs and brain structural variations in adults and neonates[31,32], in line with the pivotal role of *DISC1* in neurodevelopment. On the other hand, *DISC1* variability has been found to affect cognitive performance in domains such as attention and working memory[33-35], and the brain functional response to executive function and memory tasks[36-40]. From functional magnetic resonance imaging (fMRI) studies, among the regions where *DISC1* functional effects have been typically described, we can highlight the hippocampal region[39,40] and the prefrontal cortex[38,41-43]. These areas, indeed, have been critically involved in both the functional response to memory and attention tasks[44,45], and SZ itself[46-48]. Considering all the above mentioned together with GWAS data showing that several genomic regions associated with SZ have been related to working memory[18], this cognitive domain is a recognised intermediate phenotype to study SZ's neurobiological basis. Accordingly, studying the *DISC1* correlates of working memory at a brain level through fMRI might shed light on the disorder's brain functional changes.

Compared with the amount of research based on single polymorphic *DISC1* variants (reviewed Johnstone et al. 2011 and Duff et al. 2013[32,49]), the research based on *DISC1* haplotypic variability is scarce. To the best of our knowledge, only two studies have related *DISC1* haplotypes to cortical grey matter reductions in healthy subjects and patients with SZ[27,43]. Similarly, its haplotypic variability was associated with short- and long-term memory impairments[27]. Nonetheless, there is no data on the role of *DISC1* haplotypes on fMRI brain phenotypes which may help bridge the gap between the previously detected effects at the brain structural and cognitive level and the altered neurobiological basis in patients.

Therefore, in this study, we aimed to investigate the brain activity correlates of *DISC1* haplotypic variants associated with SZ through a neuroimaging (fMRI) genetics study. First, we conducted a case–control approach to identify the haplotypes associated with SZ in our sample. Then, we explored whether these haplotypes exerted their effect by differentially modulating working memory cognitive processes during the performance of the n-back task depending on health/disease status.

Results

Genetic association study. First, in our sample of 138 healthy subjects (HS) and 238 subjects with a diagnosis of SZ, our analysis at *DISC1* haplotypic level revealed three haplotypes associated with the risk for the disorder. The haplotypes HEP1-CTG and HEP3-GA were more frequent in HS than in patients, while the HEP3-AA haplotype was significantly overrepresented in patients (Table 1).

Neuroimaging association study. Based on the *DISC1* haplotypes associated with SZ, we performed the neuroimaging analysis with the haplotypes HEP1-CTG, HEP3-GA and HEP3-AA in a subsample of 70 HS and 70 patients (groups matched for age, sex and estimated IQ). The haplotypes were dichotomised, and each
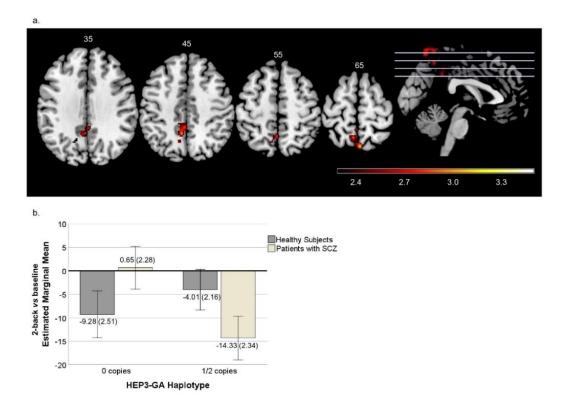
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Figure 1. (**a**) Brain regions showing the axial view of the cluster with significant diagnosis × HEP3-GA interaction in 2-back vs baseline contrast. The right side of the image represents the right side of the brain. The MNI coordinates are given for each slice. Units of the bar are the corresponding β values from the regression standardised to Z-scores. (**b**) Plot with the corresponding estimated marginal mean activity scores and ± 2 standard error (SE) for HEP3-GA haplotype in healthy subjects (42.90% with 0 copies and 57.10% with 1 or 2 (1/2) copies) and patients with SZ (51.40% with 0 copies and 48.60% with 1or 2 (1/2) copies).
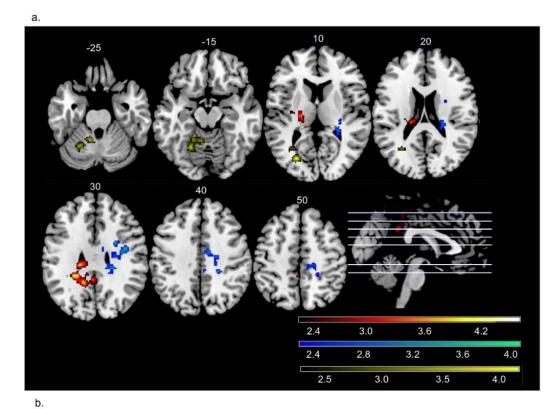
subject was defined as a carrier of 0 or 1/2 copies of the protective/risk haplotypes. Subsequently, the diagnosis x haplotype interactions were tested on n-back functional response and behavioural performance.

N-back functional response. While the haplotype × diagnosis status interactions were assessed in all the n-back contrasts (1-back vs baseline, 2-back vs baseline and 2-back vs 1-back), we focused on the 2-back vs baseline and 2-back vs 1-back findings because these contrasts are the ones better depicting working memory networks[50].

The HEP1-CTG × diagnosis interaction revealed no significant results. Concerning the HEP3, we found that both haplotypic combinations interacted with diagnosis and modulated n-back functional response. In the case of the HEP3-GA haplotype, the interaction was significant in the 1-back vs baseline (fully described in Supplementary Information and Supplementary Fig. S1) and the 2-back vs baseline contrasts.

As regards the 2-back vs baseline contrast, one significant cluster of interaction was seen, involving the cuneus and precuneus medially and the right middle cingulate cortex and the superior parietal cortex (735 voxels, peak activation at Montreal Neurological Institute coordinates system (MNI) [-4,-66,72], $Z = 3.2$, $p = 0.0182$). For interpretation of the direction of the interaction results, the mean activation scores were estimated from the areas where significance was detected, and the mean values were plotted. The mean activations of the region of interest (ROI) indicated that the patients with SZ carrying no copies of the protective HEP3-GA exhibited higher activation scores than those with 1or 2 copies. In contrast, the HS showed the opposite pattern (Fig. 1).

When the HEP3-AA haplotype was assessed, we found a significant interaction with the diagnosis in all the analysed contrasts (for 1-back vs baseline contrast results, see Supplementary Information and Supplementary Fig. S2). In the 2-back vs baseline, the diagnosis and HEP3-AA interaction was significant in three clusters: cluster (1) was in the left middle and posterior cingulate cortex, extending to the cuneus, precuneus, the thalamus and the paracentral lobule (850 voxels, peak activation at MNI [-22,-40,28], $Z = 4.10$, $p = 0.008$); cluster (2) was in the right hemisphere including the postcentral and supramarginal gyrus, the middle cingulate cortex, the paracentral lobule and also reaching, the hippocampus (930 voxels, peak activation at MNI [38,-4,28], $Z = 3.70$, $p = 0.00464$); and, cluster (3) involved regions of the lingual and fusiform gyri on the left, the calcarine sulcus and the cerebellum (1348 voxels, peak activation at MNI [-28,-72,10], $Z = 4.06$, $p = 0.000333$). In this contrast, ROI analysis revealed that for the three clusters, the HS and the patients with SZ showed similar activity profiles when they had no copies of the HEP3-AA risk haplotype. Conversely, among individuals with 1 or 2 copies of

a.

b.
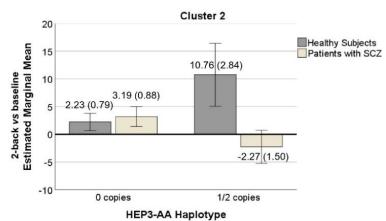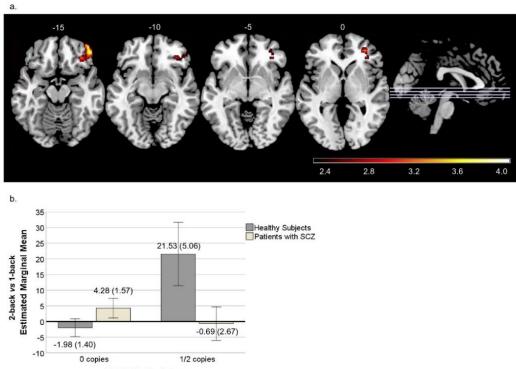
Figure 2. (a) Brain regions showing the axial view of the clusters with significant diagnosis × HEP3-AA interaction in 2-back vs baseline contrast. Cluster one is shown in red, cluster two in blue and cluster three in yellow. The right side of the image represents the right side of the brain. The MNI coordinates are given for each slice. Units of the bar are the corresponding β values from the regression standardised to Z-scores. (**b**) Plot corresponding to the 2nd cluster's estimated marginal mean activity scores ± 2 standard error (SE) for the HEP3-AA haplotype in healthy subjects (92.9% with 0 copies and 7.10% with 1 or 2 (1/2) copies) and patients with SZ (74.30% with 0 copies and 25.70% with 1 or 2 (1/2) copies).

the risk haplotype, HS showed increased activation than patients with SZ, who deactivated these regions. The mean activation scores for cluster 2 are shown in Fig. 2.

In the 2-back vs 1-back contrast, a significant interaction emerged in one cluster located in the right superior and middle frontal cortex, the middle and inferior orbitofrontal cortex and the dorsolateral and ventrolateral prefrontal cortices (585 voxels, peak activation at MNI [48,44,-16], Z = 3.83, p = 0.0441). Within individuals with no copies of the risk HEP3-AA haplotype, there were barely any differences between the HS and the SZ patients. However, among individuals with 1 or 2 copies of the HEP3-AA risk haplotype, the response was in opposite

Figure 3. (a) Brain regions showing the axial view of the cluster with significant diagnosis × HEP3-AA interaction in 2-back vs 1-back contrast. The right side of the image represents the right side of the brain. The MNI coordinates are given for each slice. Units of the bar are the corresponding β values from the regression standardised to Z-scores. (b) Plot corresponding to the estimated marginal mean activity scores ± 2 standard error (SE) for the HEP3-AA haplotype copies in healthy subjects (92.9% with 0 copies and 7.10% with 1 or 2 (1/2) copies) and patients with SZ (74.30% with 0 copies and 25.70% with 1 or 2 (1/2) copies).

directions depending on the diagnosis: HS with 1 or 2 risk copies responded with an activity increase, whereas patients showed minimal changes (Fig. 3).

N-back behavioural performance. The signal detection theory index of sensitivity (d') was the behavioural measure used (d'1 for 1-back and d'2 for 2-back). Higher values of d' indicate a better execution of the task. The comparison between HS and patients with SZ revealed significant differences at both n-back difficulty levels (d'1: $F = 12.85$, $p < 0.001$, d'2: $F = 37.31$, $p < 0.001$). Patients' performance was significantly worse than the HS' one, and the differences were more pronounced for d'2 scores (d'1 estimated marginal mean (SE) for HS: 4.24 (0.11) and for patients: 3.71 (0.11); d'2 estimated marginal mean (SE) in HS: 3.30 (0.09) and patients: 2.48 (0.09)). The interaction between the associated haplotypes and diagnosis revealed no significant results.

Discussion

This study explored whether *DISC1* haplotypic variability exerted differential effects on working memory-related brain activity. We evidenced the association of three *DISC1* haplotypes with SZ (HEP1-CTG, HEP3-GA and HEP3-AA) and subsequently the modulating role of HEP3-GA and HEP3-AA on brain activations during the performance of the n-back task depending on the health/disease status.

Our genetic association results add to previous research on the involvement of *DISC1* haplotypic variability in the risk for SZ and other psychotic disorders[13,25,27,28,51]. On the one hand, our data revealed that the HEP1-CTG (rs6675281, rs1000731 and rs999710) was associated with a protective effect (*i.e.*, less frequent in patients than in HS). In line with these data, a HEP1 haplotype containing rs6675281-C and rs1000731-T alleles was identified to be underrepresented in patients with a schizoaffective disorder through a case–control study[28]. Contrary, from a family-based approach, the opposite combination (rs6675281-T allele and rs1000731-C allele) was over-transmitted to the patients with SZ[27]. On the other hand, our findings also indicated the protective effect of the HEP3-GA (rs751229, rs3738401) and the risk effect of the HEP3-AA (*i.e.*, more frequent in patients as compared to HS). In this view, previous studies have likewise reported HEP3-AA to be more frequent in patients with a psychotic disorder than in their relatives[13,27]. Nonetheless, it is of note that the allelic variants conforming HEP1 and HEP3 and the relative frequencies observed in patients and HS are not always in consensus across

studies[25,51]. Such divergencies could be due to the sample origin (closely related to the haplotypic structure), the association designs, and the differences in the diagnostic criteria at inclusion. Still, what became evident from a study aiming to retrieve consistent results on how *DISC1* variability contributes to SZ's liability was that the locus contains both risk and protective SNPs and haplotypes[14].

Our genetic association analyses identified the haplotypic combinations related to SZ in our sample, leading to the assessment of their influence on brain functional differences in HS and patients with SZ. Through the fMRI analyses, we found no significant interaction between diagnosis and HEP1-CTG on n-back brain function. Given the scarce previous studies, a direct comparison of our results with others is not possible. However, it can be noted that one study reported changes in cortical thickness in the left supramarginal gyrus associated with a rare *DISC1* haplotype containing the rs6675281-C allele[43].

With reference to HEP3, the two haplotypic combinations revealed significant interactions with the diagnosis on n-back brain response. The interaction between the HEP3-GA and diagnosis in both 1-back vs baseline and 2-back vs baseline implicated the cuneus, the precuneus and the middle cingulate cortex. We observed that HS deactivated such regions and that patients with no copies of the protective GA haplotype had a less marked deactivation or even failed to deactivate in the most difficult level (2-back vs baseline). Considering that our association findings related the GA haplotype to a protective effect towards the disorder, this neuroimaging result seems to be in the same direction. The precuneus forms part of the so-called default mode network, a network of regions that HS deactivate during the performance of a wide range of cognitive tasks[52] and its failure of deactivation during the performance of the n-back and other tasks has also been reported in several studies in SZ[53]. Additionally, changes in the precuneus structure and functional connectivity in SZ have been previously related to *DISC1* genetic variability[54].

As regards the HEP3-AA, the interaction has been observed in all the n-back contrasts analysed. This suggests that the HEP3-AA haplotype modulates the different cognitive requirements engaged during the n-back[50]. Concerning the 2-back vs baseline contrast, the interaction was found in regions related to the previously described HEP3-GA interaction, comprising the precuneus, the posterior and middle cingulate cortex and the cuneus. This suggests that, regardless of the haplotypic combination, the HEP3 haplotype may be involved in the functional response of these brain regions. In detail, we observed that among patients with SZ, those without the protective GA haplotype and those with the risk AA haplotype were the ones presenting activation patterns in opposite directions compared to the rest of the individuals. Since the haplotypes were dichotomised, eight individuals had 1 copy of each of the two haplotypes (1 of the protective HEP3-GA and the other of the risk HEP3-AA). To overcome this haplotypic overlap, we retested the interactions with the estimated mean activity scores once these subjects were removed from the analyses, and the results remained unchanged.

In the 2-back vs 1-back contrast, the regions with significant HEP3-AA interaction with diagnosis included the right ventrolateral and the dorsolateral prefrontal cortices. Previously, *DISC1* variability has been found to modulate the dorsolateral prefrontal cortex activation in response to working memory in healthy subjects[55]. Likewise, a functional neuroimaging meta-analysis of different executive and working memory tasks found that the dorsolateral prefrontal cortex bilaterally and the right ventrolateral and premotor cortex were involved in these cognitive demanding tasks and also that their activation was reduced in SZ[48]. Considering the HEP3-AA neuroimaging results together, the most distinctive pattern occurred within HS carrying 1 or 2 copies of this risk haplotype compared to the others (HS without it and all the patients). This pattern arises from the larger absolute degree of brain activity change observed between HS carriers and non-carriers of the risk haplotype, compared to the degree of change detected within patients. Such differential effect of diagnosis has already been highlighted by Crespi & Badcock[56] when reviewing the complex relationship between genetic factors and SZ intermediate phenotypes.

About the n-back behavioural analyses, we did not detect significant interaction effects between the diagnosis and either of the haplotypes. In this sense, the comparability of the results is hampered because previous studies assessing *DISC1* variability on working memory do not report *DISC1* behavioural analyses evaluated during fMRI protocols[37,38]. However, one fMRI study is partially in line with our data, as they did not detect an effect of the *DISC1* on behavioural performance when analysing one SNP at HEP1 (rs6675281) and a different working memory task[43]. Beyond functional studies, neurocognitive evidence has associated a rare 4-SNP haplotype (including the HEP3) with visuospatial working memory[27]. Then, the results in our sample could be interpreted from the perspective that the genetic variability effect at the behavioural level is less penetrant than at the brain activity level[57], and further analyses in larger samples will be needed to furtherly explore the relationship between fMRI and behavioural data.

Regarding the effects of HEP3 haplotype on gene expression, it has been highlighted that the regions covered by this haplotype are highly conserved after human and mouse divergence, and the fact that these noncoding regions have such evolutionary conservation may be indicative of some functional significance and/or a potential regulatory role[25]. Furthermore, the rs3738401-G/A polymorphism, located in exon 2, is a missense variant that causes an Arg264Gln aminoacidic substitution. It has been reported that this polymorphism has a biological impact on Wnt signalling transduction pathways affecting neurogenesis[58], suggesting a putative mechanism for its role in decisive neurodevelopmental processes leading to psychiatric disorders. So, our results on the modulation effect that *DISC1* haplotypic variability has on brain function would link the evidence highlighting the role of *DISC1* in neurogenesis with the pathophysiological mechanisms underlying SZ.

Finally, some limitations of the current study need to be considered. First, for the genetic association analysis, our sample could be regarded as quite small; nonetheless, the fact that we inspected the haplotypic instead of single SNP variability adds power to our approach. Also, with 70 patients and 70 controls, our sample is large for functional imaging standards considering that most of the previous studies are focused exclusively on HS[37–41] or include a reduced group of patients[39,59]. On the other hand, the fact that the neuroimaging analyses were based on our haplotypic association results represents a strength of the study. Notwithstanding, future studies

		Healthy Subjects	Patients with SZ	
Neuroimaging Association sample (HS:70, SZ:70)	Sex	48:22 (0.68)	48:22 (0.68)	$\chi^2 = 0.00, p = 1$
	Age	38.86 (11.34)	39.05 (11.31)	$U = 2433, p = 0.944$
	Estimated IQ (TAP)	103.03 (7.84)	102.03 (8.54)	$U = 2282, p = 0.482$
	Illness duration[a]	–	15.93 (11.63)	–
	PANSS Total[b]	–	60.40 (30.85)	–
	PANSS Positive[b]	–	18.55 (6.01)	–
	PANSS Negative[b]	–	23.75 (8.57)	–
	PANSS general psychopathology[b]	–	30.22 (12.68)	–
	CPZ equivalents[b]	–	533.21 (433.93)	–

Table 2. Sample description. Information on the healthy subjects (HS) and patients with SZ included in the neuroimaging association study. Sex description includes male:female count (frequency in males). The clinical description of patients includes Illness duration (in years), the PANSS scores, and chlorpromazine (CPZ) equivalent dose (mg/day). All the quantitative variables include the mean value and (standard deviation). [a]Data of illness duration was available for 67 patients. [b]Data of PANSS scores and CPZ equivalents were available for 65 patients.

performed in larger samples and higher resolution scanners would be desirable. Finally, we must consider that variables related exclusively to the illness status could not be included in the interaction analysis. With this in mind, we checked within patients the possible impact of PANSS score or medication on the mean activity and the d' scores through regressions, with none of them reaching significance.

In conclusion, our data add to previous findings of an association of the HEP1-CTG, HEP3-GA and HEP3-AA haplotypes with SZ susceptibility. Additionally, this study shows, for the first time, evidence of the effect of *DISC1* haplotypic variability on brain functional differences between patients affected by SZ and HS. Although further studies are needed, our data suggest a putative role of the *DISC1* gene in the altered functional and behavioural substrates of SZ associated with n-back task performance. This might, in turn, contribute to closing the gap between the role of this gene in neurodevelopment and the pathophysiological underpinnings of schizophrenia.

Methods

Sample. The genetic association analysis to identify *DISC1* haplotypes related to SZ was conducted in a sample of 138 healthy subjects (HS) and 238 subjects with a DMS-IV-TR diagnosis of SZ (based on interviews by two psychiatrists). All participants were of European ancestry, between 19 and 65 years old. There were group differences regarding sex ($\chi^2 = 15.85$ $p < 0.001$, 72% males within patients with SZ and 51% within HS) and age (t = − 2.65 $p = 0.008$, mean age (SD) for patients with SZ = 41.98 (11.81) and for HS = 38.65 (11.64)). The HS had no personal or family history of psychiatric disorders or treatment. All participants met the same exclusion criteria: co-existent neurological disorder or medical illness affecting brain function, history of head trauma with loss of consciousness and history of drug abuse or dependence.

The neuroimaging analyses were performed in a subsample of 70 HS and 70 patients matched for age, sex, and estimated IQ (premorbid IQ in the patients), as assessed using the Word Accentuation Test (*Test de acentuación de palabras,* TAP[60]) (Table 2). In addition to the previous inclusion criteria, all participants in this part of the study were right-handed and had an estimated IQ ≥ 70. Symptoms were evaluated with the Positive and Negative Symptoms Scale (PANSS[61,62]).

Ethical approval was obtained from the Germanes Hospitalàries Research Ethics Committee, and all participants provided written informed consent about the study procedures and implications. All procedures were carried out according to the Declaration of Helsinki.

Genotyping and haplotype estimation. Genomic DNA was extracted for all individuals either from buccal mucosa through cotton swabs using ATP Genomic Mini Kit Tissue (Taknokroma Analitica, S.A., Sant Cugat del Vallès, Span) or peripheral blood cells using Realpure SSS kit (Durviz, S.L.U., Valencia, Spain). The set of SNPs was selected according to previous studies in which *DISC1* haplotypes associated with SZ were described[25,28]. Two SNPs within the HEP3 haplotype (rs751229 and rs3738401) and three SNPs within the HEP1 haplotype (rs6675281, rs1000731 and rs999710) were genotyped (Table 3). The allelic discrimination was performed using a fluorescence-based procedure (Applied Biosystems Taqman 5 '-exonuclease assays) using standard conditions, and the polymerase chain reaction plates were read on ABI PRISM 7900HT instrument with SDS v2.1 software (Applied Biosystems). The genotyping call rate was > 0.97, and the method's accuracy was retested by running in duplicate 10% of the samples and confirming all the repeated genotypes. All SNPs were in Hardy–Weinberg equilibrium in both diagnostic groups. The minor allele frequencies in our sample were similar to that described for the European population in the 1000 Genomes Project. There were no differences between the SNPs/haplotype frequencies from the whole sample and the neuroimaging subsample. For the neuroimaging approach, the estimation and tabulation of the individual haplotype phases were performed using PLINK 1.07[63].

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	SNP #rs	Chromosomal Position	Region	Alleles	1000G European MAF	Whole sample MAF
HEP3	rs751229	231632793	Intron 1	A/G	0.397	0.418
	rs3738401	231694549	Exon 2	G/A	0.339	0.352
HEP1	rs6675281	231818355	Exon 9	C/T	0.124	0.125
	rs1000731	231827745	Intron 9	C/T	0.263	0.191
	rs999710	231875197	Intron 9	G/A	0.393	0.394

Table 3. Haplotype description. The description includes the #rs of the *DISC1* SNPs, the chromosomal and gene position (GRCh38), the alleles of each SNP (major/minor allele), the minor allele frequency (MAF) observed in the European population from the 1000 Genomes Project (1000G), and the MAF observed in the genetic association sample (138 HS and 238 patients with SZ).

N-back task description and behavioural response. Functional magnetic resonance images (fMRI) were obtained while participants performed a sequential-letter version of the n-back task[64]. This functional paradigm engages storage and executive processes related to attention and memory[65]. The task had two levels of memory load (the 1-back and the 2-back), and as the difficulty load increases, higher-order executive functions like working memory become more relevant[66,67]. Since working memory is a cognitive dimension where patients affected by SZ exhibit affectations[48,68–71], we focused on the contrasts better characterising the working memory network, which, according to recent independent component analysis, are the 2-back vs baseline and the 2-back vs 1-back contrasts[50].

The two memory load levels were presented in a blocked design manner. Each block consisted of 24 letters that were shown every 2 seconds (1 second on, 1 second off). All blocks contained five repetitions (one letter beforehand in the 1-back version and two letters beforehand in the 2-back version) located randomly within the blocks. Individuals had to indicate repetitions by pressing a button. Four 1-back and four 2-back blocks were presented in an interleaved way, and between them, a baseline stimulus (an asterisk flashing with the same frequency as the letters) was presented for 16 seconds. Characters were shown in green and red for 1-back and 2-back, respectively, to identify which task had to be performed. The same day, before the scanning session, all participants underwent a training session outside the scanner.

The behavioural measure used was the signal detection theory index of sensitivity, d'[72]. Higher values of d' indicate a better ability to discriminate between targets and distractors, while negative values indicate that subjects are not performing the task. All the individuals included in the analyses had positive d' values (d'1 for 1-back and d'2 for 2-back).

Neuroimaging data acquisition. In each scanning session, 266 volumes were acquired from a GE Sigma 1.5-T scanner (General Electric Medical Systems, Milwaukee, Wisconsin, USA). A gradient echo-planar imaging sequence depicted the blood oxygen level-dependent signal. Each volume contained 16 axial planes acquired with the following parameters: repetition time = 2000 ms., echo time = 20 ms., flip angle = 70°, section thickness = 7 mm, section skip = 0.7 mm, in-plane resolution = 3 × 3 mm. To avoid T1 saturation effects, the first 10 volumes were discarded.

Statistical analyses. *Genetic association study.* We tested all the possible allelic combinations for the two haplotypes assessed (HEP1 and HEP3) for association with SZ through a logistic regression model, including sex as a covariate (PLINK). The given *p* values are those obtained after 10,000 permutations procedure. Only those haplotypes significantly associated with the disorder were furtherly examined in the neuroimaging association study.

Neuroimaging association study. Based on our genetic association results, we performed the neuroimaging analysis with the HEP1-CTG, the HEP3-GA and the HEP3-AA in the matched subsample of 70 HS and 70 patients. Because of the haplotypic frequencies in our sample, the analyses were conducted considering all haplotypes as dichotomous variables and each subject was defined as a carrier of 0 or 1/2 copies of the protective/risk haplotypes.

The fMRI analyses were performed with the FEAT tool from FSL software (FMRIB Software, University of Oxford, Oxford, UK[72]). Images were corrected for movement and co-registered to a common stereotactic space (the Montreal Neurological Institute (MNI) template). Subjects with an estimated maximum absolute movement > 3.0 mm or an average absolute movement > 0.3 mm were a priori excluded from the study to minimise unwanted movement-related effects. Normalised volumes were spatially smoothed using a Gaussian filter of 5 mm full-width at half maximum, and general linear models were fitted to generate individual activation maps for three different contrasts: 1-back vs baseline, 2-back vs baseline, and 2-back vs 1-back. The movement variables were added to the model as nuisance variables to control for movement in the scanner. All statistical tests were performed at the cluster level with a corrected *p* value of 0.05 and an initial height threshold of 2.3 (equivalent to an uncorrected *p* value of 0.01, using the Standard Field Theory correction implemented in FSL[73]). Afterwards, the interaction effect on brain function between the diagnosis and the three haplotypes was tested using regression models (whole-brain corrected and controlled for age, sex and estimated IQ). For interpretation of the direction of the interaction results, the mean activation scores were estimated from the areas where significance was detected with the FSLSTATS tool in FSL, and the mean values were plotted using SPSS (IBM SPSS Statistics,

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version 23.0, released 2015, IBM Corporation, Armonk, New York). The mean activity scores obtained from the 2-back vs 1-back contrast do not represent the mean activation per se, but the mean activation change occurring from 1-back to 2-back levels.

Analyses of the behavioural data were carried out using SPSS. First, n-back task performance (d'1 and d'2) was compared between HS and patients using an ANOVA (controlling for age, sex and estimated IQ). Next, the interaction between diagnosis and the three haplotypes was tested through full-factorial ANOVAs (including the diagnosis and haplotype main effects and controlled for age, sex and estimated IQ). These analyses were corrected for multiple comparisons (Bonferroni).

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Author contributions

Competing interests

The authors declare no competing interests.

Additional information

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SUPPLEMENTARY DATA

Supplementary Results:

The 1-back *vs* baseline contrast evidenced significant results concerning HEP3-GA and HEP3-AA.
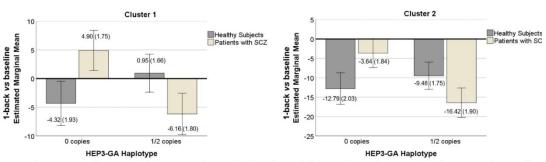
As regards HEP3-GA, two significant clusters emerged located at: i) the right and left lingual gyrus, the left cerebellum, extending bilaterally to the hippocampal region, the left amygdala and the left frontal suborbital cortex (1414 voxels, peal activation at Montreal Neurological Institute coordinates system (MNI) [10,-36,-8], Z=4.36, p=8.08e-05) and; ii) the cuneus and precuneus medially, the left middle cingulate and the right postcentral gyrus extending to the right inferior parietal cortex (1585 voxels, peal activation at MNI [0,-68,62], Z=3.66, p=2.76e-05). Mean activation in regions of interest (ROIs) based on these two clusters revealed that in both clusters, within patients with SZ, those carrying no copies of the protective HEP3-GA exhibited higher activation scores than those carrying 1 or 2 copies, who showed deactivation scores. In contrast, the opposite pattern was observed within the HS (Supplementary Figure S1).

Supplementary Figure S1
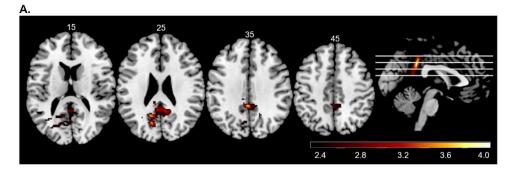
A.

Guardiola-Ripoll et al., 2022 – Combining fMRI and *DISC1* gene haplotypes to understand working memory-related brain activity in schizophrenia

B.

Supplementary Figure S1.A. Brain regions showing the axial view of the clusters with significant diagnosis x HEP3-GA interaction in 1-back *vs* baseline contrast. The red-coloured voxels represent the 1st cluster, and the blue-coloured voxels represent the 2nd cluster. The right side of the image represents the right side of the brain. MNI coordinates are given for each slice. Units of the bar are the corresponding β values from the regression standardised to z-scores. **B.** Plots with the corresponding estimated marginal mean activity scores and ± 2 standard error (SE) for HEP3-GA haplotype copies for HS (42.90% with 0 copies and 57.10% with 1/2 copies) and patients with SZ (51.40% with 0 copies and 48.60% with 1/2 copies).
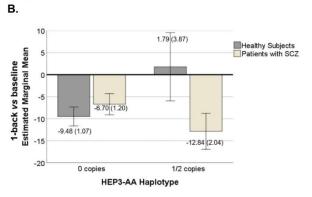
When the HEP3-AA haplotype was analysed, one cluster showed significant results. The cluster was located medially in the precuneus and at the middle and posterior cingulate cortex and extended to the left towards the cuneus, the calcarine sulcus, the middle cingulate cortex, the middle occipital cortex and the middle temporal cortex (1780 voxels, peak activation at MNI [0,-44,32], Z=3.92, p=8.94e-06). ROI analysis showed that individuals with no copies of the risk AA haplotype presented similar activity patterns irrespective of diagnosis. However, the subjects with 1/2 copies of the risk HEP3-AA haplotype showed opposite responses depending on the diagnostic status (Supplementary Figure S2).

Supplementary Figure S2.

A.

Guardiola-Ripoll et al., 2022 – Combining fMRI and *DISC1* gene haplotypes to understand working memory-related brain activity in schizophrenia

B.

Supplementary Figure S2.A. Brain regions showing the axial view of the cluster with significant diagnosis x HEP3-AA interaction in 1-back *vs* baseline contrast. The right side of the image represents the right side of the brain. MNI coordinates are given for each slice. Units of the bar are the corresponding β values from the regression standardised to Z scores. **B.** Plot with estimated marginal mean activity scores and ± 2 standard error (SE) for HEP3-AA haplotype copies for HS (92.9% with 0 copies and 7.10% with 1/2 copies) and patients with SZ (74.30% with 0 copies and 25.70% with 1/2 copies).

Study 3.

A functional neuroimaging association study on the interplay between two schizophrenia genome-wide associated genes (*CACNA1C* and *ZNF804A*).

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ORIGINAL PAPER

A functional neuroimaging association study on the interplay between two schizophrenia genome-wide associated genes (*CACNA1C* and *ZNF804A*)

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Abstract

The *CACNA1C* and the *ZNF804A* genes are among the most relevant schizophrenia GWAS findings. Recent evidence shows that the interaction of these genes with the schizophrenia diagnosis modulates brain functional response to a verbal fluency task. To better understand how these genes might influence the risk for schizophrenia, we aimed to study the interplay between *CACNA1C* and *ZNF804A* on working memory brain functional correlates. The analyses included functional and behavioural N-back task data (obtained from an fMRI protocol) and *CACNA1C*-rs1006737 and *ZNF804A*-rs1344706 genotypes for 78 healthy subjects and 78 patients with schizophrenia (matched for age, sex and premorbid IQ). We tested the effects of the epistasis between these genes as well as of the three-way interaction (*CACNA1C* × *ZNAF804A* × diagnosis) on working memory-associated activity (N-back: 2-back vs 1-back). We detected a significant *CACNA1C* × *ZNAF804A* interaction on working memory functional response in regions comprising the ventral caudate medially and within the left hemisphere, the superior and inferior orbitofrontal gyrus, the superior temporal pole and the ventral-anterior insula. The individuals with the GWAS-identified risk genotypes (*CACNA1C*-AA/AG and *ZNF804A*-AA) displayed a reduced working memory modulation response. This genotypic combination was also associated with opposite brain activity patterns between patients and controls. While further research will help to comprehend the neurobiological mechanisms of this interaction, our data highlight the role of the epistasis between *CACNA1C* and *ZNF804A* in the functional mechanisms underlying the pathophysiology of schizophrenia.

Keywords *CACNA1C* gene · *ZNF804A* gene · Epistasis · Schizophrenia · fMRI · Working memory

Introduction

Schizophrenia (SZ) is a severe and disabling psychiatric disorder whose heritability has been estimated to be up to 80%, highlighting its strong genetic component [1]. Genome-wide association studies (GWAS) have provided

compelling evidence of the polygenic architecture of SZ, with probably thousands of genetic variants with additive effects [1, 2]. The organisational complexity that involves such polygenicity is further complicated by the fact that genes are not functioning alone, and many of them interplay with each other, the so-called genetic epistasis. Since then, the challenge has been to study the modifying effect that one allele may exert over another at a different locus, an effect related to the dependencies within molecular pathways to ensure biological function [3]. As well, the convergence of GWAS data has allowed highlighting relevant pathways in the pathophysiology of SZ, such as synaptic plasticity [4, 5]. The *CACNA1C* and *ZNF804A* genes are part of these pathways, and they map to two of the most robustly associated loci with the susceptibility

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for the disorder [4–8]. However, how they increase the vulnerability for SZ remains relatively unknown, and their epistatic effect has been scarcely studied.

On the one hand, the *CACNA1C* gene encodes for the α-1C subunit of the Cav 1.2 voltage-dependent L-type calcium channel, an ion channel that regulates the calcium influx into the cell upon the polarisation of the membrane and represents the predominant calcium channel in the brain [9–11]. The location of this channel in neuronal bodies, dendritic spines and shafts is indicative of critical roles in the regulation of postsynaptic signalling pathways, neurotransmitter release, neuronal excitability, synaptic plasticity and cell survival [12]. Within *CACNA1C* variability, the rs1006737-G/A has been associated with the risk of SZ through GWAS and meta-analysis [8, 13] and with the modulation of *CACNA1C* mRNA levels in the dorsolateral prefrontal cortex in human prenatal post-mortem brain samples [14]. Reinforcing the relevance of this genetic variant, a study based on human-induced neurons has reported the association between the SZ's risk allele and an increased mRNA expression and Cav1.2 current density [15]. Additionally, studies on post-mortem human brain samples have related this same risk allele to *CACNA1C* expression changes [14, 16].

On the other hand, the *ZNF804A* gene encodes for the zinc-finger protein 804A. While its exact function remains unclear, the presence of the zinc-finger domain suggests a role as a transcription factor and as a gene-expression regulatory element of genes related to synaptic plasticity processes, such as cell adhesion, neurite outgrowth and dendritic branching [17–19]. To these data, recent studies also add evidence on its implication in mRNA processing and RNA translation [20, 21]. Indeed, among genes regulated by *ZNF804A*, there are *RBFOX1*, *DRD2* and *COMT,* which have also been associated with the risk for SZ [20, 22]. The *ZNF804A* is expressed throughout foetal development and in the adult human brain [18, 23], and its dysregulation may contribute to altered neuronal and synaptic structures related to psychotic disorders [24]. Regarding the genetic variability of *ZNF804A*, the rs1344706-A/C has been associated with psychosis [2, 4, 6, 25, 26] and with higher schizotypy scores, a risk phenotype associated with the susceptibility for psychosis distributed in the general population [27, 28]. Moreover, from a molecular point of view, it has been seen that the rs1344706-A allele is associated with reduced *ZNF804A* expression in prenatal and adult post-mortem human brain [18], which was later confirmed by another post-mortem foetal brain study evidencing a reduced expression of the most abundant *ZNF804A* splice variant in A risk homozygotes [23]. For both, *CACNA1C*-rs1006737 and *ZNF804A*-rs1344706, the A allele has been identified as the risk variant associated with SZ through candidate gene, GWAS and meta-analytic approaches [2, 6, 8, 26, 29, 30].

To get a comprehensive overview of how genetic variability contributes to SZ, functional MRI (fMRI) is considered a powerful tool to assess the relationship between genetic and biological mechanisms underlying the cerebral activation patterns and cognitive features in psychiatric disorders [31]. In this regard, there is extensive research on the role of *CACNA1C* and *ZNF804A* in the modulation of brain function using multiple approaches and paradigms. Nonetheless, the previous studies are mainly based on healthy participants [32–37]. Focusing on working memory, several studies have reported independent associations for both genes with changes in the connectivity between the dorsolateral prefrontal cortex and the hippocampus in healthy subjects [32, 34, 38]. Regarding the *CACNA1C,* there is only one study that reported the effect of the rs2007044 variability (a variant in linkage disequilibrium with rs1006737) on working memory brain activity response in a case–control sample of Chinese origin [39]. On the other hand, most of the *ZNF804A*-fMRI data on SZ come from studies based on resting-state paradigms or evaluating different cognitive dimensions [40–43]. Only one study showed that within affected individuals, the rs1344706 modulated the connectivity between the right dorsolateral prefrontal cortex and the left hippocampal formation during the N-back task performance [40].

Based on this evidence, a common downstream physiological pathway for *CACNA1C* and *ZNF804A* genes has been suggested [44] since genes that disrupt the same molecular pathway are more likely to influence similar phenotypes [45]. For this reason, inspecting epistatic effects in quantifiable and brain-based phenotypes may add relevant data on their joint role. Indeed, previous evidence points towards an interplay between these two genes on brain function during a verbal fluency task [46], showing that carrying both risk genotypes (*CACNA1C*-AA/AG and *ZNF804A*-AA) could be associated with opposite effects in fMRI response in individuals with SZ and healthy subjects. Also, from structural approaches in bipolar disorder, which has a substantial shared background with schizophrenia [47, 48], there are data suggesting a *CACNA1C* and *ZNF804A* epistasis on white matter microstructure alterations [49]. In this sense, further neuroimaging studies analysing the epistasis between *CACNA1C* and *ZNF804A* in healthy controls and patients are needed, and they could benefit from using more homogeneous samples to overcome some of the limitations resulting from the disorder's epidemiological characteristics.

According to the above mentioned, our main goal was to investigate the *CACNA1C* and *ZNF804A* epistatic effects concerning brain function during the performance of a working memory task in a matched sample of healthy subjects and patients with SZ. Secondly, we aimed to assess whether this putative epistatic effect exerted a differential modulation depending on the health/disease status. We hypothesised that the effect of the genetic variability at the *CACNA1C* gene

on the brain response to the N-back task would be modulated by the variability at the *ZNF804A* gene, or vice versa, and that this epistatic effect would be different regarding the diagnosis.

Methods and materials

Sample

The sample consisted of 78 healthy subjects (HS) and 78 patients with a confirmed diagnosis of SZ according to DMS-IV-TR (based on an interview by two psychiatrists). All participants were of European ancestry with ages comprised between 18 and 65 years old, had a current IQ > 70 (WAIS-III) [50] and were right-handed. The HS had no personal or family history of psychotic disorders or treatment. All participants met the same exclusion criteria, which included: major medical illness affecting brain function, neurological conditions, history of head trauma with loss of consciousness and present or history of drug abuse or dependence. The patients were evaluated with the Positive and Negative Symptoms Scale (PANSS) [51, 52]. The premorbid IQ in patients (and the corresponding estimated IQ in controls) was assessed using the Word Accentuation Test [53]. Healthy subjects and patients with SZ were matched for age, sex, and premorbid IQ to conduct the neuroimaging association analyses. The description of the sample is summarised in Table 1.

Ethical approval was obtained from the Germanes Hospitalàries Research Ethics Committee, and all participants provided written informed consent about the study procedures and implications. All procedures were carried out according to the Declaration of Helsinki.

Molecular analysis

Genomic DNA was extracted for all individuals either from buccal mucosa through cotton swabs using ATP Genomic Mini Kit Tissue (Teknokroma Analitica, S.A., Sant Cugat del Vallès, Spain) or from peripheral blood cells using Realpure SSS kit (Durviz, S.L.U., Valencia, Spain). Two SNPs were genotyped, the rs1006737-A/G at *CACNA1C* gene (12p13.33) and the rs1344706-C/A at *ZNF804A* gene (2q32.1). The allelic discrimination was performed using a fluorescence-based procedure (Applied Biosystems Taqman 5′-exonuclease assays) using standard conditions, and the polymerase chain reaction plates were read on ABI PRISM 7900HT instrument with SDS v2.1 software (Applied Biosystems). The genotyping call rate was > 0.99, and the accuracy of the method was tested by running in duplicate the 10% of the samples and confirming all the repeated genotypes. The minor allele frequency in our sample (rs1006737-A = 0.30 and rs1344706-C = 0.42) was similar to the one described for the European superpopulation in the 1000 Genomes Project (rs1006737-A = 0.32 and rs1344706-C = 0.38), and the genotype frequencies were in Hardy–Weinberg equilibrium in both diagnostic groups.

Table 1 Demographic, clinical and genetic description of the sample included in the study

	Healthy subjects ($n = 78$)	Patients with SZ ($n = 78$)	
Age	37.31 (10.09)	37.56 (9.80)	$t\text{-}student = -0.16, p = 0.88$
Sex	53:25 (67.9%)	53:25 (67.9%)	$\chi^2 = 0.00, p = 1.00$
Premorbid IQ	104.13 (7.23)	102.33 (7.94)	$t\text{-}student = 1.38, p = 0.14$
Illness duration[a]	–	13.91 (9.99)	–
PANSS total[b]	–	72.62 (21.28)	–
PANSS positive[b]	–	17.23 (6.37)	–
PANSS negative[b]	–	20.97 (8.16)	–
PANSS general psychopathology[b]	–	34.41 (10.05)	–
CPZ equivalents[c]	–	569.56 (447.0.2)	–
CACNA1C Acar + *ZNF804A* AA	16 (0.21)	17 (0.22)	$\chi^2 = 0.12, p = 0.99$
CACNA1C Acar + *ZNF804A* Ccar	20 (0.26)	21 (0.27)	
CANCA1C GG + *ZNF804A* AA	12 (0.15)	11 (0.14)	
CACNA1C GG + *ZNF804* Ccar	30 (0.38)	29 (0.37)	

All the quantitative variables include mean and standard deviation (sd). The sex description includes male/female count (% of males) for both healthy subjects and patients with schizophrenia (SZ). The clinical description of patients includes: illness duration (in years), PANSS scores, and Chlorpromazine (CPZ) equivalents (mg/day). The count (frequency) of each genotype combination is given

[a]Data of Illness duration were available for 73 patients

[b]Data of PANSS scores were available for 73 patients

[c]Data of CPZ equivalent doses were available for 76 patients

To maximise the power and given the small number of individuals carrying *CACNA1C*-AA and *ZNF804A*-CC genotypes, all the analyses were carried out by grouping the minor and the heterozygous genotypes (Table 1), following the same criteria as previously [46]. Then, the resulting dichotomised genotypes were used in all the analyses: *CACNA1C*-GG homozygotes *vs CACNA1C*-AA/AG (A-allele carriers, Acar); *ZNF804A*-AA homozygotes *vs ZNF804A*-AC/CC (C-allele carriers, Ccar).

N-back task

Functional images were acquired while participants performed a sequential-letter version of the N-back task [54], which engages many storage and executive processes related to attention and working memory. The task had two levels of memory load (1-back and 2-back) presented in a blocked design manner. Each block consisted of 24 letters that were shown every 2 s (1 s on, 1 s off), and all blocks contained five repetitions located randomly within the blocks. Individuals were told to indicate repetitions by pressing a button. Four 1-back and four 2-back blocks were presented in an interleaved way, and between them, a baseline stimulus (an asterisk flashing with the same frequency as the letters) was presented for 16 s. Characters were shown in green for 1-back blocks and red for 2-back blocks. The same day and before the scanning session, all participants underwent a training session outside the scanner.

fMRI acquisition parameters

The fMRI data acquisition was performed with a GE Sigma 1.5T scanner (General Electric Medical Systems, Milwaukee, Wisconsin, USA) at Hospital Sant Joan de Déu (Barcelona, Spain). The fMRI scanners included 266 volumes for each individual and a gradient echo-planar imaging sequence depicting the blood oxygen level-dependent (BOLD) signal. Each volume contained 16 axial planes acquired with the following parameters: repetition time = 2000 ms., echo time = 20 ms., flip angle = 70°, section thickness = 7 mm, section skip = 0.7 mm, in-plane resolution = 3 × 3 mm. The first 10 volumes were discarded to avoid T1 saturation effects.

Brain functional data analysis

The fMRI image analyses were performed using FEAT tool included in FSL Software (FMRIB Software, University of Oxford, Oxford, UK) [55]. In the first-level analysis, images were corrected for movement and co-registered to a common stereotaxic space [Montreal Neurologic Institute (MNI) template]. To minimise unwanted movement-related effects, subjects with an estimated maximum

absolute movement > 3.0 mm or an average absolute movement > 0.3 mm were previously excluded from the study. Normalised volumes were spatially smoothed using Gaussian filter with a full-width at half-maximum of 5 mm, and general linear models were fitted to generate individual activation maps for three different contrasts: 1-back *vs* baseline, 2-back *vs* baseline and 2-back *vs* 1-back. Additionally, to control for the movement parameters, the movement variables were added to the model as nuisance variables. All the statistical tests were performed using a cluster-wise correction method for multiple comparisons. The initial set of clusters was defined with a cluster-forming threshold of $Z = 2.6$ (equivalent to a p value < 0.005) using the standard field theory correction implemented in FSL. Afterwards, only those clusters with a p value < 0.05, family-wise corrected for multiple comparisons using Gaussian random field methods, were considered and reported (according to standard procedures in FSL). Subsequently, in the second-level analysis, we tested in the whole sample (healthy subjects and patients): (i) the *CACNA1C* × *ZNF804A* epistasis and, (ii) the *CACNA1C* × *ZNF804A* × diagnosis three-way interaction. This was conducted through a full-factorial ANOVA, including the main effects of diagnosis, *CACNA1C* and *ZNF804A* and all the two-way interactions (whole-brain corrected and adjusted by age, sex, and premorbid IQ). This was tested in the 2-back vs 1-back contrast to specifically assess working memory functional response [56]. Afterwards, to interpret the direction of the results, using the FSLSTATS tool in FSL, individual mean activity scores were estimated from the areas where significant effects were detected, and these values were plotted using SPSS (IBM SPSS Statistics, version 27.0, released 2020, IBM Corporation, Armonk, New York). It must be acknowledged that the mean activity scores obtained from the 2-back *vs* 1-back contrast do not represent mean activity per se, but the mean activity change occurred between 1-back and 2-back levels.

To assess the diagnostic relevance of these results, we first evaluated the diagnostic differences in 2-back *vs* 1-back contrast by employing an ANOVA model (whole-brain corrected) comparing brain activity between HS and patients (adjusted for age, sex, and premorbid IQ). The results retrieved the clusters with higher activation in HS as compared to patients and the clusters with higher activation in patients as compared to HS (described in detail in Supplementary Material). These regions were then transformed into two brain masks. Afterwards, we repeated the above-explained full-factorial ANOVA tests within these two brain masks.

N-back behavioural measures

The behavioural measure used was the signal detection theory index sensitivity, d' score [57]. Higher values of the d'

score indicate a better ability to discriminate between targets and distractors, while negative values indicate that subjects are not performing the task. Therefore, all the individuals included in the analyses had positive d' values (both, $d'1$ for 1-back and $d'2$ for 2-back).

Statistical analyses

Demographic and clinical data were analysed using SPSS. First, in the complete sample, the effect of the $CACNA1C \times ZNF804A$ epistasis on sex, age and premorbid IQ was examined through χ^2 and ANOVA. Second, we tested the epistasis in relation to the risk of the disorder by means of $\chi 2$. Finally, within patients, we assessed the epistatic effect on the clinical variables (PANSS score and Chlorpromazine equivalents) using ANOVA tests. No significant results were derived from these analyses (Table 1).

The statistical analyses conducted for the fMRI data have been described previously in the fMRI data analysis section.

Regarding the N-back behavioural analysis, we studied both: (i) the $CACNA1C \times ZNF804A$ epistasis and, (ii) the three-way interaction ($CACNA1C \times ZNF804A \times$ diagnosis), on the variability between $d'1$ and $d'2$ performance using a full factorial repeated measures ANOVA (SPSS). In this model, the two d' values were considered as the within-subjects two-level factor and the diagnosis, $CACNA1C$ and $ZNF804A$ as the between-subjects factors (adjusted by age, sex, and premorbid IQ).

Results

Brain functional data

We tested the $CACNA1C \times ZNF804A$ epistasis and three-way interaction ($CACNA1C \times ZNF804A \times$ diagnosis) on the brain activity patterns during working memory (2-back vs 1-back contrast of the N-back task).

On the one hand, we observed a significant $CACNA1C \times ZNF804A$ epistasis in one cluster (445 voxels, peak activation at MNI [− 2, 6, − 6], Zmax = 4.15, p value = 0.0149) (Fig. 1). This cluster was located medially at the ventral caudate and the olfactory cortex and, within the left hemisphere, extended to the superior and inferior orbitofrontal gyrus, the superior temporal pole and reached the ventral-anterior insula. To better describe this result, we extracted and plotted the mean activity scores of the cluster separately for HS and patients. We observed that the epistatic effect worked in the same direction in both groups (see the dashed arrows in Fig. 1b). Beyond the epistatic effect, it is of note that all individuals, except the patients carrying both risk alleles ($CACNA1C$-Acar + $ZNF804A$-AA), responded to the increased difficulty of the task by

decreasing the mean activity (as indicated by the negative values of the mean activity change in Fig. 1b). On the contrary, the patients carrying both risk alleles presented a mean activity change in the opposite direction compared to the rest of the subjects (see the positive values of the mean activity change in Fig. 1b).

On the other hand, the three-way interaction was non-significant.

To assess the relevance of the detected effect in relation to the SZ's diagnosis, we extracted the clusters with significant activity differences between patients and controls (described in Supplementary Material). Within these regions, the analyses of the $CACNA1C \times ZNF804A$ epistasis and three-way interaction confirmed the previously explained results. The same cluster where the epistasis was detected in the whole-brain analysis, albeit reduced in size (encompassing the medial caudate and the olfactory cortex), remained significant (187 voxels, peak activation at MNI [− 2, 6, − 6], Zmax = 4.15, p value = 0.0149).
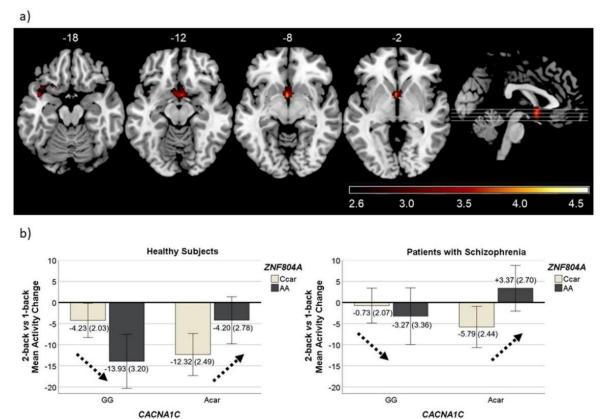
N-back behavioural data

The epistasis showed a trend effect on the performance differences between the two levels of the task ($d'1$ and $d'2$) ($F = 3.52$, p value = 0.063). Independently of the diagnosis, individuals carrying $CACNA1C$-GG + $ZNF804A$-Ccar genotypes, and also those with both risk genotypes ($CACNA1C$-Acar + $ZNF804A$-AA), showed less ability to adapt to the task increased difficulty (Fig. 2). The three-way interaction did not retrieve significant results on N-back performance.

Discussion

Besides the extensive research done on the role of $CACNA1C$-rs1006737 and $ZNF804A$-rs1344706 in brain functional phenotypes, there is only one previous fMRI study exploring the genetic epistasis between these two genes. This study reports an epistatic effect in healthy subjects and a three-way interaction with the diagnosis on verbal fluency's functional correlates [46]. Our study adds evidence on the interaction between these genes on another cognitive domain affected in schizophrenia, as is working memory, and describes a $CACNA1C \times ZNF804A$ epistasis on N-back associated functional response across patients with SZ and healthy subjects.
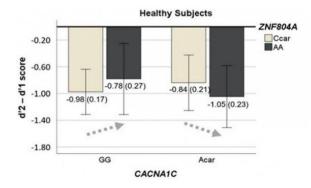
The analysis assessing the interplay between $CACNA1C$ and $ZNF804A$ on brain activity associated with N-back performance (2-back vs 1-back contrast) revealed significant epistasis between these two SZ risk genes. The epistasis was found in regions comprising the caudate, the inferior frontal gyrus, the superior temporal pole, and the insula. The fact that the epistasis worked in the same

Fig. 1 **a** Axial view of the cluster with significant *CACNA1C* × *ZNF804A* epistasis at 2-back vs 1-back contrast, resulting from the analysis including both healthy subjects and patients with schizophrenia (Zmax = 4.15, *p* value = 0.0149). The right side of the image represents the right side of the brain. The MNI coordinates are given for the shown slices. Units of the bar are the standardised Z scores (Z threshold = 2.6, *p* value < 0.05). **b** Bar plots with corresponding mean activity change for the significant 2-back vs 1-back cluster. Estimated marginal means and ± 2 standard errors (se) are plotted separately for healthy subjects in the left and patients with schizophrenia in the right by *CACNA1C* × *ZNF804A* genotypes. The black dashed lines indicate the directionality of the significant *CACNA1C* × *ZNF804A* epistasis detected. Based on these values, the effect size was estimated ($\eta_p^2 = 0.01$)
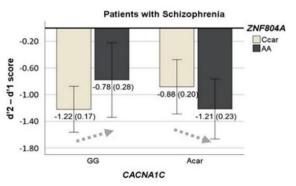
Fig. 2 Plots representing the *d′*2–*d′*1 score (N-back behavioural measures), which is used to evaluate performance differences between the two levels of the task (according to Egli et al. [56]). The bars correspond to the estimated marginal means and ± 2 standard errors (se) for healthy subjects in the left and patients with schizophrenia in the right by *CACNA1C* × *ZNF804A* genotypes. The grey dashed lines indicate the directionality of the *CACNA1C* × *ZNF804A* epistasis trend (*F* = 3.52, *p* value = 0.063)

direction in both diagnostic groups explains why the $CACNA1C \times ZNF804A \times$ diagnosis three-way interaction was not detected in this cluster. However, the estimation of the mean activity change showed that the directionality of the mean activity shift in patients with SZ carrying the risk genotype combination ($CACNA1C$-Acar + $ZNF804A$-AA) was opposite as compared to the rest of the individuals. This is indicative of an increase in brain activity in response to the task difficulty, which is contrary to the activity decrease observed in the rest of the subjects.

The areas where the epistasis was found have been previously associated with divergent brain function between patients and controls in response to the N-back task. On the one hand, regions such as the superior temporal pole were previously associated with N-back differences between HS and patients with SZ, in a study performed by our group in a partially overlapping sample [58]. Also, through meta-analytic approaches, regions such as the left insula have been related to SZ's distinctive functional activity in response to this task [59]. On the other hand, when we conducted the analysis within the regions with diagnostic differences, the epistasis was also significant. The diagnostic relevance of the implicated regions and the directionality of the effect driven by the previously identified risk alleles point towards the biological plausibility of the finding. However, despite the biological meaningfulness of our data obtained through a cluster-wise correction method, which helps in type I error control [60], we must acknowledge that our results have to be interpreted cautiously because the detection of epistatic effects in other regions or even three-way interactions could be hampered by our limited sample size.

Framing our results with previous evidence, our data are partially aligned with Tecelão et al. [46]. This study found that healthy individuals carrying both risk genotypes ($CACNA1C$-Acar and $ZNF804A$-AA) showed reduced activation in the precuneus, the posterior cingulate cortex, the calcarine sulcus and the thalamus. In contrast, they did not describe any effect on subjects with SZ. Unlike the preceding data [46], we did not find the diagnosis to modulate the directionality of the genetic epistasis. While our study and the previous one used a comparable sample, the same scanner's magnetic field and similar acquisition parameters, this dissimilarity could be due to other methodological differences. First, the three-way epistasis previously described modulated the functional response to verbal fluency, while we assessed working memory. Also, distinct results could arise from differences in the characteristics of the samples. In the preceding study, the sample included individuals from different ethnical origins and with demographic differences across diagnostic groups. In contrast, our sample included only individuals of European ancestry and the putative effect of age, sex and premorbid IQ on brain function was controlled by matching HS and patients with SZ.

Considering the behavioural results, the epistasis did not reach significance. This result could be understood from the perspective that behavioural phenotypes are further from the genetic background, and therefore, genetic variability at this level is considered less penetrant [61]. Nonetheless, considering together both behavioural and functional results, it must be mentioned that the individuals who showed higher modulation in the functional response were also the ones whose performance was least affected by the change at behavioural difficulty ($CACNA1C$-GG + $ZNF804A$-AA and $CACNA1C$-Acar + $ZNF804A$-Ccar). This might suggest a link between the observed epistatic effect on brain activity modulation and the putative effect on behavioural response. Together our and previous data indicate the interest of the analyses of epistatic effects on the brain and behavioural phenotypes. While the assessment of epistatic and three-way interactions on neuroimaging phenotypes has been typically conducted in samples sizes comparable to ours [62–65], advances towards a better understanding of inter-individual differences in brain function require the reproducibility in samples of thousands of participants and meta-analytical evidence [66].

Lastly, some limitations of our study should be acknowledged. The main one is accounted for the sample size. Although our sample of 78 HS and 78 patients with SZ is larger than the median sample size of brain-wide association studies according to a recent revision [66] and, also, than the previous fMRI study reporting $CACNA1C$ and $ZNF804A$ epistasis [46], neuroimaging genetic association studies conducted in samples with less than 100 individuals may be conditioned by type I and type II errors [60]. On the one hand, considering type I error, there are several methodological aspects in our analyses that have been used to prevent it, such as our hypothesis-driven approach and the polymorphic variants selection based on SZ's GWAS significance; the homogeneity of our sample in terms of ethnicity, demographic variables, and general cognitive abilities, derived from the use of matched groups; and the stricter significance threshold together with the cluster-wise correction method. On the other hand, type II errors could have impeded the detection of epistatic effects in other regions or even three-way interactions. While we are aware that the lack of power statistical analyses limits this interpretation, their implementation in our study was difficulted by the statistical model used for the neuroimaging analysis (whole-brain three-way interaction). To our knowledge, the available power tools are focused on ROI-based approaches and two-sample T test. Also, we considered that *post hoc* power analyses have been repeatedly discouraged and regarded as uninformative [67–69]. Finally, we must consider that, as in our statistical model patients with SZ and HC were both included, variables related exclusively to the illness status could not be considered. Bearing this in mind, we examined

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the possible effect of illness duration, PANSS total score, estimated medication dose through Chlorpromazine (CPZ) equivalents on the estimated brain mean activity and the d' difference ($d'2$–$d'1$ score) through bivariate correlations within patients. Whereas no effects were detected neither in relation to mean brain activity (illness duration $r = 0.164$, $p = 0.17$; PANSS score $r = 0.09$, $p = 0.47$; medication dose $r = 0.07$, $p = 0.56$; medication type $F = 0.23$, $p = 0.80$), nor on the task performance (illness duration $r = 0.05$, $p = 0.68$; PANSS score $r = 0.01$, $p = 0.97$; medication dose $r = 0.13$, $p = 0.28$; medication type $F = 0.92$, $p = 0.41$), we cannot completely rule out the modulatory effects of patients' clinical conditions and medication on these phenotypes. Finally, data from larger samples (ideally including thousands of individuals to ensure the reproducibility of the results), the assessment of larger genetic variability, (two SNPs do not represent the polygenic nature of working memory and SZ), and higher resolution scanners (with higher sensitivity for detecting changes in brain activation), are needed to compare these results and replicate thereof.

In conclusion, our study adds novel evidence on the interplay between *CACNA1C* and *ZNF804A*, two of the variants most strongly associated with SZ, on working memory functional response, evaluated with the N-back task during an fMRI protocol. Furthermore, we observed an opposite activity pattern between patients and healthy subjects when considering only those carrying the GWAS-identified risk genotypes. While further studies are needed to comprehend the neurobiological mechanisms by which these two genes interact, the converging evidence suggests the role of this epistatic effect in the altered functional mechanisms underlying the pathophysiology of schizophrenia and encourages new research on their putative common pathway.

Declarations

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Guardiola-Ripoll et al., 2022 – A functional neuroimaging association study on the interplay between two schizophrenia genome-wide associated genes (*CACNA1C* and *ZNF804A*)

Supplementary Material

S1. Supplementary Results
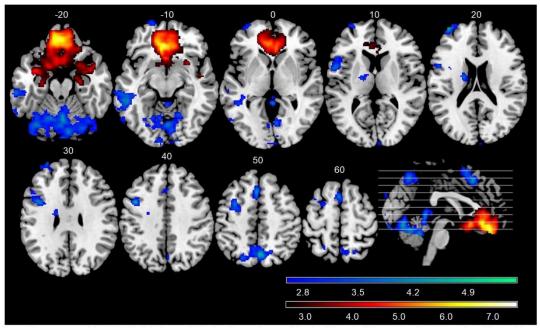
S1.1 Diagnosis main effect at 2-back vs 1-back contrast

When the main effect of diagnosis was tested on 2-back *vs* 1-back contrast, the analysis revealed differences in: i) six clusters representing higher activation in healthy subjects (HS) as compared to patients with schizophrenia (SZ) and, ii) one cluster representing higher activation in patients with SZ as compared to healthy subjects (Supplementary Figure S1).

Clusters more activated in HS were located: cluster 1) medially comprising regions of the medial superior frontal cortex, supplementary motor area (389 voxels, peak activation at MNI coordinates [-2,22,46], Zmax=4.45, p-value=0.0293; cluster 2) at the left hemisphere within the middle and superior orbitofrontal cortex and the middle and superior frontal cortex (484 voxels, peak activation at MNI coordinates [-36,64,18], Zmax=5.39, p-value=0.00964); cluster 3) medially at the precuneus, superior parietal cortex bilaterally, (878 voxels, peak activation at MNI coordinates [4,-64,50], Zmax=4.63, p-value=0.000171); cluster 4) at the left hemisphere comprising the inferior and middle temporal cortex (1107 voxels, peak activation at MNI coordinates [-62,-30,-16], Zmax=4.65, p-value=2.0e-05); cluster 5) also at the left hemisphere within the opercular and triangular part of the inferior frontal gyrus, the rolandic operculum, the precentral gyrus, the middle frontal cortex and reaching the left thalamus and some voxels from the posterior caudate (1353 voxels, peak activation at MNI coordinates [-42,6,38], Zmax=4.82, p-value=2.8e-06); and cluster 6) at the cerebellum and extending medially towards the lingual gyrus reaching the calcarine sulcus and comprising bilaterally the fusiform gyrus and the inferior occipital cortex (4197 voxels, peak activation at MNI coordinates [0,-74,-14], Zmax=4.55, p-value=2.54e-14).

On the other hand, the cluster more activated in patients with SZ comprised regions of the medial frontal cortex, the anterior cingulate cortex and the rectus, the fusiform gyrus, the middle temporal pole and the inferior temporal pole, the olfactory cortex and the superior and inferior orbitofrontal cortex bilaterally and ventrally the parahippocampal region, the hippocampus and the amygdala from both hemispheres extending towards the superior temporal pole bilaterally (11037 voxels, peak activation at MNI coordinates [-4,36,-12], Zmax=7.22, p-value=4.64e-28).

1

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Supplementary Fig. S1 Axial view of the brain regions with significant differences between healthy subjects (HS) and patients with schizophrenia in 2-back *vs* 1-back contrast. The six clusters that HS significantly activate more than patients are shown in blue (cluster 1 Zmax=4.45, p-value=0.0293; cluster 2 Zmax=5.39, p-value=0.00964; cluster 3 Zmax=4.63, p-value=0.000171; cluster 4 Zmax=4.65, p-value=2.0e-05; cluster 5 Zmax=4.82, p-value=2.8e-06, and cluster 6 Zmax=4.55, p-value=2.54e-14). The cluster that patients activate more than healthy subjects is shown in red (Zmax=7.22, p-value=4.64e-28). The right side of the image represents the right side of the brain. The MNI coordinates are given for the shown slices. Units of the bars are the standardized Z-scores (Z threshold=2.6, p-value<0.05).

2

Study 4.

A systematic review of the Human Accelerated Regions in schizophrenia and related disorders: where the evolutionary and neurodevelopmental hypotheses converge.

Guardiola-Ripoll M, Fatjó-Vilas M.

Review

A Systematic Review of the Human Accelerated Regions in Schizophrenia and Related Disorders: Where the Evolutionary and Neurodevelopmental Hypotheses Converge

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Abstract: Schizophrenia is a psychiatric disorder that results from genetic and environmental factors interacting and disrupting neurodevelopmental trajectories. Human Accelerated Regions (HARs) are evolutionarily conserved genomic regions that have accumulated human-specific sequence changes. Thus, studies on the impact of HARs in the context of neurodevelopment, as well as with respect to adult brain phenotypes, have increased considerably in the last few years. Through a systematic approach, we aim to offer a comprehensive review of HARs' role in terms of human brain development, configuration, and cognitive abilities, as well as whether HARs modulate the susceptibility to neurodevelopmental psychiatric disorders such as schizophrenia. First, the evidence in this review highlights HARs' molecular functions in the context of the neurodevelopmental regulatory genetic machinery. Second, brain phenotypic analyses indicate that HAR genes' expression spatially correlates with the regions that suffered human-specific cortical expansion, as well as with the regional interactions for synergistic information processing. Lastly, studies based on candidate HAR genes and the global "HARome" variability describe the involvement of these regions in the genetic background of schizophrenia, but also in other neurodevelopmental psychiatric disorders. Overall, the data considered in this review emphasise the crucial role of HARs in human-specific neurodevelopment processes and encourage future research on this evolutionary marker for a better understanding of the genetic basis of schizophrenia and other neurodevelopmental-related psychiatric disorders. Accordingly, HARs emerge as interesting genomic regions that require further study in order to bridge the neurodevelopmental and evolutionary hypotheses in schizophrenia and other related disorders and phenotypes.

Keywords: evolution markers; human accelerated regions; HARs; human neurodevelopment; brain configuration; cognitive abilities; schizophrenia; autism

1. Introduction

Schizophrenia (SCZ) is a complex neuropsychiatric disorder characterised by alterations in perception and behaviour. Although the specific aetiological and pathophysiological mechanisms have not yet been fully elucidated, the amount of evidence regarding environmental and genetic risk factors converging in neurodevelopmental pathways [1–7], has led to the neurodevelopment hypothesis of the origin of the disorder and its posterior revisions. Currently, this hypothesis states that the joint and interacting effect of the polygenic background and environmental stressors disrupt the neurodevelopment and brain maturation trajectories and underlie the later emergence of schizophrenia [8–11].

It has been proposed that, depending on the neurodevelopmental patterns and the resulting brain functional deviations, different mental disorders may emerge [12]. Apart

from the shared symptoms, there is also an important genetic overlap across psychiatric disorders, which converges in common biological pathways related to transcriptomic regulation and synaptic plasticity [12–16]. These commonalities in behavioural patterns and biological pathways have led to the placement of different mental disorders—such as schizophrenia, other psychotic disorders, and autism spectrum disorders—along the same continuum [12].

Genetic diseases and disorders that arise from the cumulative effect of many genetic variants are thought to persist in human populations due to a subtle balance between mutation, genetic drift, and natural selection. Thus, the alleles conferring risk are introduced in the population by mutation, persist due to random genetic drift, and are eventually eliminated by purifying selection [17–20]. The epidemiological characteristics of SCZ—such as its onset in late adolescence or early adulthood and the reduced fitness presented by affected patients, especially in males [17,20]—should lead to the eventual removal of genetic risk factors from the genetic pool. Having said this, SCZ is still a relatively common disorder with a similar prevalence across countries and regions whereby it is estimated to be present in 0.28% of the global human population [21]. The world-wide homogeneous SCZ prevalence [21] is also related to the question of when the genetic liability for SCZ emerged. In this regard, genome-wide association studies (GWAS) in SCZ that were conducted in African, Asian, and European populations identify the involvement of mostly the same genes and pathways that are related to neurodevelopment [2,22,23]. This implies that the genetic variability underlying SCZ is common in all current human populations, and that evolutionary changes underlying the emergence of neurodevelopmental human specificities would probably precede the divergence of human populations.

Therefore, the next question is why the genes that increase the likelihood of suffering from SCZ have persisted in the human genome. This evolutionary paradox has been discussed through various models and evolutionary mechanisms, such as balancing selection, fitness trade-offs, fluctuating environments, sexual selection, mutation–selection balance, and background selection [24–27]. While the details have been reported elsewhere [26,27], these evolutionary scenarios are not mutually exclusive and converge in the idea that SCZ's genetic underpinnings emerged as a costly by-product in relation to the evolution of the ontogenetic mechanisms sustaining human-specific neurodevelopment and higher-order cognitive abilities [28–30].

It is believed that the expansion of the cortex during primate evolution, especially in terms of the human lineage, has contributed to the assembly of more complex neuroarchitectures, which are able to withstand higher cognitive abilities—such as abstraction, language, memory, attention, awareness and thought [31–35]. Although the neurodevelopmental patterns, cytoarchitecture, cell type composition, and neurogenic gene expression programs of humans, macaques, and chimpanzees are remarkably similar [36,37], the genes involved in the respective neurodevelopmental trajectories exhibit tempo-spatial expression differences between humans and chimpanzees [36]. Additionally, human and macaque culture assays point towards an environmental independence of the progenitor neurons' developmental timing [35], thus suggesting that the corresponding neurodevelopmental processes (and particularly neurodevelopmental timings) are highly genetically determined.

Therefore, the study of human-specific genomic changes as compared to our closest living relatives may lead to a better comprehension of human-specific phenotypic traits [38]. In this sense, the results indicate that human particularities could be intimately related to non-coding DNA variability, thus leading to a differential control of the transcriptional networks [39]. Indeed, comparative expression studies describe differences in gene expression patterns between humans and primates during critical neurodevelopmental periods in regions such as the prefrontal cortex [36,40].

To identify the evolutionarily relevant genomic regions that are responsible for these differences, it is possible to take advantage of comparative genomics, which enables the identification of genomic loci that are highly divergent between different species. Based on this idea, Pollard et al. [41] identified the Human Accelerated Regions (HARs). HARs

are evolutionarily conserved genomic elements across mammals' evolution that have rapidly accumulated human-specific DNA sequence changes since the divergence from the human–chimpanzee ancestor. After the first discovery of HARs, several studies described other HAR sets based on different methodologies [42–46]. Nonetheless, the accelerated divergence of HARs between humans and their ancestors is suggested to reflect their role in human evolution, as well as in respect of their association with certain human-specific traits [47–49], such as neurodevelopment mechanisms and outcomes [50]. Most of these HARs do not code for proteins and are in intergenic regions or within introns near protein-coding genes, transcription factors, and DNA binding proteins, thus pointing towards their role as regulatory elements and RNA genes [41,50,51].

Indeed, HARs' role appears to be mediated through their predicted function as developmental enhancers, whereby some of them operate with human-specific activity [50]. For example, the enhancer activity testing of several non-coding HARs drew attention to HAR238, which showed spatial activity differences between humans and chimpanzees during forebrain development. Such inter-species tissue-differential expression patterns are proposed to be responsible of the changes in the enhancer effect of HAR238 on the nearby gene *GLI2* [50], a zinc-finger transcription factor in the Sonic Hedgehog signalling pathway that is critical for the induction of neural tube formation. These results guide the view of how small sequence changes in HARs may modify the complex patterns of gene expression that are necessary for proper development in a human-specific manner.

All the HARs in a person are estimated to contain an average of 1273 genetic variants and have a two-fold depletion of rare variants when compared to coding, noncoding conserved, and randomly selected loci [52]. Thus, the variants that endowed HARs their human specificity are not the same that provide human variability because the human-specific sites account for only around 2% of HAR variants [52]. Moreover, comparative genomics data suggest that the rare mutations in HARs are generally deleterious, which is reflected in a paucity of recent alleles (non-ancestral alleles 8.3%) and the fixation of 96% of ancestral alleles [52,53]. As such, some authors have suggested that HARs could have gone from positive selection along human evolution to a possible switch back to negative selection within human populations [53].

Inspecting the role of HARs and the associated genes could provide novel insights into the human brain's uniqueness and the pathogenesis of mental disorders. Although there are reviews on HARs' role in the genome, these are mainly focused on their role during neurodevelopment [54–57]. Moreover, in the past two years, HAR-based studies on human-specific brain traits and psychiatric disorders have been a burning issue. In addition, there is a need to review the role they play in human brain configuration, particularities, cognitive abilities, and neurodevelopmental-related psychiatric disorders. Accordingly, we aimed to conduct a systematic review of the literature with respect to HARs' role in neurodevelopment, brain structure and function, cognition, and psychiatric disorders susceptibility.

Starting with evidence regarding HARs' biological function as neurodevelopmental regulatory elements, we continue to review their modulatory role in the context of human-specific brain configuration, able to sustain higher-order cognitive abilities, such as intelligence or sociability. Lastly, we summarise the findings that directly relate HARs' genetic variability with SCZ, as well as other neurodevelopment-related disorders and syndromes. Therefore, our ultimate objective is to join data in order to evaluate whether the study of these evolutionary markers could help to shed light on the understanding of the genetic component of neurodevelopment and major psychiatric disorders.

2. Methods

We conducted a systematic search from April 2021 to November 2022 in the PubMed and Web of Science (Web of Science Core Collection) databases, searching for articles from inception to 14th November 2022. For the search strategy, we used the following terms: ("human accelerated regions" or "accelerated regions" or "accelerated gene" or "human

accelerated genes") and ("schizophrenia" or "psychiatric disorder" or "neurodevelopment" or "brain development" or "development" or "cognition"). The inclusion criteria were English-written peer-reviewed original articles and studies that aimed to assess the role of Human Accelerated Regions, or their related genes, on neurodevelopment, brain-based phenotypes, or psychiatric disorders. The studies that did not fulfil these criteria were excluded.

The papers were initially screened based on the title and abstract. Those meeting the inclusion criteria were analysed by the two researchers who independently performed the data extraction using a form. This form included the main article aims, the HARs approach (candidate vs. whole-genome), the main methodology, findings, strengths and limitations, and the classification of the article according to three major topics (HARs in neurodevelopment, HARs in the brain and cognitive phenotypes, and HARs in psychiatric disorders). The Preferred Reporting Items for Systematic Reviews and Meta-analyses (PRISMA) guideline was followed to guarantee the good quality of the systematic review.

3. Results

3.1. Literature Search and Study Selection

Following the systematic search strategy and study selection, 16 publications that met the inclusion criteria were included. Additionally, six articles that also met the inclusion criteria were found from cross-referencing and were additionally reviewed. Thus, a total of 22 articles were considered in the final qualitative synthesis (Figure 1).
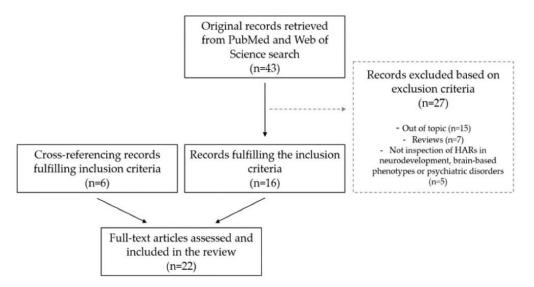
Figure 1. Flow diagram of the literature search and article selection.

A summary of the main findings is presented around three different topics. The first topic encompasses the role of HARs during neurodevelopment. This includes the articles that have assessed the role of HARs and the nearby and interacting genes (HAR genes) during neurodevelopment. This was achieved using genome-wide approaches to conduct histone profiling, chromatin conformation data, DNase I sensitivity, and massively parallel reporter assays [52,58–60] (Table 1), and studies that assessed the role of several candidate HARs and genes associated with HARs (HAR genes) concerning their neurodevelopmental expression [47,52,58,60–65] (Table 2). The second topic includes research on the role of HARs in the context of adult brain structure, function, and cognitive and behavioural phenotypes [66–69] (Table 3). Lastly, the third topic comprises research on HARs' involvement in psychiatric disorders. Most of the studies are focused on SCZ [66,69–76], but

there is also research on other neurodevelopmental psychiatric disorders and phenotypes, such as autism spectrum disorders (ASD) [52,58,66], bipolar disorder [76], and delirium syndrome [77] (Table 4).

Table 1. Summary of the main findings of whole-genome-based studies assessing the role of HARs in neurodevelopment.

Main Objective (Methodology)	Main Results
Doan et al. [52] To characterise the functions of HARs in neurodevelopment (in silico analyses)	• HARs are enriched in regulatory elements of neurodevelopmental processes in adult and foetal brain samples. • Comparative genomics show HARs' human-specific role during cortical development by altering the sequence of transcription factor motifs.
Won et al. [58] To map and characterise HAR expression patterns, tissue, and cell specificity (in silico analyses)	• HARs are enriched in putative regulatory elements active prenatally and in enhancers accessible in cortex neurogenic zones. • HAR genes regulate corticogenesis and cortical lamination, are upregulated during neurogenesis, and are related to outer radial glia in the developing cortex and astrocytes in adult prefrontal cortex.
Uerbbing et al. [59] To study the effect of HAR variability in human neurogenesis (massively parallel reporter assay (MPRA) in human neural stem cells)	• Human-specific substitutions within HARs interact with each other and with background sequences to modify enhancer activity and modify transcription factor binding sites.
Girskis et al. [60] To study HARs' effect on the recent evolution of the human cerebral cortex (Capture MPRAs in human neural stem cells and neurospheres)	• Half of HARs act as brain enhancers, preferentially in neurodevelopment, with critical roles in corticogenesis. • Most of the HARs with enhancer activity show increased enhancer activity in humans vs. chimpanzees.

Table 2. Summarised results of the functional validation of HAR regulatory activity on candidate genes.

HAR	Gene—Function	Validation Methodology	Main Results
HAR1 [47]	*HAR1F and HAR1R* (*Highly Accelerated Region 1A and 1B,* 20q13.33) Non-Protein-Coding RNAs. Unknown function.	Expression assay on human embryonic and adult brain. Comparative expression analysis in embryonic macaque, mouse, and human brains	• HAR1 is part of two RNA genes: *HAR1F* and *HAR1R,* both expressed in the developing cortex and in the adult frontal cortex, hippocampus, thalamus, and hypothalamus. *HAR1R* is expressed in an attenuated way compared to *HAR1F.* • There are differences in *HAR1F* and *HAR1R* expression ratios between humans and mice.
HACN96, HAR202, 2xHAR142, HAR89, 2xHAR223, 2xHAR157, 2xHAR122, HAR96, HACNS658, HAR189, HACNS553, HAR21, HACNS221, HAR173 [62,63]	*NPAS3* (*Neuronal PAS Domain Protein 3,* 14q13.1) Transcription factor involved in the control of neurosignalling pathways during neurogenesis.	Expression assays in transgenic zebrafish and mice. Comparative expression of human, chimpanzee, and mouse HAR orthologs. Hybridisation in transgenic mice	• Most HARs (11/14) in *NPAS3* act as transcriptional enhancers during brain development (in transgenic zebrafish and mice), and all human-specific substitutions produce gain or loss of transcription factor binding sites. • Human HARs have different expression patterns in location and intensity compared to chimpanzee and mouse orthologs. • *NPAS3* and 2xHAR142 expression patterns overlap in the forebrain regions with neural progenitor cells in transgenic mice.
HAR31, HACNS174, HACNS369 [64]	*AUTS2* (*Autism Susceptibility Candidate 2,* 7q11.22) Transcription factor involved in neurodevelopmental regulation, axon and dendrite elongation, and neuronal migration.	Targeted expression assays in transgenic zebrafish and mice	• HAR31 and HAR369 show regulatory effects on *AUTS2* expression in the brain of zebrafish and mice.
HARE5 [65]	*FZD8* (*Frizzled Class Receptor 8,* 10p11.21) Receptor in the WNT pathway implicated in cortical development.	Comparative expression of human and chimpanzee HAR orthologs in transgenic mice	• Human-specific changes in HARE5 result in different transcription factor binding sites and drive an earlier and more robust brain expression of *FZD8* at the onset of corticogenesis.

Table 2. *Cont.*

HAR	Gene—Function	Validation Methodology	Main Results
HAR-HSTR1 [61]	*HSTR1* (*Human-Specific Tandem Repeat 1*, 20p) Non-Protein-Coding RNA. Unknown function. Non-annotated gene.	Targeted expression assays in HEK293T cells. Comparative expression of human, chimpanzee, gorilla, and orangutan HAR orthologs through luciferase reporter assays	• This HAR-*HSTR1* acts as the promoter of the *HSTR1* gene. • Human HAR sequence has higher promoter activity than the other primate orthologs.
HAR426 [52]	*CUX1* (*Cut Like Homeobox 1*, 7q22.1) Transcription factor involved in the control of neuronal differentiation.	Mutant and wild-type HAR mutation effect through luciferase reporter assays in mouse neural-precursor-like cells	• A rare mutation in HAR426 is associated with a three-fold increased *CUX1* enhancer activity and promoter expression.
HAR169 [52]	*PTBP2* (*Polypyrimidine Tract Binding Protein 2*, 1p21.3) RNA-binding protein and brain-specific splicing regulator essential for neuronal differentiation.	Mutant and wild-type HAR mutation effect through luciferase reporter assays in mouse neural-precursor-like cells. Massively parallel reporter assays in mouse neurospheres	• The mutation in HAR169 alters transcription factor binding sites and causes a 50% reduction in *PTBP2* enhancer activity in neural-like mouse cells. • The mutation in HAR169 produces a 40% reduction in *PTBP2* enhancer activity in mouse neurospheres.
HAR1325 [52]	*GPC4* (*Glypican Proteoglycan 4*, Xq26.2) Protein essential for excitatory synapse development in mice and dosage-sensitive gene in adult human brain.	Mutant and wild-type HAR mutation effect through luciferase reporter assays in mouse neural-precursor-like cells. Massively parallel reporter assays in mouse neurospheres	• Two rare mutations in HAR1325 alter transcription factor binding sites and cause a 20–30% reduction in the *GPC4* enhancer activity in neural-like mouse cells. • One of the mutations in HAR1325 produces a 30% reduction in *GPC4* enhancer activity in mouse neurospheres.
HAR4 [58]	*GLI2* (*GLI family zinc finger 2*, 2q14.2) Transcription factor in the Sonic Hedgehog (Shh) pathway critical for neural tube formation. Involved in cell growth and specialisation.	Targeted expression in primary human neural progenitor cells	• Compared to *GLI2* expression without HAR4, the presence of HAR4 increases by 60% the expression of *GLI2* in primary human neural progenitor cells.
HAR1225 [58]	*GLI3* (*GLI family zinc finger 3*, 7p14.1) Transcription factor in the Shh pathway critical for neural tube formation. Essential for dorsal–ventral patterning of telencephalon and cortex formation in humans.	Targeted expression in primary human neural progenitor cells	• Compared to *GLI3* expression without HAR1225, the presence of HAR1225 increases between 30–40% the expression of *GLI3* in primary human neural progenitor cells.
HAR342 [58]	*TBR1* (*T-Box Brain Transcription Factor 1*, 2q24.2) Transcriptional factor repressor involved in neuronal migration, laminar and areal identity, and axonal projection.	Targeted expression in primary human neural progenitor cells	• Inconclusive results for HAR342's effect on *TBR1* expression.
HAR2635, HAR2636 [60]	*PPP1R17* (*Protein Phosphatase 1 Regulatory Subunit 17*, 7p14.3) Phosphatase inhibitor involved in neural progenitor cell proliferation and expression regulation in the developing human cortex.	Targeted chromatin conformation capture 3C interaction analysis	• HAR2635 and HAR2636 interact with promoter of *PPP1R17* in cultured neural cells, suggesting the regulating role of these HARs.

Table 3. Summarised findings of the studies assessing the role of HARs in brain and cognitive phenotypes.

Main Objective	Main Methodology	Main Results
Wei et al. [66] To study the evolutionary genetics of cortical expansion using HAR gene expression	Correlation analyses of HAR genes expression with cortical expansion differences from human vs. chimpanzee (sMRI data) and human vs. primates comparative gene expression. Association analyses of HAR and HAR brain genes with DMN variability (resting state fMRI data), intelligence, and sociability (based on GWAS data)	• The expression profiles of HAR genes and HAR-brain genes correlate with human cortical expansion (more expansion, more expression). The highest HAR genes and HAR brain genes expression is observed at the DMN. • Humans display an upregulated HAR brain gene expression in cognitive networks compared to primates. • HAR brain genes modulate the DMN individual variability. • HAR genes and HAR brain genes are associated with individual intelligence variability and sociability.

Table 3. *Cont.*

Main Objective	Main Methodology	Main Results
Li et al. [67] To study the evolutionary genetics of brain connectivity using HAR brain gene expression	Correlation analyses of HAR brain gene expression with functional connectivity data (resting state fMRI data)	• HAR brain gene expression positively correlates with functional connectivity variability. • HAR brain gene expression is higher in limbic, default mode, dorsal, and ventral attentional and frontoparietal networks. The most correlated genes are involved in synapse development, neurogenesis, and neuron differentiation.
Luppi et al. [68] To study the evolutionary genetics of redundant and synergistic information organization using HAR brain gene expression	Correlation analyses of HAR brain gene expression with the spatial distribution of synergistic and redundant brain interactions (resting state fMRI data)	• Redundant interactions predominate in the somatomotor, salience subnetworks, and visual regions, while synergy predominates in higher-order association cortices affiliated with the DMN, frontoparietal network, and limbic subnetwork. • HAR brain gene expression positively correlates with the regional distribution of synergistic interactions. • Spatial variation of HAR brain gene expression explains up to 30% of the regional synergy-redundancy variance.
Cheung et al. [69] To test whether genes associated with intelligence are enriched in HARs	Enrichment analyses on HARs, brain expression, and their interaction on intelligence (based on GWAS data)	• Polymorphisms in HAR genes are more likely associated with intelligence than polymorphisms in other regions. • The expression of HAR genes across five developmental stages is associated with intelligence variability.

Default mode network (DMN); functional magnetic resonance imaging (fMRI); genome-wide association study (GWAS); structural magnetic resonance imaging (sMRI).

Table 4. Summarised findings of the studies assessing the role of HARs in psychiatric disorders.

Main Objective	Main Methodology	Main Results
Schizophrenia		
Xu et al. [71] To study HAR enrichment on common variability associated with SCZ	HAR enrichment analysis in SCZ (based on GWAS data) and gene co-expression network analyses	• SCZ-associated loci are enriched in genes near HARs, specifically in recently evolved HARs (pHARs). • pHAR gene associated with SCZ converge in GABA-related pathways, neuron differentiation, cell adhesion, plasma membrane, and cadherin-binding processes. • pHAR gene associated with SCZ are hub genes in regulatory networks of the human prefrontal cortex.
Srinivassan et al. [73] To study HAR enrichment of common variability associated with SCZ	HAR enrichment analysis in SCZ (based on GWAS data)	• Common genetic variants associated with SCZ are enriched in HAR regions. • HAR brain genes are enriched in SCZ-associated single nucleotide polymorphisms compared to other brain genes or other HARs (not brain-specific).
Wei et al. [66] To study the association of HAR genes and HAR brain genes with SCZ	Examination of potential associations of HAR and HAR brain genes with SCZ variability (based on GWAS data)	• HAR genes and HAR brain genes are associated with genetic variants in SCZ.
Cheung et al. [69] To study HAR gene enrichment on genes associated with neuropsychiatric disorders conditional to developmental gene-expression patterns	HAR gene enrichment analyses in five neuropsychiatric disorders (SCZ, BPD, ASD, MDD, and ADHD, based on GWAS data) conditional to gene expression in five developmental stages	• Single nucleotide polymorphisms in HAR genes are more likely associated with the risk for SCZ and MDD than polymorphisms in other regions. • HAR genes highly expressed in whole brain across development are more likely associated with the risk for SCZ.
Erady et al. [76] To investigate nORF associated with HARs in the genetic architecture of SCZ and BPD	Assess the overlap between nORF and nORF differentially expressed in SCZ and BPD and HARs. nORD and HARs overlap enrichment analyses in SCZ and BPD (based on GWAS data)	• There is an overlap between nORF and HARs regions, and some of these regions are associated with differential expression in SCZ and BPD. • Some of these nORF and HAR overlap regions harbour loci associated with SCZ and BPD through GWAS.

Table 4. *Cont.*

Main Objective	Main Methodology	Main Results
Tolosa et al. [70] To study the association of common variants in *HAR1F* gene with SCZ risk and AH in SCZ	Case–control association study (285 SCZ-spectrum disorders [221 AH and 64 no AH] and 337 HC) of *HAR1F* gene (six variants genotyped) with SCZ risk	• No allelic, genotypic, or haplotypic associations are found with the risk for SCZ. • A six-variant haplotype increases the risk for AH in patients (OR = 2.83).
González-Peñas et al. [72] To study the association of common and rare variants in *NPAS3* HARs with SCZ risk	Case–control association study (538 SCZ and 539 HC) of *NPAS3* gene (26 variants genotyped) with SCZ risk	• None of the analysed variants are associated with SCZ at allelic, genotypic, or haplotypic level.
Bhattacharyya et al. [74,75] To assess the association of variants in HARs with SCZ and cognitive performance	Case–control association study (Discovery: 494 patients and 436 healthy controls (HC); Replication: 552 patients and 551 HC) of HARs (49 variants genotyped) with SCZ risk. Case–control association study in a subsample (331 patients and 235 HC) of HARs (49 variants genotyped) with cognition variability	• Four variants are significantly associated with SCZ. Three of them interfere with transcription factor binding sites (TFBS) and have methylation marks of active promoters, repressors, or enhancers in the brain. • Five variants significantly modulate cognitive performance within controls (13 variants) or patients (six variants). All these variants interfere with TFBS, and five had methylation marks of active promoters, repressors, or enhancers.
Other neurodevelopmental psychiatric disorders and related syndromes		
Doan et al. [52] To evaluate the mutational landscape of HARs and their contribution to ASD	HAR gene mapping through in silico chromatin interaction data. Assessment of copy number variants (CNVs) in 2100 ASD-sibs sample. Assessment of rare mutations in HARs through whole-genome sequencing in 218 ASD families	• HAR genes are dosage-sensitive and enriched for associations with ASD and SCZ, especially the neurally active HAR genes. • The rare de novo CNVs are more prevalent in HARs than in other genomic regions and are associated with ASD. • Individuals with ASD have an excess of rare HAR mutations compared to non-affected individuals. The HARs harbouring these rare mutations are enriched for transcription factor binding sites. Among the genes flanking the rare HAR mutations, 70% are expressed in the brain and associated with ASD.
Won et al. [58] To study the role of HAR genes in the susceptibility for neurodevelopmental disorders	HAR enrichment analysis with genes harbouring loss-of-function variants in ASD, SCZ, and DD data	• HAR genes are enriched for loss-of-function-intolerant genes that harbour de novo mutations associated with ASD and developmental delay.
Wei et al. [66] To study the association of HAR genes and HAR brain genes with ASD variability and brain structural changes found in psychiatric disorders	Examination of potential associations of HAR and HAR brain genes with genes associated with ASD (based on rare variants of brain disorders). Correlation analyses of HAR brain gene expression with structural alterations across psychiatric disorders (sMRI data on SCZ, BPD, ASD, MDD, OCD)	• HAR genes and HAR brain genes are enriched for genes with rare variants associated with ASD compared to other regions in the genome. • Changes in brain structure observed across psychiatric disorders correlate with HAR brain gene expression patterns.
Takahashi et al. [77] To identify the molecular pathways associated with delirium and test the enrichment of HAR genes	Functional enrichment analysis of HAR genes in delirium-associated genes (obtained from the toxicogenomics database)	• The top networks associated with delirium include genes enriched in HAR brain genes.

Auditory hallucinations (AH); autism spectrum disorder (ASD); bipolar disorder (BPD); healthy controls (HC); major depressive disorder (MDD); novel open reading frames (nORF); obsessive-compulsive disorder (OCD); schizophrenia (SCZ). HARs defined based on conservation in non-human primates (pHARs); structural magnetic resonance imaging (sMRI).

3.2. HARs and Neurodevelopment

3.2.1. HARs' Function in Neurodevelopment

The first attempts to uncover the role of HARs during neurodevelopment from a genome-wide perspective were through in silico tools (Table 1). These were designed to search for specific genomic motifs that are associated with histone marks, active chromatin, and transcription factor binding sites (TFBS).

First, it was pointed out that HARs showed significant enrichment for foetal and adult brain histone profiles. These histone profiles were (i) accessible in neurogenic zones of the developing cortex and (ii) specific to neural progenitor cells and maturing neurons, thereby suggesting their activity both in the developing and adult brain [52,58,60]. Analyses indicated that >75% of HARs possessed marks of active chromatin states, of which more than 45% were active in neural and foetal brain tissue [52,58,60]. On the one hand, HAR

regions were enriched in TFBS (DNA regions where transcription factors bind to promote transcription). Indeed, many of them were associated with transcription factors specifically involved in neurodevelopment (e.g., *MEF2A, SOX2, ZNF333, REST, CTCF,* and *NFIA* [52]) and with roles during cortical neurogenesis (e.g., *RBPJ, TBR2, PAX6, ATOH1,* and *TED* [60]). In this regard, the HAR sequence comparison between humans and chimpanzees suggested that the human-specific genetic changes in HARs altered the TFBS motifs (with losses or gains of them), thus pointing towards species-differential regulation of neurodevelopmental processes [52]. On the other hand, specific assays evidenced the role of certain HARs as transcription factors per se, with a precisely regulated activity during the different neurodevelopmental periods. For example, certain HARs with enhancer activity in neural progenitor cells were enriched for transcription factor (TF) motifs belonging to cortical patterning and cell maintenance. Other HARs active in maturing neurons presented TF motifs associated with cell fate determination and cell differentiation [60].

Later, massively parallel reporter assays (MPRAs) and capture MPRAs (CaMPRA), which allow targeting HARs to evaluate their enhancer activity in vitro, have contributed to validating in silico predictions. These analyses evidenced that between 13% and 49% of HARs possessed enhancer activity in human neural cells. Additionally, they showed that between 28% and 61% of HARs presented different enhancer activity in humans when compared to chimpanzees [59,60]. While further data are needed, these HAR orthologs comparisons indicated that human-specific HAR sequence changes were mostly associated with increased enhancer activity in humans [60]. Interestingly, those HARs that presented differential activity between humans and chimpanzees overlapped with regions with different chromatin accessibility. This, therefore, suggested that human-specific sequence changes could modify chromatin conformation and accessibility, thereby producing changes in the enhancer activity [59].

3.2.2. Genes Associated with HARs and Their Expression and Functional Patterns

The genes associated with HARs (HAR genes) have been defined according to different methodologies, such as positional mapping, proximity, or functional interaction (Table 1). Positional mapping has allowed the identification of genes that include HARs within their introns or near the 5′ and 3′ UTRs. Other HAR genes, however, have been retrieved from chromatin interaction data and chromosome conformation capture [52,58]. The biological processes and pathways in which these HAR genes participate include neurodevelopmental processes, neuronal differentiation, and axonogenesis [52].

Chromatin interaction analyses in human fibroblasts evidenced that many HARs interacted with the promoters of the flanking genes and allowed the mapping of 21% of HARs to 700 target genes [52]. Nonetheless, chromatin interactions are predicted to be of high tissue- and cell-type specificity, thus suggesting that more accurate methods for HAR gene identification are required. In this sense, with the use of three-dimensional chromatin interaction data in the developing human cortex, Won et al. [58] were able to map 38% of HARs to 1648 genes that were active in the cortical plate and the germinal zone. These results again evidenced the fact that HAR genes were related to neurodevelopmental pathways. Moreover, more specific biological processes emerged, such as neural regionalisation, dorsal–ventral patterning, cortical lamination, and the proliferation of neuronal progenitors. This approach indicated that while proximity can be a good indicator of the target genes, it should not be considered the only factor. This is important since the interactions between HARs and genes are cell-type-specific. Additionally, it was described that HAR genes (such as *SOX2, PAX6, POU3F2, GLI3, EN1,* and *TBR2*) played major roles in cerebral cortex development and dorsal–ventral/anterior–posterior pattern specification [58]. Convergently, data from chromosome conformation in neural progenitor cells and human neocortical stem cells also implicated HAR genes in functions related to cell differentiation and development [59].

From a functional point of view, HAR genes identified through chromatin interaction data presented elevated dosage sensitivity levels (defined as a significantly elevated

haploinsufficiency), especially in those that were neurally active, when compared to the rest of the genes in the genome. This trait could suggest that their pathogenicity may be mediated by alterations in the expression levels [52]. In relation to this, HAR genes present loss-of-function intolerance levels that are typical of genes undergoing strong purifying selection [58]. Following on from this, the expression modulation role of HARs has been conceptualised as a human-specific source of dosage regulation [52].

Expression analyses across developmental stages evidenced that HAR genes were highly expressed during prenatal development and sharply upregulated during neurogenesis—peaking near mid-gestation, which is a period marked by neuronal migration, early neuronal phenotype definition and dendritic arborisation [58]. In addition, HAR genes were predominately expressed in cells from the outer radial glia, which is a major class of neural stem cells in the germinal layer that shows substantial expansion in the primate lineage [58]. Analyses in adult prefrontal cortex samples suggested that HAR gene expression was enriched in neuronal cell types, rather than in glia. Additionally, HAR gene expression was enriched in superficial cortical layers, which form the inter- and intra-hemispheric connections between cortical regions and are significantly expanded in primates [58].

3.2.3. Regulatory Effect of Candidate HARs on Their Proximal Genes

Complementary to whole-genome HAR analyses, different experiments have focused on validating the regulatory activity of specific HARs on candidate genes and have contributed to unravelling their role during neurodevelopment (Table 2).

While the functional analyses were conducted using different methodologies, as well as in cellular or animal models, all candidate HARs analysed thus far are expressed in neural-like cell types [47,52,58,60–64]. Interestingly, HAR1 was found to be part of two previously unknown RNA genes, *HAR1F* and *HAR1R,* which are expressed in the foetal and adult human brains, albeit with still unknown functions. In the foetal brain, *HAR1F* (also known as *HAR1A*) is expressed in the neocortex, specifically in Cajal–Retzius neurons and co-detected with reelin; meanwhile, *HAR1R* (also known as *HAR1B*) expression is less localised and intense. In the adult brain, *HAR1F* is expressed in the frontal cortex, the hippocampus, the thalamus, and the hypothalamus, while, again, *HAR1R* expression is blunted [47].

The vast majority of the 29 HARs validated operate as transcriptional enhancers of genes related to neurodevelopmental processes [52,58,60,62,63,65]. Only one HAR, HACNS174, associated with the *AUTS2* gene, did not evidence conclusive enhancer function [64]. Meanwhile, two other HARs performed distinct roles: HAR1 was an integral part of two RNA genes [47], and the HAR flanking the *HSTR1* gene functioned as its promoter [61].

From a functional point of view, the analysed HARs were found to interact with genes that perform crucial roles for brain development and wiring, such as being associated with developmental signalling pathways and homeostasis maintenance, such as *NPAS3*, *AUTS2*, and *GLI2* [62–64]; being related to neuronal differentiation and proliferation, such as *PTBP2* or *PPP1R17* [52,60]; and being implicated in synaptic and cortical development, such as *FZD8*, *CUX1*, *GPC4*, *GLI3*, and *TBR1* [52,58,65]. Indeed, genes such as *PPP1R17*, which regulates the neural progenitor cell cycle progression, as well as the slowing down and lengthening of the neural progenitor cell cycle, are of particular interest for human-specific cortical development. As described by Girskis et al. [60], the *PPP1R17* expression in humans was restricted to foetal development, driven by cortical neural progenitor cells in contraposition to the macaques' expression, which continued in adulthood in cortical astrocytes.

The studies that evaluated the effect of HARs' human-specific substitutions describe that those genetic changes that had functional consequences were related either to higher promoter activity [61] or to a gain or loss of transcription factor binding sites [62,63,65]. Additionally, the studies that compared HAR ortholog sequences among humans, primates, and mice also reported human expression differences during neurodevelopment compared

to the other species [61–63,65]. For instance, analyses in transgenic mice evidenced that the human-specific substitutions in the enhancer HARE5 associated with the *FZD8* gene produced faster cell cycles when compared to the HARE5 ortholog sequences in chimpanzees and mice. Furthermore, this, in turn, resulted in greater cortical gyrification and larger mice cortices [65]. Moreover, transgenic mice expression analyses also showed that while both the human and chimpanzee HARE5 orthologs were expressed in the developing telencephalon, human HARE5 expression occurred earlier and more intensely [65].

3.3. HARs and Brain and Cognitive Phenotypes

If HARs participate in the neurodevelopmental gene expression machinery, it appears obvious to investigate their involvement in shaping brain architecture and the cognitive traits derived from it (Table 3).

Under this rationale, after describing that the frontoparietal and the default mode networks were the cortical networks that experienced the larger expansion in humans when compared to chimpanzees, Wei et al. [66] analysed the implication of HAR genes. First, they showed that the spatial expression trajectories of HAR genes and the patterns of cortical expansion positively correlated. Second, the regions of higher-order cognitive networks with the larger expansion in humans (which included the frontoparietal, ventral–attentional, and default mode networks) were also the same networks with the higher expression of HAR genes. With respect to this, the highest correlation was observed within regions of the default mode network (DMN). These results remained significant even when the HAR gene set was limited to those specifically related to brain processes (HAR brain genes) [66]. Indeed, the comparative expression analyses in humans, chimpanzees, and macaques evidenced the fact that the elevated expression of HAR brain genes observed in higher-order cognitive networks in humans was not found to be as high in chimpanzees and macaques. This points towards the fact that, in humans, HAR genes are upregulated in brain areas related to higher-order cognitive functions. Additionally, functional magnetic resonance imaging (fMRI) analyses in healthy subjects revealed that the genetic variability of HAR brain genes affected the functional modulation of the DMN, in contrast to the results in other functional networks [66].

The use of HAR genes and HAR brain genes expression patterns to unravel the genetic determinants of other brain phenotypes is a strategy followed onward in other studies. On the one hand, a study investigating individual differences in functional connectivity described that the highest functional variability across individuals was observed within higher-order cognitive modules such as the frontoparietal network, the dorsal–ventral attentional network, and the DMN [67]. Subsequent analyses on how functional connectivity spatial variability covaried with gene expression patterns revealed that HAR brain gene expression positively correlates with functional variability. An increasing HAR brain gene expression was described from the subcortical regions and primary areas (which showed the lowest individual variability) to the association cortices (with the highest individual variability and the highest HAR expression levels). Actually, HAR brain gene expression accounted for up to 31% of functional connectivity interindividual variability. In line with the findings from Wei et al. [66], HAR brain gene expression was found to be higher in the DMN, dorsal–ventral attentional network, and frontoparietal network. In addition, the most correlated genes were related to the development of the synapses, neurogenesis, and neuron differentiation.

On the other hand, in a cutting-edge study that utilised multimodal neuroimaging analyses and information decomposition methods, Luppi et al. [68] studied the brain's neural information processing in terms of redundant (or shared information, which provides robustness to the system) and synergistic (or complementary information, which provides integration to the system) brain interactions. Their analyses evidenced that synergistic and redundant brain interactions showed a regional gradient, whereby the redundant patterns were prominent in the brain's somatomotor, salience subnetworks, and visual regions. Meanwhile, the synergy was predominant in higher-order association cortices that were

affiliated with the DMN, the frontoparietal network, and the limbic subnetwork. Human and macaque comparisons highlighted high evolutionary stability but also showed the exceptionality of the prefrontal cortex, which was a synergy-dominated region in humans. This high human prefrontal cortex synergy also correlated with the human-specific cortical expansion previously reported [66], thus suggesting that the additional cortical tissue in humans may be dedicated to synergistic rather than redundant interactions. However, the relevance of these findings for our matter comes when the authors described that the regional predominance of synergy correlated with the regional expression of HAR brain genes, which explained up to 30% of the regional synergy–redundancy variance.

Regarding cognitive phenotypes, the study by Wei et al. [66] took advantage of GWAS data to investigate HARs' implications in cognitive and social abilities. First, gene-set analyses of HAR genes and HAR brain genes evidenced their association with intelligence. Second, the same gene-set effects modulated a proxy of sociability, which derived from a single question evaluating the frequency of friend and family visits. Also, using intelligence GWAS data, Cheung et al. [69] described that the variability within HAR genes was more likely to be associated with intelligence than SNPs not affiliated with HARs. Additionally, thanks to brain gene expression data across neurodevelopmental stages (from early prenatal to adulthood), their findings highlighted that HAR genes highly expressed in the brain across the different neurodevelopmental stages are associated with intelligence.

3.4. HARs and Psychiatric Disorders

3.4.1. HARs in Schizophrenia

While using different methodological frameworks, many attempts to investigate HARs' link with susceptibility to SCZ have been conducted through enrichment analyses. Enrichment methodologies assess whether SNPs associated with SCZ significantly cluster in HAR regions [73], HAR genes [69,71], and HAR brain genes [69] (Table 4).

First, Xu et al. [63] compared the overlap between the GWAS loci that were associated with SCZ, and the genes associated (within 100kb flanking regions) with recently evolved HARs. These recently evolved HARs, named pHARs, were defined based on conservation among non-human primates. Moreover, other ancient accelerated regions, PARs (primate accelerated regions based on conservation among non-primate mammals), and mHARs (based on the conservation among non-human mammals) were investigated. The results indicated that all the SNPs associated with the disorder at p-value $< 1 \times 10^{-7}$ were significantly enriched within the genes in pHARs as compared to PARs and mHARs. This means that pHARs harboured more SCZ-associated SNPs than would be expected by chance. Subsequent pathway and biological processes analyses in pHARs highlighted the involvement of GABAergic pathways, which, as pointed out by the authors, have been consistently described as dysregulated in SCZ [78]. Moreover, pHAR variability converged in genes related to neuron differentiation, cell adhesion, plasma membrane, and cadherin binding, categories related to brain development and synapse formation, following previous findings [52,58]. Network analysis on gene expression profiles from the human cortex revealed that the pHAR genes associated with SCZ were more connected than other SCZ genes. This, therefore, entails the fact that these pHAR genes were hub genes in the human cortex expression network [71].

Second, Srinivasan et al. [65], through fold enrichment plots, also showed that SNPs within HARs (and in linkage disequilibrium with them) were enriched in susceptibility variants for SCZ. The same analysis but specifically focused on HAR brain genes delivered the same conclusion.

Third, Cheung et al. [69] also evidenced that the genetic variability within HAR genes was significantly enriched with respect to associations with SCZ. In addition, the HAR genes highly expressed in the brain across different neurodevelopmental stages were also associated with this disorder. These enrichment findings would align with previous gene-set results demonstrating that HAR genes and HAR brain genes were associated with genetic variants underlying SCZ [66].

Beyond HAR enrichment findings through genome-wide approaches, there are also data linking subsets of HARs with SCZ. Novel open reading frames (nORF) are genomic loci able to encode for uncharacterised transcripts and protein products. A slight overlap between nORF regions and HARs has recently been described. Some of these overlapping regions have been found to harbour loci associated with SCZ and bipolar disorder through GWAS studies [76]. Furthermore, focusing on the nORFs differentially expressed between healthy controls and patients with SCZ, a HAR overlap was also described. As such, these findings relate HARs with novel sources of transcription and with altered expression in SCZ.

Lastly, candidate HAR genes have also been investigated in SCZ susceptibility through association studies in European and Indian populations. While common and rare single nucleotide variants in the *HAR1A* and *NPAS3* genes were not significantly associated with the risk for SCZ [70,72] in European samples, a six-SNP haplotype in *HAR1F* was associated with the presence of auditory hallucinations in SCZ patients [70]. Conversely, four SNPs in candidate HARs were significantly associated with the disorder's risk in two independent case–control samples from a north-Indian origin [75]. Additionally, 15 SNPs significantly modulated cognitive performance within either controls or patients from a subsample with cognitive assessments including attention, spatial and working memory, sensory-motor and emotional processing, and abstract and mental flexibility [74]. Nearly all the associated SNPs interfered with TFBS and possessed methylation marks of active promoters, repressors, or enhancers in different brain regions [74,75].

3.4.2. HARs in Other Neurodevelopment-Related Psychiatric Disorders and Syndromes

Although most of the genetic association and enrichment studies of HARs have been conducted in SCZ, if we consider the etiological and pathophysiological continuum across different psychiatric disorders [12] it is inevitable to investigate the role of HARs in other neurodevelopment-related disorders that have previously shown certain genetic and symptomatic overlap with SCZ (Table 4).

In contrast to SCZ studies, the analyses of HARs on ASD have been mainly focused on rare and de novo variability [52,58,66]. When using a sample of 2100 sibling–ASD dyads, Doan et al. [52] observed that rare, previously unidentified (de novo) copy number variants containing HARs were more frequent in ASD probands than in healthy siblings. Afterwards, the impact of mutations in HARs was assessed in 218 consanguineous ASD families (first-cousin marriages, with higher ASD genetic load). In addition, the sequencing of the HARome revealed that affected individuals presented a significant excess of rare mutations, which remained significant even when the analysis was limited to neurally active HARs. These results converge with findings from studies examining the enrichment of HAR genes and HAR brain genes in rare, damaging, and de novo variants. Not only were the rare variants associated with ASD more prevalent within HAR genes and HAR brain genes [66], but rare de novo loss-of-function mutations associated with ASD and developmental delay were also more prevalent within HAR genes [58]. In parallel, analyses on the functional impact of several HAR mutations in ASD-affected individuals through MPRA assays in mouse embryonic cortex neurospheres revealed a higher prevalence of mutations in conserved loci with predicted regulatory function when compared to non-conserved loci. This suggests that variability in HARs may contribute to altering pathophysiological mechanisms in ASD [52].

HAR genes have also been associated with brain structural changes across psychiatric disorders. In particular, it was described that HAR brain gene expression correlated with cortical volume changes that were found across SCZ, ASD, bipolar disorder, major depression disorder, and obsessive-compulsive disorder [66].

Finally, HAR brain genes have been associated with delirium, which is a clinical syndrome characterised by fluctuating disturbances in attention, consciousness, and cognition [79,80]. Through protein–protein interaction network analyses that were used to prioritise

genetic pathways associated with a certain condition, it was observed that HAR brain genes were hub genes and among the top network modules associated with delirium [77].

4. Discussion

The present review offers an overview with respect to the role of an evolutionary marker, the HARs, as gene expression regulatory elements during human neurodevelopment. This role is supported by results not only showing HARs' impact in the context of brain architectural and functional configuration of human-specific traits, but also in the inherent cognitive and behavioural human diversity. Also, data on the association of genetic variants in HARs with the vulnerability for schizophrenia and other psychiatric disorders strengthen the idea that HARs may contribute to brain development and function, thus eventually leading to mental disorders.

Recalling the findings on the role of HARs in neurodevelopment, many pieces of evidence converge on the idea that HARs possess paramount roles—presumably as enhancers, but also as other gene expression modulators—concerning genes that guide processes, such as neural proliferation and differentiation [52,58–60]. Nonetheless, not all HAR sequences have been functionally validated and thus, unequivocal conclusions are still missing. In addition, as seen by the temporal and tissue specificity of HAR expression [52,58,60], it has been possible to infer the temporal window in which HARs operate, the early neurodevelopment, and the tight expression regulation to which they are subject. Still, as previously suggested [50,81], new research is required to assess the role of HARs in other developmental tissues, stages, and in their native environment.

Focusing on the mechanisms by which HARs exert their effects, certain findings indicate that HARs potentially act as binding sequences of transcription factors that are specifically involved in neurodevelopment and cortical neurogenesis. However, they may also function as transcription factors per se, which are related to cortical patterning, cell fate determination, maintenance, and differentiation [52,60]. Likewise, the fact that between 30 and 60% of HARs show enhancer activity differences between humans and chimpanzees seems to indicate how HARs would have contributed to the human neurodevelopmental uniqueness. Indeed, human-specific substitutions in HARs would change the transcription factor binding landscape [59,60] and would lead, in turn, to changes in gene expression timing, intensity, and patterns and cell cycle lengthening. These expression changes would even result in major phenotypic changes, such as greater cortical gyrification and enlargement, because certain studies on HAR gene expression profiles show such effects [58,66]. Such evidence reinforces the view of the HARome as a contributor to the emergence of human-specific traits and underlying human behavioural diversity.

Beyond the human interindividual variability associated with HARs, there is evidence highlighting their role in human-specific disorders intimately related to brain and conduct traits. Among the genetic determinants shared across neurodevelopmental psychiatric disorders such as SCZ and ASD, the genomic regions that comprise transcription factors regulatory sites stand out, identifying them as a genetic mechanism of interest [82]. In addition, it is remarkable that these regulatory elements are described as potential coregulators of genes that are related to nervous system development, transcription, and synaptic transmission [82]. Therefore, by their neurodevelopmental guidance role, HARs may harbour common genetic determinants shared between different brain phenotypes and psychiatric disorders.

Moving on to the studies directly inspecting HARs' role with respect to brain phenotypes, all results describe correlations between HAR expression and (i) the human organisation of higher-order cognitive networks [66], (ii) individual functional connectivity variability [67], and (iii) the brain's synergistic and redundant information processing patterns [68]. These findings point towards the HARs' involvement in the cortex structural organisation, brain information integration and, ultimately, the brain's functional and cognitive responses. Indeed, the functional networks highlighted by the different studies have been described not only to be altered in psychiatric disorders [83–86], but also to sustain

complex cognitive processes and social cognition [87,88]. Indeed, these are core traits of humankind [89] that are, in turn, commonly compromised in SCZ and other psychiatric disorders such as ASD [90,91]. Also, we should also consider the results on human-specific brain network organization and its association with brain dysconnectivity in SCZ [92]. It was evidenced that the transcription profile of HAR genes significantly correlated with cortical areas that display human-specific connections when compared to chimpanzee connection features. In addition, the identified human-specific cortical connections mirrored the regions where SCZ patients presented lower fractional anisotropy [92]. Therefore, indirectly, these observations add to the key role of HARs in shaping the architecture of these networks and sustaining human cognitive abilities and the alterations in SCZ and other neurodevelopmental psychiatric disorders.

As is the case with investigations on the association of HARs with psychiatric phenotypes—while genome-wide based studies have consistently reported an enrichment of SCZ-associated variants in HAR regions [52,58,66,71,73]—the candidate HAR approaches have shown less consistent findings [70,72,74,75]. Conversely, other studies have successfully highlighted the role of candidate HARs as susceptibility loci for SCZ and other mental disorders, as is the case of the *NPAS3* [93–95]. In line with this, the *AUTS2* gene has been associated with ASD and other disorders, such as attention deficit hyperactivity disorder, epilepsy, and dyslexia [96–98]. Thus, future association studies would greatly benefit from the selection of different candidate HARs, such as those that function as neurodevelopmental enhancers of the *AUTS2, CUX1, GPC4, GLI2, GLI3,* and *TBR1* genes, which have been related to the biological roots of ASD and SCZ [52,93,99–103]. However, apart from the implication of common genetic variability in the susceptibility for these disorders, there are also data directly involving rare and more penetrant variants [52,58,66]. Independently of the frequency of the genetic determinants, there appears to be a shared HAR-related genetic variability underlying psychiatric disorders, especially SCZ and ASD. As studies on the shared symptomatology and genetic variability suggest [13], if we understand neuropsychiatric disorders in a neurodevelopmental continuum [12], rather than as discrete clinical entities, we could consider HARs as overlapping genetic signals that disrupt common biological mechanisms. These biological mechanisms would converge in neurodevelopmental pathways and involve processes guiding neural proliferation, differentiation, architectural organization, and functioning. Supporting this view, there are results showing that among human diseases, HAR genes are mostly associated with cognitive disorders and nervous system diseases [104].

All these data can be integrated under the umbrella of the human brain evolution hypotheses that postulate that the larger human neocortex could arise from an increasing number of cortical neurons generated in the germinal zones during foetal development [35,37,105,106]. This neocortical expansion would be driven by a greater and prolonged proliferative capacity rather than due to the differentiation capacity of the human neural stem and progenitor cells, which are differences indicative of a species-specific transcriptomic regulation of neocortex development [37,107]. In this sense, findings regarding the interaction between HARs and genes that are related to neuronal differentiation and proliferation—such as *PTBP2* or *PPP1R17* [52,60]—together with the specific enrichment of HAR genes in radial glia during neurodevelopment [58], are highly relevant. The radial glia is a major class of neural stem cells, common progenitors of neurons and oligodendrocytes that give rise to neurons and glial cells [108], which show several unique human features that pave the way for the human-specific expansion of the cortex [109]. Hence, if HARs somehow contribute to guiding the expression mechanisms of radial glia cells, these evolutionarily relevant genomic regions would be part of the neurodevelopmental program that renders the human neocortex expansion unique. Indeed, among the characteristics of human-specific cortical expansion—as compared to our closest relatives, the chimpanzees—there are differences in the proliferative capacity of neural progenitors during cortical development [37]. This is, in turn, what the radial unit hypothesis states: that the expansion of the cortical surface area is driven by the proliferation of neural progen-

itors, while the thickness is determined by the number of their neurogenic divisions [110]. Certainly, this hypothesis has been supported by the latest ENIGMA Consortium GWAS results [111], which describe different developmental mechanisms behind surface area expansion and cortical thickness increase. Then, the surface area would be influenced by genetic variants that alter gene regulatory activity in neural progenitor cells during foetal development. On top of that, surface area genetic determinants positively correlate with those of cognitive functioning and educational attainment, which thus entails the fact that the genetic background underlying these phenotypes shows evidence of bidirectional causation [111]. Moreover, radial-glia-specific SCZ polygenic risk has been related to neuroplasticity processes in the hippocampus [112], which is a brain structure with critical roles in terms of memory and associated cognitive dimensions [113,114]. All this evidence together suggests that sequence changes in HARs could display critical roles in radial glial neurodevelopmental features. Furthermore, it also opens the possibility that part of the variability in HAR regions could influence the brain and cognitive phenotypes as well as the SCZ-related brain structural changes.

Globally, the relationship between HARs and human-specific gene regulation fits with the hypothesis of "human evolution as a non-coding revolution" [115]. Moreover, it also aligns with the evidence that remarks the importance of gene expression regulatory mechanisms, rather than the genetic products themselves, in the pathophysiology of neurodevelopmental disorders [116–119]. Therefore, future studies assessing the role of HARs in mental disorders and the associated neurobiological pathways may help to jointly address two hypotheses on the origins of these disorders—both the neurodevelopmental and the evolutionary.

5. Future Perspectives

The increasing knowledge regarding the functions of HARs and the biological mechanisms in which they are involved opens new investigation venues. First, as we have underlined, many HARs have not been successfully validated, and while their biological functions are presumed based on motif-prediction algorithms, there is a need for new functional studies in order to deepen our understanding regarding their specific role. In this sense, the use of models closer to the biological reality, such as iPSC or brain organoids that can model corticogenesis, could be beneficial not only to validate HARs' function in human neurodevelopment, but also to investigate the neurodevelopmental ontogenetic differences in patients suffering from neurodevelopmental-related psychiatric disorders. Second, to gain insights into the pathogenesis of neurodevelopmental disorders, especially with respect to SCZ or ASD, HARs' functional data should be combined with results on rare genetic variants, such as those coming from whole-genome sequencing approaches. Therefore, the prioritisation and interpretation strategies in whole-genome sequencing approaches should consider not only exonic or promoter variants, but also regulatory regions such as HARs. Third, it should be considered that more than 30% of the approved drugs, especially those for neurological disorders, target at least one HAR gene [104]. This opens novel investigation possibilities and puts HARs in the spotlight for developing novel therapeutic strategies. Notwithstanding, HARs should not be the only evolutionary relevant markers being inspected in the context of human-specific neurodevelopment and psychiatric susceptibility [58,120–123]. Similarly, in order to further understand the role of HARs in the architectural and information processing basis of the human brain, new structural and fMRI studies to evaluate whether HARs' genomic variability also influences specific cognitive and psychopathological processes dominated by the DMN and the frontoparietal network should be encouraged.

6. Conclusions

The human brain responds adaptively and exhibits sophisticated control, purposeful behaviour, and complex cognitive abilities due to the neurodevelopmental processes, which have evolved such that they guarantee a brain architecture supporting neural integrations

to, in turn, sustain these abilities. During this evolutionary process, HARs have emerged as genomic regions that have accumulated human-specific genetic changes. In this review, we have compiled the data showing that HARs participate in the genomic regulatory machinery of genes that are related to neural proliferation, differentiation, and axonogenesis, which converge in neurodevelopmental pathways. In addition, we have shown that human-specific changes in HARs endow this neurodevelopmental machinery with unique characteristics. Moreover, studies converge into the idea that HARs underlie, to a certain extent, human-specific cortical expansion, and neural integration, especially with respect to the regions within higher-order functional networks.

Despite the tight neurodevelopmental control needed that is required for proper brain function, there is still room for not only the individual differences in neurodevelopmental trajectories—which, in turn, sustain the inherent variability in cognition, intelligence, behaviour, and sociability traits found in humans—but also the eventual dysfunctions leading to neurodevelopmental disorders. As such, in this review, we have shown that HARs modulate individual differences in the context of brain functioning, but also that common and rare genetic variability in HARs is associated with neurodevelopmental psychiatric disorders, such as SCZ and ASD (Figure 2).
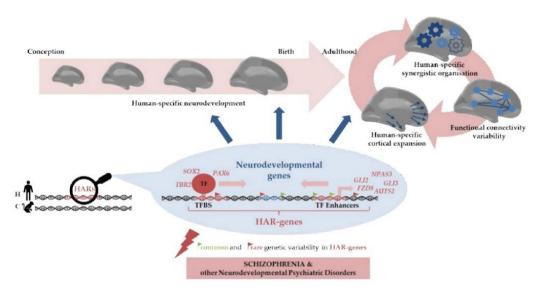
Figure 2. Graphical summary of Human Accelerated Regions' (HARs) impact on neurodevelopment, brain configuration, and associated psychiatric disorders. Middle panel: HARs are evolutionarily conserved genomic regions across mammals' evolution that accumulated human-specific (H) changes since the divergence from chimpanzees (C). Molecular studies evidence that HARs function as transcription factor binding sites (TFBS) or transcription factors (TF) of genes involved in neurodevelopmental pathways [52,58–60]. Top panel: The key roles of HARs in the neurodevelopmental gene regulatory machinery underlie the human-specific neurodevelopmental characteristics sustaining human-specific brain architecture, information processing, and variability in neural integration, brain traits, which in turn are associated with different psychiatric disorders [66–68]. Bottom panel: Common and rare genetic variability in HARs and HAR genes is related to changes in the neurodevelopmental trajectories, and therefore, sustains the inherent human variability in cognition, intelligence, behaviour, and sociability traits [66,69], but also the neural dysfunction observed in the neurodevelopmental disorder continuum. Additionally, the described effect of HAR genomic variability on the genetic determinants underlying schizophrenia and other neurodevelopmental psychiatric disorders [52,58,66,69,71,73] strengthens the paramount role of HARs in the neurodevelopmental machinery.

Thus, HARs may be the genomic regions that deserve further investigation to bridge the gap between the neurodevelopmental and evolutionary aetiological hypothesis of human-specific disorders such as schizophrenia. Future research into the molecular mechanisms that are associated with HARs will help us to understand the evolutionary changes underlying brain configuration as well as the susceptibility to human brain disorders. Achieving this will most likely result in fundamental effects on the disciplines of neuroscience and biomedicine.

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Study 5.

Human-specific evolutionary markers linked to foetal neurodevelopment modulate brain surface area in schizophrenia.

Guardiola-Ripoll M, Almodóvar-Payá C, Arias-Magnasco A, Latorre-Guardia M, Papiol S, Canales-Rodríguez EJ, García-León MA, Fuentes-Claramonte P, Salavert J, Tristany J, Torres L, Rodríguez-Cano E, Salvador R, Pomarol-Clotet E, Fatjó-Vilas M.

.

1 **Human-specific evolutionary markers linked to foetal neurodevelopment modulate brain surface area**

2 **in schizophrenia.**

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1

29   **ABSTRACT**

30   Schizophrenia (SZ) is hypothesised to represent a costly trade-off in the evolution of the neurodevelopmental

31   ontogenetic mechanisms associated with human-specific cognitive capacities. Human Accelerated Regions

32   (HARs) are evolutionary conserved genomic regions that have accumulated human-specific sequence

33   changes. These evolutionary markers function as neurodevelopmental transcription enhancers and have

34   been associated with the brain's cortical expansion and connectivity, the processing of neural information,

35   and the risk for SZ. We sought to investigate whether HARs' polygenic load influenced neuroanatomical

36   measures.

37   Our sample consisted of 128 patients with SZ and 115 healthy controls with high-resolution structural T1 MRI

38   and genome-wide genotyping data. We extracted the cortical thickness (CT) and surface area (SA) for the

39   34 Desikan-Killiany regions per hemisphere. We calculated four polygenic risk scores (PRS): SZ genetic load

40   (Global PRS_{SZ}), HARs' specific variability (HARs PRS_{SZ}), HARs' variability associated with transcriptional

41   regulatory elements uniquely active in the foetal brain (FB-HARs PRS_{SZ}) and in the adult brain (AB-HARs

42   PRS_{SZ}). Through linear regression analyses, we explored whether these four PRSs modulated CT and SA

43   within diagnostic groups and the PRSs and diagnostic interaction on the cortical measures.

44   Results indicate that FB-HARs PRS_{SZ} influenced patients' right SA on the lateral orbitofrontal cortex, the

45   superior temporal cortex, the pars triangularis and the paracentral lobule and that a higher SZ risk load in

46   FB-HARs is associated with lower SA values.

47   These findings evidence the involvement of the HARs-foetal gene regulatory activity in SA architecture and

48   the evolutionary component of this regulation in SZ. These data emphasise the importance of HARs in the

49   transcriptional regulatory machinery from early neurodevelopment and their role as the bridging point

50   between the neurodevelopmental and evolutionary hypotheses in SZ.

51   **Key words:** Human Accelerated Regions; Evolution; Schizophrenia; Polygenic Risk Score; MRI; Cortical

52   Surface Area; Cortical Thickness

2

1. INTRODUCTION

Schizophrenia (SZ) is a complex neuropsychiatric disorder characterised by symptoms that alter the perception and behaviour, such as hallucinations and delusions, and affectations of higher-order cognitive functions. These symptoms intimately relate the disorder with traits that distinguish humans as a species: abstraction, social cognition, language and thinking (Polimeni & Reiss, 2003). Accordingly, while the foundations of this multifactorial and complex disorder are not entirely understood, multiple pieces of evidence straightforwardly point towards a neurodevelopmental and evolutionary origin.

On the one hand, the strong genetic background of SZ, with heritability estimates up to 80% and a polygenic architecture with thousands of genetic variants with additive effects (Hilker et al., 2018; Legge et al., 2021; Purcell et al., 2009; Sullivan et al., 2003), converge with recent molecular data showing the role of developmental, neuronal, and synaptic differentiation pathways in the aetiology of the disorder (Gulsuner et al., 2013; O'dushlaine et al., 2015; Trubetskoy et al., 2022). The neurodevelopment processes and the neuroplasticity of the human brain are tightly orchestrated and involve gene expression regulatory mechanisms that are of paramount importance (Davidson, 2006). In line with the neurodevelopmental hypothesis of SZ, among the different environmental factors associated with an increased risk, prenatal and early adverse events occurring during crucial periods of brain development have been highlighted from epidemiological and clinical population-based studies due to their higher prevalence among people who later develop SZ (Rapoport et al., 2005). Also, the presence of prenatal complications was correlated with a higher genomic risk for the disorder (Ursini et al., 2018), and the placenta-associated genomic load has been linked to reduced brain volumes in neonates and poorer cognitive development during the first two years of life, emphasising the importance of the prenatal period in neurodevelopmental paths of risk (Ursini et al., 2021). In this line, delayed developmental milestones, considered the reflection of early deviances in neurodevelopmental trajectories, are associated with the disorder and predict psychotic symptoms in childhood and adulthood (Cannon et al., 2002; Niemi et al., 2003; Sørensen et al., 2010). Another aspect to be taken into account, is that prior to the onset of the psychotic symptoms, patients with SZ already present brain structural differences, such as reduction of grey matter, disrupted white matter integrity, and widespread cortical thinning (Haijma et al., 2013; Harrison et al., 2003), which latter remain once the diagnosis is settled (Jauhar et al., 2022; van Erp et al., 2018). Among the neuroanatomical measures used to characterise brain cortical morphology, cortical thickness (CT) and cortical surface area (SA) are highly heritable and influenced by largely independent genetic factors related to adult and mid-foetal active regulatory elements, respectively, but both related to neurodevelopmental genetic control (Grasby et al.,

84   2020; Panizzon et al., 2009; Strike et al., 2019). All this evidence sustains the prevailing hypothesis that SZ

85   results from environmental and genetic interactions modulating and deviating neurodevelopmental

86   trajectories during the intrauterine and perinatal periods as well as during childhood and early adolescence

87   (Birnbaum & Weinberger, 2017; Kahn et al., 2015) that disrupt the ontogenetic plan guiding brain

88   architecture, brain configuration, and brain functioning.

89   On the other hand, the common prevalence of the disorder (nearly 1% (Charlson et al., 2018; J. McGrath et

90   al., 2008; Perälä et al., 2007), and the fact that people affected, particularly males, have a reduced rate of

91   reproduction (fitness) compared with the non-affected population (Haukka et al., 2003; J. J. McGrath et al.,

92   1999) raise a question on why the genetic variants that increase the likelihood of suffering from SZ have

93   persisted in the human genome? These, together with the close relationship between several clinical aspects

94   of the disorder and human-specific cognitive traits (Polimeni & Reiss, 2003), have boosted the

95   evolutionary view of the disorder. Accordingly, the evolutionary hypothesis of SZ suggests that the disorder

96   emerged as a costly trade-off in the evolution of the ontogenetic mechanisms guiding human-specific

97   neurodevelopment and sustaining complex cognitive abilities (Burns, 2004, 2006; Crow, 2000; Wynn &

98   Coolidge, 2011).

99   While the evolutionary traces of SZ are difficult to follow, comparative genomics may be advantageous. The

100   study of human-specific genomic changes may lead to a better comprehension of human-specific phenotypic

101   traits and increased knowledge of what genetic changes contributed to making us human (O'Bleness et al.,

102   2012). In this sense, Human accelerated regions (HARs) might be helpful. HARs are evolutionary conserved

103   genomic regions that have experienced significant changes after human and chimpanzee divergence (Bird

104   et al., 2007; Bush & Lahn, 2008; Gittelman et al., 2015; Lindblad-Toh et al., 2011; Pollard, Salama,

105   King, et al., 2006; Prabhakar et al., 2006). This accelerated divergence of HARs is suggested to reflect

106   their role in some human-specific characteristics. Most HARs are intergenic, within introns near protein-

107   coding genes, transcription factors and DNA binding proteins (Capra et al., 2013; Doan et al., 2016;

108   Hubisz & Pollard, 2014; Pollard, Salama, Lambert, et al., 2006; Won et al., 2019). All the studies that

109   intended to characterise HARs' functional role converge in highlighting them as transcription factors binding

110   sites, transcription factors on their own and participants in the neurodevelopmental gene expression

111   machinery (Doan et al., 2016; Girskis et al., 2021; Uebbing et al., 2021; Won et al., 2019).

112   Recently, studies inspecting the expression patterns of HARs-associated genes (HARs-genes) show their

113   implication in human-specific cortical expansion, brain functional connectivity and the brain's neural

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114   information processing. First, in a comparative study exploring the cortical expansion in humans and

115   chimpanzees, it was described that the expression profiles of HARs-genes correlated with the expansion of

116   higher-order cognitive networks, such as the frontoparietal and the default mode networks (Wei et al., 2019).

117   The same study also revealed that the genetic variability in HARs-genes expressed in the brain (HARs-brain

118   genes) was associated with the DMN functional variation in healthy subjects (Wei et al., 2019). Second,

119   HARs-brain genes expression patterns have been related to the individual variability in functional

120   connectivity and to the brain's information processing (Li et al., 2021; Luppi et al., 2022). Remarkably, these

121   studies report that HARs-brain genes show the highest expression in higher-order cognitive networks, such

122   as the frontoparietal and the default mode networks; the ones with the greatest functional heterogeneity

123   across individuals and also the ones with predominant synergistic interactions.

124   The evidence on HARs' contribution to human-specific brain architectural configuration, functioning and

125   information processing is also accompanied by studies that describe that HARs' genetic variability influences

126   the risk for SZ. For example, the investigation into the overlap between HARs and SZ GWAS SNPs showed

127   that SZ's polygenic background was enriched in genes associated with these evolutionary regions (Xu et al.,

128   2015). In line, subsequent findings also described that SNPs in HARs or in linkage disequilibrium with them

129   were more likely associated with the disorder (Srinivasan et al., 2017). Notwithstanding, to our knowledge,

130   HARs modulation of brain measures in SZ has been scarcely explored, and further studies using brain-

131   based phenotypes to assess their role in the disorder are necessary.

132   Considering the polygenic nature of both cortical structural configuration and SZ's susceptibility, studies

133   using measures summarising this complex genetic architecture, such as Polygenic Risk Scores (PRSs),

134   would be helpful to disentangle the genetic roots not only of the disorder but of complex brain traits. The

135   PRS is a quantitative measure of the genetic burden of a trait based on GWAS data. It can be calculated at a

136   whole-genome level, but also within subsets of SNPs defined based on their involvement in particular

137   biological pathways of interest. Therefore, based on the evidence of HARs' role in neurodevelopment, brain

138   configuration and susceptibility for SZ, we aimed to investigate the modulatory effect HARs' polygenic load

139   on neuroanatomical measures through a neuroimaging genetics approach in healthy control and patients

140   with SZ. We generated different PRSs summarising HARs genetic variability, specifically including HARs

141   SNPs related to active regulatory elements in the foetal and adult brain. We explored whether the PRSs

142   modulated cortical thickness and surface area differently depending on the health/disease condition.

5

143   **2. MATERIAL AND METHODS**

144   **Sample**

145   The initial sample consisted of a case-control dataset of 378 individuals, of which 284 passed both the

146   genetic and neuroimaging quality control (see details in the corresponding *Molecular analyses* and *MRI data*

147   *acquisition* sections). Patients were recruited from the inpatients and outpatients at the Hospital Benito

148   Menni, Sant Boi de Llobregat (Barcelona, Spain) and healthy controls were recruited from the same area.

149   The patients' diagnosis was confirmed according to DSM-IV-TR based on an interview with two psychiatrists.

150   All participants were of European ancestry, between 18 and 65 years old, right-handed and had an

151   estimated intelligence quotient (IQ) (premorbid IQ in patients), higher than 70, as assessed using the Word

152   Accentuation Test (Gomar et al., 2011). All participants met the same exclusion criteria, which included

153   suffering from major medical illness, conditions affecting cognitive or brain function, neurological conditions,

154   history of head trauma with loss of consciousness and present or history of drug abuse or dependence.

155   Additionally, for healthy controls (HC), exclusion criteria also included personal or family history of psychiatric

156   service contact or treatment.

157   Individuals in both diagnostic groups were matched by age and sex to minimise age and sex differences

158   between them, and the analyses were conducted in a sample of 115 HC and 128 patients with a SZ

159   diagnosis (**Table 1**).

160   All subjects signed a written consent after being fully informed about the procedures and implications of the

161   study, approved by the Germanes Hospitalàries Research Ethics Committee, and performed following its

162   guidelines and in accord with the Declaration of Helsinki.

163   **Table 1.** Sample characteristics, including demographic and clinical description. All the quantitative variables include
164   mean and standard deviation (sd). Sex description includes male/female count (% of males). Illness duration is given in
165   years and Chlorpromazine (CPZ) equivalents in mg/day.

	Healthy Controls	**Patients with SZ**	
Age	38.44 (11.98)	40.15 (10.96)	t-test=-1.17, p=0.24
Sex	60/55 (52.20%)	82/46 (64.10%)	$\chi 2$=3.53, p=0.06
Premorbid IQ	103.52 (8.43)	101.05 (9.16)	t-test=2.18, p=0.03
Illness duration[1]	–	17.30 (10.74)	
CPZ equivalents[2]	–	581.28 (573.64)	

166   [1]Illness duration was estimated by subtracting the age at onset to the current age and was available for 122 patients.

167   [2]CPZ equivalent dose data were available for 126 patients.

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168   **Molecular analyses**

169   Genomic DNA was extracted either from buccal mucosa through cotton swabs using ATP Genomic Mini Kit

170   Tissue (Teknokroma Analitica, S.A., Sant Cugat del Valles, Spain) or from peripheral blood cells using

171   Realpure SSS kit (Durviz, S.L.U., Valencia, Spain).

172   A genome-wide genotyping was performed using the Infinium Global Screening Array-24 v1.0 (GSA)

173   BeadChip (Illumina, Inc., San Diego California, U.S) at the Spanish National Cancer Research Centre, in the

174   Human Genotyping lab (CeGen-ISCIII), resulting in the genotyping of 730,059 SNPs. After QC, a dataset of

175   447,035 SNPs with the following characteristics was obtained: Hardy-Weinberg equilibrium in patients and

176   healthy controls, SNP call rate higher than 98% and minor allele frequency (MAF) higher than 0.005.

177   Individuals with an SNP missingness higher than 2% were excluded. In addition, through a principal

178   component analysis (PCA), those individuals found to be related or not of European ancestry were also

179   excluded. Next, re-phasing and imputation were performed using, respectively, Eagle (Durbin, 2014) and

180   Minimac4 (Das et al., 2016) and the Haplotype Reference Consortium dataset (HRC version r1.1) (McCarthy

181   et   al.,   2016)   hosted   on   the   Michigan   Imputation   Server   (Das   et   al.,   2016)

182   (https://imputationserver.sph.umich.edu/). A MAF value of > 1% and an imputation quality of $R^2 > 0.3$ were

183   required for the inclusion of the variants into further analyses. Finally, our final SNP dataset included

184   7,606,397 genetic markers.

185

186   **Polygenic Risk Score Estimation**

187   Using SZ GWAS 2022 summary statistics from the European subsample (Trubetskoy et al., 2022) we

188   estimated four different PRS using PLINK 1.90 software (Chang et al., 2015) based on the PRS-C+T

189   methodology (Privé et al., 2019), in a similar fashion as several recent GWAS studies (Grasby et al., 2020;

190   Mullins et al., 2021; Trubetskoy et al., 2022). This method is defined as the sum of allele counts, weighted by

191   estimated effect sizes obtained from the GWAS, after two filtering steps: LD clumping (based on the

192   European population from the phase 3 1,000 Genomes reference panel) and p-value thresholding.

193   First, we calculated the whole-genome PRS (**Global PRS$_{SZ}$**). The LD filtering was conducted by including the

194   most significant SNP from any pair showing an LD $r^2 > 0.015$ within 1,000kb windows, resulting in a set of

195   informative linkage-disequilibrium independent markers (98,121 SNPs). Subsequently, for the p-value

196   thresholding, we considered a range of thirteen p-value thresholds: $p < 5 \times 10^{-8}$, $p < 5 \times 10^{-7}$, $p < 5 \times 10^{-6}$, $p < 5 \times 10^{-5}$,

197    $p < 5 \times 10^{-4}$, $p < 5 \times 10^{-3}$, $p < 0.05$, $p < 0.1$, $p < 0.2$, $p < 0.3$, $p < 0.4$, $p < 0.5$, $p < 1.0$. Through logistic regression, we

7

198   established the best threshold in $p<5 \times 10^{-3}$ as the better predictor of the diagnosis status () based on the

199   Nagelkerke's pseudo R^2 ($p=4.75 \times 10^{-12}$, $R^2=0.22$).

200   Second, we estimated a PRS accounting for HARs genetic variability (**HARs PRS$_{SZ}$**), exclusively including

201   SNPs within the 3,070 autosomal HARs sequences compiled by Girskis et al., 2021 (*Supplementary Table*

202   *1*). Using Bedtools 2.30.0 (Quinlan & Hall, 2010) we selected the genetic markers in our sample within these

203   HARs sequences. Considering the number of SNPs within the HARs, the PRS estimation was conducted

204   following the same PRS-C+T methodology but adjusting LD clumping parameters. Following PLINK default

205   options, we selected the most significant SNPs within 250kb windows and with LD $r^2>0.5$ and the unique p-

206   value threshold was set at $p<1.0$. The final set of variants was composed of 2,114 SNPs.

207   Third, to assess the effect of HARs SNPs specifically affiliated with foetal brain (FB) or adult brain (AB) gene

208   regulatory elements, we estimated two additional PRS scores (**FB-HARs PRS$_{SZ}$** and **AB-HARs PRS$_{SZ}$**) with

209   the same procedure and parameters as for PRS-HARs. Following the methodology used in the latest

210   ENIGMA human cerebral cortex GWAS by (Grasby et al., 2020), we downloaded ChromHMM chromatin

211   states (core 15 state model) from the Epigenomics Roadmap (Roadmap Epigenomics Consortium et al.,

212   2015). We selected two foetal tissues (E081=foetal brain female and E082=foetal brain male) and four adult

213   tissues (E067=brain angular gyrus, E069=brain cingulate gyrus, E072=brain inferior temporal lobe and

214   E073=brain dorsolateral prefrontal cortex) and for each tissue, the genomic regions comprising active

215   regulatory elements (TssA, TssAflnk Enh and EnhG). We combined the foetal (E081 and E082) and adult

216   (E067, E069, E072 and E073) annotations and selected only those regions non-overlapping between them

217   as foetal brain-specific and adult brain-specific. With the selected **HARs PRS$_{SZ}$** SNPs, we selected the

218   genetic variants allocated within these foetal and adult brain-specific regions. The final set of variants

219   included in the **FB-HARs PRS$_{SZ}$** and **AB-HARs PRS$_{SZ}$** estimations were 112 and 81 SNPs, respectively.

220

221   **MRI data acquisition**

222   The MRI neuroimaging data were obtained from two scanners: 58% (70 HC, 72 patients) of the sample was

223   scanned in a 1.5T GE Sigma scanner (General Electrical Medical Systems, Milwaukee, Wisconsin, USA)

224   and the other 42% (45 HC, 56 patients) in a 3T Philips Ingenia scanner (Philips Medical Systems, Best, The

225   Netherlands) at Hospital Sant Joan de Déu (Barcelona, Spain).

8

226   High-resolution structural-T1 MRI data in the 1.5T scanner was obtained using the following acquisition

227   parameters: matrix size 512 x 512; 180 contiguous axial slices; voxel resolution 0.47 x 0.47 x 1 mm^3; echo

228   time (TE) = 3.93 ms, repetition time (TR) = 2,000 ms; and flip angle = 15º. At the 3T scanner, structural T1-

229   weighted sequences were acquired as follows: matrix size 320 × 320 x 250; voxel resolution 0.75 x 0.75 x

230   0.80 mm3; TE= 3.80 ms, TR = 8.40 ms; and flip angle = 8º. All images were visually inspected to exclude

231   those with artefacts and movement.

232

233   **Surface-based morphometry**

234   Structural   MRI   data   were   processed   using   the   FreeSurfer   image   analysis   suite

235   (http://surfer.nmr.mgh.harvard.edu/). Image pre-processing included removal of non-brain tissue, automated

236   Talairach transformation, tessellation of the grey and white matter boundaries and surface deformation

237   (Fischl et al., 2004). Several deformation procedures were performed in the data analysis pipeline, including

238   surface inflation and registration to a spherical atlas. This method uses both intensity and continuity

239   information from the entire three-dimensional images in the segmentation and deformation procedures to

240   produce vertex-wise representations of CT and SA. The CT was defined as the measure of the distance

241   between the white matter surface and the pial surface, and cortical SA was calculated as the area of the

242   white matter surface. With FreeSurfer we automatically performed the segmentation of 34 cortical regions of

243   interest for each hemisphere using the Desikan-Killiany cortical atlas (Desikan et al., 2006). Mean values of

244   CT and SA were quantified for each individual within these defined regions.

245   All subjects included in this study passed the standardised quality-control protocols from the ENIGMA

246   consortium (https://enigma.ini.usc.edu/protocols/imaging-protocols/) that have previously been applied in

247   large-scale multi-centre studies (Grasby et al., 2020; Hibar et al., 2018).

248

249   **Statistical analyses**

250   Demographic and clinical data were processed and analysed using SPSS (IBM SPSS Statistics, version

251   29.0, released 2022, IBM Corporation, Armonk, New York, USA).

252   We compared the SZ polygenic load of patients and controls based on the four different PRS estimations

253   (Global PRS$_{SZ}$, HARs PRS$_{SZ}$, FB-HARs PRS$_{SZ}$ and AB-HARs PRS$_{SZ}$) by means of block-wise logistic

9

254   regressions with the two diagnostic groups with SPSS. For each PRS, two statistical models with

255   case/control status as outcome were compared, one testing the covariates alone (age, sex and the two first

256   ancestry-specific principal components as a baseline model), and the other testing the covariates plus the

257   corresponding PRS (full model). We report the R^2 values as the differences in Nagelkerke's pseudo-R^2

258   between these two nested models as an indicator of explained variance (Smigielski et al., 2021; Smith &

259   Mckenna, 2013).

260   Next, we examined to which extent different PRSs explain CT and SA measures from the 34 cortical regions.

261   We applied linear regression models (R software) within diagnostic groups (separately in HC and patients

262   with SZ) to test the effect of each PRS on CT/SA. In order to assess whether the PRS effect was modulated

263   by the diagnostic status, we conducted linear models using the whole sample and tested the PRS x

264   diagnosis interaction. Sex, age, premorbid IQ, intracranial volume, and scanner effect were included in the

265   statistical models in order to control for their potential effects. In the linear regression within patients with SZ,

266   the antipsychotic dose was as well included as a covariate (Scherk & Falkai, 2006; van Erp et al., 2018).

267   According to the findings, the SNPs included in the FB-HARs PRS$_{SZ}$ were furtherly mapped and functionally

268   annotated using FUMA (Watanabe et al., 2017) with the *SNP2GENE* and the *GENE2FUNC* tools. The

269   positional mapping parameters were left as default. The eQTL mapping was conducted in PsychENCODE,

270   ComminMind and BRAINEAC tissues filtering by PsychiENCODE and brain open chromatin atlas

271   annotations. The 3D chromatin interaction mapping was conducted with PsychENCODE, adult and foetal

272   cortex, dorsolateral and hippocampus and neural progenitor cells data filtering by PsychENCODE and brain

273   open chromatin atlas annotations.

274   The p-values resulting from each one of the before mentioned statistical tests were adjusted by using the

275   false discovery rate (FDR) method, specifically the Benjamini-Hochberg procedure, to control for multiple

276   comparisons at level q=0.05. Accordingly, only those results with a corrected FDR-pval<0.05 were

277   considered statistically significant.

278   The significant results on the cortical regions were plotted using the *ggseg* library in R (Mowinckel & Vidal-

279   Piñeiro, 2020) and the regressions with the direction of the results were plotted using SPSS (**Figure 1**).

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280   **3. RESULTS**

281   **PRS comparison between diagnostic groups**

282   Diagnostic PRS comparisons revealed differences in the Global PRS_{SZ}, with patients presenting higher SZ

283   genetic load than HC. The HARs-derived PRSs did not show between-groups differences (**Table 2**).

284   **Table 2.** Polygenic risk score (PRS) comparisons between healthy controls (HC) and patients with schizophrenia (SZ).
285   PRS means and standard deviations (sd) are given for both diagnostic groups for the four estimated PRSs (Global
286   PRS_{SZ}, HARs PRS_{SZ}, FB-HARs PRS_{SZ}, AB-HARs PRS_{SZ}). The logistic regression results include the β and standard
287   error (se), the logistic regression statistic (Wald, W), the Nagelkerke's pseudo R^2 (R^2), and the adjusted p-values after
288   FDR correction (FDR-pval).
289

	HC mean (sd)	SCZ mean (sd)	β (se)	W	R^2	FDR-pval
Global PRS_{SZ}	-201.14 (1.23)	-200.10 (1.02)	0.79 (0.14)	32.97	0.20	3.75×10^{-8}*
HARs PRS_{SZ}	-5.45 (0.17)	-5.41 (0.19)	1.32 (0.73)	3.28	0.02	9.33×10^{-2}
FB-HARs PRS_{SZ}	-0.53 (0.05)	-0.52 (0.04)	2.07 (2.96)	0.49	0.003	4.85×10^{-1}
AB-HARs PRS_{SZ}	-0.43 (0.04)	-0.42 (0.04)	7.69 (3.60)	4.55	0.02	6.57×10^{-2}

290   *Significant findings at FDR-pval<0.001.

291   **PRS associations with morphometric measures**

292   Regarding CT measures, no PRS estimates modulated thickness either within HC or patients with SZ. In the

293   same line, the PRSs x diagnosis interactions on CT evidenced no significant effects.

294   In contrast, the linear regression analyses revealed that among patients with SZ, the FB-HARs PRS_{SZ}

295   significantly affected SA in different regions in the right brain hemisphere. FB-HARs PRS_{SZ} modulated the

296   patients' SA of the lateral orbitofrontal cortex (Standardised β=-0.234, SE=440.443, adjusted R^2=0.491,

297   FDR-pval=0.008), the superior temporal cortex (Standardised β =-0.235, SE=545.898, adjusted R^2=0.488,

298   FDR-pval=0.008), the pars triangularis (Standardised β=-0.242, SE=438.928, adjusted R^2=0.322, FDR-

299   pval=0.019) and the paracentral lobule (Standardised β=-0.233, SE=282.910, adjusted R^2=0.264, FDR-

300   pval=0.030) (**Figure 1**). In these regions, a higher SZ risk load in FB-HARs was associated with lower SA

301   values. Conversely, no other associations were observed between the other estimated PRSs and SA neither

302   within HC nor in interaction with diagnosis.

303   The inspection of the genomic context of the SNPs in FB-HARs PRS_{SZ}, showed that 54.1% of the SNPs

304   were in intergenic regions, 26.5% in introns and 16.6% in intronic non-coding RNA. They mapped into 223

305   genes (2). The subsequent functional annotation results highlighted that these genes were enriched in

306   several Gene Ontology (GO) categories. The biological process retrieved were neuron differentiation,

307   neurogenesis, neuron development, and forebrain development, among others. Also, the only cellular

308   component category enriched was cell junction (*Supplementary Table 3*).

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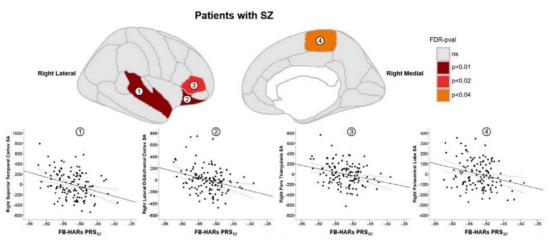
Figure 1. **Brain and scatter plots with significant FB-HARs PRS$_{SCZ}$ effect on surface area in patients with schizophrenia (SZ)**. Brain plots include the lateral and medial sagittal views for the right hemisphere. The coloured regions are the ones with significant FB-HARs PRS$_{SCZ}$ effect on surface area (SA) after FDR correction (FDR-pval < 0.05). The scatter plots show the relationship between the FB-HARs PRS$_{SCZ}$ (on the X-axis) and the unstandardised SA residuals (on the Y-axis in cm^2, estimated regressing out the covariates) and evidence their negative correlation (black solid line and black dashed lines representing mean 95% confidence intervals). Each region is numerically labelled as following: 1) superior temporal cortex; 2) lateral orbitofrontal cortex; 3) pars triangularis; and 4) paracentral lobule.

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310

4. DISCUSSION

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312 Through a neuroimaging genetic approach, we have evaluated the SZ's polygenic load of specific human

313 evolutionary markers, such as HARs, on brain-based phenotypes closely related to the pathophysiology of

314 the disorder. This is, to the best of our knowledge, the first study assessing the effect of HARs genetic

315 variability on brain cortical measures in patients with SZ and healthy controls. Our analyses provide evidence

316 on the modulatory effect of foetal active regulatory HARs on the cortical surface area of different brain

317 regions in patients with SZ. These findings highlight the importance of human-specific genetic changes,

318 especially those affecting active regulatory elements specific to foetal neurodevelopment, in the genetic

319 machinery guiding human brain cortical structure.

320 The comparisons between the different PRS estimates across diagnostic groups show that individuals with a

321 diagnosis of SZ present higher Global PRS$_{SZ}$ than healthy individuals. Thus, the sample of patients presents

322 a higher polygenic load for SZ. This result aligns with the current view on the value of the PRS$_{SZ}$ as a highly

323 informative genetic vulnerability marker for its consistency across numerous studies, not only in patients' *vs*

324 controls comparisons (Calafato et al., 2018; Vassos et al., 2017) but through family approaches, which

325 evidence the intermediate genetic load that healthy relatives of affected patients have (Smigielski et al.,

326 2021; van Os et al., 2020).

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327   However, in our sample, the analyses indicate no significant differences across diagnostic groups using the

328   other three HARs-related PRS_{SZ}. The direct comparison of our findings with previous studies is difficult

329   because of the absence of HARs-based PRS studies; nevertheless, some previous studies have explored

330   the role of specific candidate HARs in SZ through association approaches. In this sense, the haplotypic

331   variability at the *HAR1A* gene, a novel RNA gene with a presumable neurodevelopmental role that harbours

332   the HAR with the highest substitution rate in humans as compared to chimpanzees (Pollard, Salama,

333   Lambert, et al., 2006), was associated with auditory hallucinations in patients with a schizophrenia-spectrum

334   disorder in a European sample (Tolosa et al., 2008). In line, several candidate HARs-SNPs altering

335   transcription factor binding sites and presenting methylation marks of active promoters, repressors or

336   enhancers in the brain were associated with the risk for SZ and modulated cognitive performance in a north-

337   Indian population (Bhattacharyya et al., 2021, 2022). On the other hand, genome-wide-based studies also

338   describe that HARs-genes and HARs-brain genes are associated with SZ at the GWAS level (Cheung et al.,

339   2022; Srinivasan et al., 2017; Wei et al., 2019; Xu et al., 2015). Some of these studies, indeed, went beyond

340   common variability and showed that rare variants in HARs-genes were also enriched with the disorder (Wei

341   et al., 2019); suggesting, therefore, that common and rare variants joint analyses can help disentangling the

342   role of HARs variability on the susceptibility for the disorder and its specific phenotypes.

343   In our investigation on the contribution of HARs polygenic background on cortical neuroanatomical measures

344   variability, we report a modulatory effect of FB-HARs PRS_{SZ} on the SA within patients with SZ. These

345   findings suggest that the genetic variability in HARs associated with regulatory elements uniquely active in

346   the foetal brain would specifically influence brain phenotypes in SZ. Results show that as the FB-HARs

347   PRS_{SZ} increases (i.e as more SZ risk variants accumulate in these HARs associated with foetal active

348   regulatory elements), patients present lower cortical surface area in the lateral orbitofrontal cortex, the

349   superior temporal cortex, the pars triangularis and the paracentral lobe. First, these findings converge with

350   data highlighting the importance of the foetal period (and the gene expression circumscribed to it) to

351   understand the neurodevelopmental trajectories linked to schizophrenia (Ursini et al., 2021). Also, our

352   findings line up with previous studies reporting widespread smaller surface area, with the largest effect sizes

353   in the frontal and temporal lobe regions (van Erp et al., 2018). Focusing on the regions significantly

354   modulated by the SZ genetic load in foetal brain active HARs, we can draw attention to the orbitofrontal

355   region and the temporal cortex as these are among the regions suffering the largest expansion in the human

356   cortex in comparison with chimpanzees. Wei et al., 2019 described that areas of the orbital frontal gyrus and

357   the temporal lobe experienced an x4 and x3 expansion, respectively, and evidenced that the transcription

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358   profile of 1711 HARs-genes positively correlated to the pattern of human cortical expansion, meaning that

359   the highest HARs-gene expression occurs in highly expanded areas of the human cortex.

360   A remarkable finding though is the specificity of significant PRS association with SA, as we were not able to

361   observe HARs polygenic effect on the CT variability. This could be interpreted considering that the genetic

362   influences on the two cortical measures and the underlying mechanisms are largely different, and that SA

363   and CT follow distinct developmental trajectories (Jha et al., 2018; Lyall et al., 2015; Wierenga et al., 2014).

364   As posited by the radial unit hypothesis, cortical surface area expansion would be driven by the proliferation

365   of neural progenitor cells, while thickness would be determined by the number of their neurogenic divisions

366   (Rakic, 1988). In this line, a study described that HARs-genes are highly expressed during prenatal

367   development, their expression is upregulated during neurogenesis and enriched in cells from the outer radial

368   glia (Won et al., 2019). Indeed, radial glia is a major class of neural stem cells in the germinal layer that

369   shows substantial expansion in the primate lineage and among the neurodevelopmental differences between

370   human and chimpanzees there is the proliferative capacity of neural progenitors during cortical development

371   (Mora-Bermúdez et al., 2016). Recent data shows that the genetic determinants of SA are predominately

372   related to gene regulatory activity in neural progenitor cells during foetal development while CT is influenced

373   by regulatory processes that occur after mid-foetal development (Grasby et al., 2020). Also, common genetic

374   variants explained a larger part of SA variance (SNP-h^2=34%, SE=3%) than of CT variance (SNP-h^2=26%,

375   SE=2%) (Grasby et al., 2020). However, our results could also be influenced by the reduced number of

376   SNPs in our PRS estimates, which could have hampered the capture of the genetic determinants of CT.

377   Likewise, by using HARs, we are putting the focus on regions highly stable along mammal evolution that

378   experiences rapid sequence changes in the human lineage since the divergence from our closest relatives

379   (Girskis et al., 2021), and while SA has enormously increased during the evolution of primates, cortical

380   thickness has remained relatively constant (Eickhoff et al., 2005). Therefore, other genetic evolutionary

381   markers could be more suitable for inspecting the evolutionary traces of CT.

382   Relative to the exploratory gene mapping results, it was interesting to find that the SNPs underlying cortical

383   surface area differences within patients were enriched in biological processes important for nervous system

384   development. These findings would be in line with previous studies describing that HARs associated genes

385   mainly participate in biological processes and pathways related to neurodevelopment, neural differentiation

386   and axonogenesis (Doan et al. 2016).

387   Finally, we should account for some limitations of this study. Regarding our genetic association approach,

388   the samples could be considered small; nonetheless, the focus of our study was the neuroimaging

389   association analyses. These analyses have been conducted in a sample of hundreds of individuals,

390   exceeding, therefore, the median sample size of neuroimaging association studies according to a recent

391   revision (Marek et al., 2022). In this regard also, we have to point out that our structural images were

392   obtained from two different scanners, which could represent a source of bias. Notwithstanding, we did not

393   detect differences in neuroanatomical measures based on the two scanning sites, all the images passed the

394   standardised quality-control protocols recommended by the ENIGMA consortium, which have been

395   previously applied in large-scale multi-centre studies, and the scanner site was accounted as a covariate in

396   the regressions. In terms of the genetic data, we should contemplate that our PRS estimates are pondered

397   using SZ genetic burden and the use of other GWAS sum stats such as the corresponding to the cortical

398   phenotypes could derive different effects. The PRS estimation method used in the present study, PRS-C+T,

399   is the most used, and latest SZ GWAS has been conducted using the same method; however, other PRS

400   calculation methodologies could be helpful (Ni et al., 2021). Speaking of PRS estimations, our results are

401   based on a sample of European ancestry and SZ GWAS statistics were derived from the European cohort,

402   then, although GWAS studies performed non-European samples converge in the same SZ's genes and

403   pathways (Gulsuner et al., 2020), the extrapolation of our findings to other ethnic groups should be done

404   cautiously. Finally, data from larger samples should be highly encouraged to compare our results and

405   replicate thereof. Also, the understanding of the role of HARs in the neurobiological roots of SZ would benefit

406   from analyses on other brain-based phenotypes such as structural connectivity, white matter microstructure

407   (Canales-Rodríguez et al., 2014, 2021; van den Heuvel et al., 2019), or MRI protocols related to social

408   cognition (Fujiwara et al., 2015).

409   In conclusion, our study adds novel evidence on the role of the genetic variability within HARs guiding foetal

410   neurodevelopment and shaping cortical surface area configuration in patients with SZ. The biological

411   plausibility of our findings highlights the paramount importance of HARs in the early developmental gene

412   regulatory machinery led us to think that these regions may contribute to bridging together the

413   neurodevelopmental and evolutionary hypotheses in schizophrenia.

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Author contributions

767 MG-R and MF-V conceived the study. MG-R conducted the DNA extraction and sample normalization for

768 genotyping. EJC-R, MAG-L and PF-C pre-processed and segmented the MRI images. EP-C, PF-C, JS, JT,

769 LT and ER-C conducted the recruitment and/or the clinical evaluation. EJC-R, RS and EP-C designed the

770 MRI protocols. MG-R, CA and AA performed the data curation. MG-R conducted the formal statistical

771 analyses and graphical representations with the help of CA-P, AA and ML-G. SP and MF-V were implicated

772 in the revision of the methodology. MG-R and MF-V interpreted the results. MG-R wrote the first draft and the

773 subsequent drafts of the paper. MF-V supervised the study activity planning and execution. MF-V and EP-C

774 participated in the funding acquisition. All the authors reviewed and approved the final manuscript.

789   **Data availability**

790   The data that support the findings of this study are available from the corresponding author upon reasonable

791   request.

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Summary of the Results

Relative to the **first hypothesis**: "*genetic variability at synaptic plasticity-related genes will sustain, to some extent, the differences between patients with **schizophrenia** and healthy individuals at the **brain functional** level in **response** to **higher-order cognitive processes**"*; three specific objectives were derived, each resulting in one article:

1.1. **Guardiola-Ripoll M et al., 2022**. New insights of the role of the *KCNH2* gene in schizophrenia: an fMRI case-control study. *European Neuropsychopharmacology*, 2022 Jul;60:38-47

1.2. **Guardiola-Ripoll M et al., 2022**. Combining fMRI and *DISC1* gene haplotypes to understand working memory-related brain activity in schizophrenia. *Scientific Reports*, 2022 May 5;12(1):7351.

1.3. **Guardiola-Ripoll M et al., 2022.** A functional neuroimaging association study on the interplay between two schizophrenia genome-wide associated genes (*CACNA1C* and *ZNF804A*). *European Archives of Psychiatry and Clinical Neuroscience*, 2022 Oct;272(7):1229-1239.

In these three studies, the following **results** have been obtained:

1.1. When testing the *KCNH2* gene effect on brain activity for the first time in patients with schizophrenia, we detected that the rs3800779 polymorphism modulates the medial prefrontal cortex response when performing the 1-back *vs.* baseline contrast of the N-back task. This functional effect suggests an association of this gene with the attentional and working-memory processes involved in the N-back execution. Interestingly, the rs3800779 interaction with diagnosis resulted in a different brain activity outcome in healthy controls and patients with schizophrenia. While healthy controls deactivated the medial prefrontal cortex following a risk-allele (A allele) dose effect, the patients presented the opposite activity pattern towards activation. Indeed, patients homozygous for the risk allele activated the medial prefrontal cortex. In contrast, no *KCNH2* and diagnosis interaction effect was observed concerning the N-back behavioural response.

1.2. We assessed the genetic landscape of *DISC1* gene (HEP1, rs6675281-rs1000731-rs999710; and HEP3, rs751229-rs3738401) in relation to schizophrenia's liability. We identified two haplotypes associated with a protective effect, HEP1-CTG (OR=0.63, 95%CI=0.41-0.98) and HEP3-GA (OR=0.63, 95%CI=0.45-0.89), and one associated with the risk, HEP3-AA (OR=2.03, 95%CI=1.17-3.53). Further analyses on N-back brain functional and behavioural responses revealed that while none of the *DISC1* haplotypes influenced N-back performance; the protective HEP3-GA and the risk HEP3-AA haplotypes differently modulated attentional and working memory-related brain activity (1-back *vs.* baseline, 2-back *vs.* baseline and 2-back *vs.* 1-back contrasts of the N-back task) depending on the health/disease status. The brain clusters involved in these interactions comprised

regions such as the cuneus, precuneus, middle and posterior cingulate cortex, frontal and orbitofrontal cortices, and ventrolateral and dorsolateral prefrontal cortices.

1.3. We described an epistatic effect (gene-gene interaction) between *CACNA1C*-rs1006737 and *ZNF804A*-rs1344706, two SNPs associated with schizophrenia at GWAS level, on working-memory brain functional response (2-back *vs*. 1-back contrast) independently of the diagnosis. The regions implicated in this epistasis comprised the medial ventral caudate, the left superior and inferior orbitofrontal gyrus, the superior temporal pole and the ventral anterior insula. Interestingly, patients with the combination of the two GWAS risk genotypes (*CACNA1C*-AA/AG and *ZNF804A*-AA) were the unique group that showed mean activity increases instead of decreases. However, these effects were not observed at the behavioural level.

Concerning the **second hypothesis**: "*recently evolved genomic regions will underly the unique neurodevelopmental features and brain traits and **harbour genetic variants associated with schizophrenia**, therefore the genetic variability within these regions will **contribute** to the **brain's cortical architecture differences** between healthy individuals and patients with schizophrenia*"; two specific objectives were derived, each resulting in one article:

2.1. **Guardiola-Ripoll M and Fatjó-Vilas M, 2023**. A systematic review of the Human Accelerated Regions in schizophrenia and related disorders: where the evolutionary and neurodevelopmental hypotheses converge. *International Journal of Molecular* Sciences, 2023 Feb; 24(4):3597.

2.1. **Guardiola-Ripoll M et al., 2023**. Human-specific evolutionary markers linked to foetal neurodevelopment modulate brain surface area in schizophrenia. *Submitted for peer review in an indexed journal and available as a preprint at medRxiv.*

In these two studies, the following **results** have been obtained:

2.1. There is convergent data on the role of HARs as paramount transcriptional regulatory elements participating in the neurodevelopmental gene expression machinery. The brain expression patterns of genes associated with HARs correlate with human-specific cortical expansion and neural information processing and are associated with variability in brain functional connectivity. Lastly, common and rare genetic variants in HARs are related to neurodevelopmental psychiatric disorders such as schizophrenia and autism. These results suggest that the same regions that endowed the neurodevelopment with human-specific characteristics sustain not only the inherent

human variability in cognition and behaviour but also the susceptibility to neurodevelopmental disorders, especially schizophrenia and autism.

2.2. We investigated whether the polygenic load of HARs towards schizophrenia influenced brain cortical architecture in patients with schizophrenia and healthy controls. Our results indicate that the genetic variability in HARs associated with active transcriptional regulatory elements specific of the foetal brain (FB-HARs PRS$_{SZ}$) influenced the surface area of the lateral orbitofrontal cortex, the superior temporal cortex, the pars triangularis and the paracentral lobule in patients with schizophrenia, but not in controls. The higher the schizophrenia risk load associated with these HAR foetal regulatory elements, the lower the surface area. Conversely, the polygenic load of the HARs associated with active transcriptional regulatory elements specific of the adult brain (AB-HARs PRS$_{SZ}$) did not modulate the surface area or the cortical thickness in patients or controls.

Discussion

This thesis is framed in the field of psychiatric genetics and seeks to contribute to a better understanding of the complex aetiological foundations of schizophrenia. We have approached this goal by means of two distinct strategies based on the analyses of candidate genes and whole-genome variability on informative gene sets and focusing on neuroimaging intermediate phenotypes.

On the one hand, we have studied the relationship between genetic changes, brain functional response, cognitive performance and schizophrenia through candidate genes with key functions in neurodevelopmental and synaptic plasticity mechanisms. However, to advance in the understanding of these complex, an evolutionary view is needed, looking at the specifically human changes in ontogenetic neurodevelopmental trajectories. Accordingly, on the other hand, we have studied how human-specific evolutionary markers such as Human Accelerated Regions (HARs) underlie human-specific neurodevelopmental signatures, brain configuration, functioning and susceptibility behind psychiatric disorders. Furtherly, we have assessed the modulatory effect of HARs polygenicity on brain cortical architectural differences in schizophrenia.

Among the primary purposes around which human genetics turns is understanding the aetiological roots of genetic diseases and disorders. Advances in this goal are paramount for developing new therapeutic strategies and contributing to the identification of informative biomarkers. Nonetheless, understanding the functional impact of genomic findings is a significant challenge. It is necessary to fit together the puzzle of results from multidisciplinary approaches. Only by integrating the findings from genetic and molecular studies, cell-biology experiments, and animal and human research will it be possible to fully understand the mechanism by which the genetic signatures underlie the alterations that ultimately cause the disorder.

Synaptic plasticity genes underlying brain functional response to higher-order cognitive process associated with schizophrenia

As regards the first question (or hypothesis) of this thesis, our findings suggest that common DNA sequence variants at neurodevelopmental and synaptic plasticity genes underlying, to a certain extent, the brain functional differences observed between patients with schizophrenia and healthy individuals. Globally, we have assessed genotypic effects from diverse approaches: analyses including one SNP at one gene to more complex additive genetic effects, several SNPs at one gene configuring different haplotypes, and even genetic interactions, two SNPs at two genes in epistasis. Our findings accentuate the value of neuroimaging genetics approaches to uncover the genetic and pathophysiological mechanisms underlying brain dysfunction in schizophrenia, especially those related to neural excitability and synaptic plasticity. At the same time, the findings outlined the intricacy of the genotype-phenotype

relationships, especially for psychiatric disorders because of the high polygenicity and the complex and heterogeneous aetiology.

In an attempt to jointly evaluate the results, we can say that *KCNH2* and *DISC1* genetic variability modulated the N-back attentional and/or the working memory-related functional responses and that these effects were diagnosis-dependant. Therefore, we have reported that common genetic variants at these genes contribute to explaining the patient-control variability in brain functional response to specific cognitive demands. On the contrary, while the *CACNAC1C* and *ZNF804A* epistasis influenced the functional ability to adapt to increases in working memory difficulty, the impact of this genetic interaction was independent of the diagnostic status. Therefore, these results suggest that the role of this epistasis is homogeneous across individuals and not affected by the disorder. Some of the regions where the genetic effects have been detected can be included within higher-order functional networks like the frontoparietal and default mode networks (such as the dorsolateral prefrontal cortex, the medial superior frontal cortex, the precuneus, or the superior temporal pole). Furthermore, these are regions where functional alterations had been previously reported in schizophrenia during the performance of cognitive tasks (Pomarol-Clotet et al. 2008; Minzenberg et al. 2009; Whitfield-Gabrieli et al. 2009; Dae et al. 2009; Hu et al. 2017; Buckner and DiNicola 2019). Overall, the detected effects align with the previously described role of these genes in the risk for schizophrenia (Hashimoto et al. 2013; Zhu et al. 2014; Ma et al. 2018; Liu et al. 2020), and in the variability underlying attentional and working memory impairments (Hashimoto et al. 2010; Hori et al. 2012; Vázquez-Bourgon et al. 2015; Carr et al. 2016; Cosgrove et al. 2017; Teng et al. 2018; Meller et al. 2019), domains widely implicated in the disorder and related to excitation and inhibition imbalances, neural activity dysfunctions and changes at synaptic plasticity level (Uhlhaas and Singer 2010; Lisman 2012; Kahn and Keefe 2013; Sutcliffe et al. 2016; Mould et al. 2021).

Interpreting the functional findings would be more straightforward if accompanied by task performance data (Egli et al. 2018; Thomas et al. 2022). However, in our studies, the detected differences at the functional level were not related to changes at the behavioural level. Part of the explanation must be sought in the fact that the candidate gene approaches restrict the biological response assessed to the physiological role of the analysed genes. While this represents a strength when intending to connect a particular biological process to closer intermediate phenotypes, it is not so powerful when extending the analyses to more distal phenotypes. In this sense, intermediate phenotypes have proven to help capture genetic effects, and in this line, neuroimaging measures are believed to be closer to the genetic background than behavioural phenotypes. Thus, while both brain function and cognition are complex and highly polygenic traits challenging to depict with a handful of genetic variants (Davies et al. 2015; Sniekers et al. 2017; Savage et al. 2018; Elliott et al. 2018), meta-analyses on candidate

genes remark the power of neuroimaging studies to detect larger genetic effects as compared to cognitive investigations (Rose and Donohoe 2013). In all, the divergent findings on function and performance reflect the coordinated response that the brain gives to different cognitive-demanding stimuli (compensating the functional imbalances) and also show the complicated relationship between brain activity and cognition (Egli et al. 2018; Thomas et al. 2022).

Overall, the effects reported herein on the implication of different synaptic plasticity genes in the functional response to higher-order cognitive functions would align with the conceptualisation of the involvement of synaptic plasticity dysfunctions in the origin of schizophrenia (Lewis et al. 2012; Foss-Feig et al. 2017; McCutcheon et al. 2020) and with results on the convergence of schizophrenia common and rare genetic variability on biological process and pathways related to neurodevelopment, neuronal excitability, synaptic organization and signalling and function (Kirov et al. 2012; Smeland et al. 2020b; Singh et al. 2022; Trubetskoy et al. 2022; Andreassen et al. 2023).

Human Accelerated Regions underpin brain functional and anatomical traits associated with schizophrenia

Concerning the second question (or hypothesis) of this thesis, our findings from the two HAR-based studies converge on the idea that given the presumable role of HARs as human-specific gene expression regulatory elements along neurodevelopment, genetic variants within these regions underlie, to some extent, the human variability in brain traits, but also the neural dysfunction observed in neurodevelopmental disorders such as schizophrenia. Moreover, the detected association between the variants within foetal active regulatory HARs and surface area in patients point out the importance of evolutionary mechanisms in brain configuration and susceptibility for disorders intimately related to the human condition. These data also outlined the importance of considering different developmental windows, not only in relation to the etiological understanding of the disorder but also in identifying particular significant time-frames for its prevention.

Regarding the results derived from the review, there is an unequivocal link between these evolutionary relevant sequences and the neurodevelopmental process, brain architecture, functioning and cognitive and psychiatric phenotypes, especially in schizophrenia. Likewise, we should highlight that the link between HARs and the susceptibility to neurodevelopmental psychiatric disorders seems to be intimately related to their role as transcriptional regulations during foetal and adult brain development (Doan et al. 2016; Won et al. 2019; Girskis et al. 2021). This link was subsequently validated by our findings on the effect of HARs linked to foetal regulatory activity on the cortical surface area in patients. Altogether, these findings could be

interpreted jointly with data underscore the importance of regulatory elements in general and of foetal-specific elements in particular in the altered gene expression trajectories of schizophrenia (Tansey and Hill 2018; Alver et al. 2022).

Despite the considerable evidence on HARs' neurodevelopmental function and the genetic background underlying associated psychiatric disorders, research on their role in neurocognitive or neuroimaging phenotypes in schizophrenia has been poorly investigated. Our case-control analyses focused on HARs PRSs on the cortical surface area and thickness contribute to understanding the evolutionary changes in this neurodevelopmental disorder. The regions where the polygenic effect of HARs has been detected in the surface area of patients are within the temporal and prefrontal cortices. These regions deserve attention for two main reasons. First, a study investigating human cortical expansion found that the bilateral orbital inferior frontal gyrus and several temporal pole regions were between three and four times more expanded in humans than in chimpanzees (Wei et al. 2019). Second, results from the ENIGMA Consortium on cortical brain alterations in schizophrenia described that even though surface area reductions seem to be widespread rather than regional-specific, the largest differences between patients and controls were observed in regions from the frontal and temporal lobes (van Erp et al. 2018). Additionally, a study investigating the structural patterns of brain dysconnectivity in schizophrenia described that the superior temporal cortex was among the regions with the most remarkable differences between patients and controls (van den Heuvel et al. 2019). Considering that convergent findings have associated HARs with the modulation of other brain-based traits such as cortical gyrification (Boyd et al. 2015), the functional modulation of the default mode and the frontoparietal networks (Wei et al. 2019), future research on other brain phenotypes, both structural and functional (including connectivity, resting-state and task-based protocols) should be encouraged.

Previous findings point towards the implication of adult gene regulatory elements in the genetic foundations of cortical architecture (Grasby et al. 2020), and others report significant correlations between cortical volume changes across psychiatric disorders and brain-expressed genes associated with HARs (Wei et al. 2019). Given these results, we would also have expected results on HARs linked to adult brain regulatory activity and the overall HAR set. Nonetheless, divergent findings could indicate the specific importance of evolutionary mechanisms in prenatal gene regulatory landscapes. However, the differences could also be related to the cortical measures and the specificity of schizophrenia compared to other studies focused on other structural measures and other data on combined diagnostics.

These findings highlight the importance of HAR polymorphisms associated with schizophrenia in the brain architectural differences at the temporal and frontal lobes and point towards the crutial role of the homeostasis at early prenatal stages to build the

cornerstones of the adult brain architecture. Likewise, the results align with the neurodevelopmental hypothesis of schizophrenia and emphasise the imprints that disturbances occurring even at early stages of neurodevelopment leave in the later brain configuration (Birnbaum and Weinberger 2017; Ursini et al. 2021; Schmitt et al. 2022).

Connecting the genetic and genomic variability findings to the schizophrenia phenotype

Genetic findings must be contextualised into the biological environment to guide the path from "bench to bedside". A good approach might be to track susceptibility genes and genetic variants based on their functional impact on specific molecular mechanisms, their relevance for brain functioning, their relationship with behavioural variability, and their role in neurobiological networks and pathways that could be of interest to the identification of novel biomarkers and therapeutic strategies (Uffelmann and Posthuma 2021; Mould et al. 2021; Wong et al. 2021; Andreassen et al. 2023). Still, this is a path hampered by the currently incomplete understanding of the functional role of most genes and proteins (Flint and Ideker 2019) and by the only intuited cellular, tissular, systematic and developmental specificity of gene expression regulation (Uffelmann and Posthuma 2021; Wong et al. 2021). However, integrating the data from this thesis with the current knowledge can offer new insights into the role of synaptic plasticity genes and Human Accelerated Regions in brain function and structure in the origin of schizophrenia (Figure 7).

Genes and products
Which are the functional implications at biological level?

Cellular and animal
Which are the effects observed at cellular/neuronal level and on animal models?

Intermediate Phenotypes
Which are the effects on brain-based phenotypes?

Therapy
Which are the mechanisms subject to be modulated by drug effects?
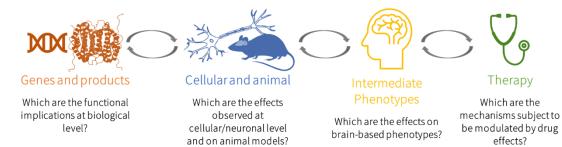
Figure 7. An approach to integrate genes in the aetiology of the disorder and encourage the development of therapeutic strategies. The identification of the mechanisms underlying the symptoms that emerge in neurodevelopmental psychiatric disorders may be traced based on evidence at different levels: based on the functional implications of the candidate genes, the impact of altered sequence or genetic products at the cellular/animal level, their implication modulating brain-based intermediate phenotypes and evidence on the impact of drug therapies in the related biological mechanisms.

Genes and products and the implicated biological pathways

Considering the hypotheses-driven approach followed, especially in the first aim of the thesis, the candidate genes were selected based on their functional implication in synaptic plasticity, neural excitability, and neurodevelopmental pathways. Likewise, while the second aim of the thesis had an open hypothesis based on whole genome variability, the polygenic load was assessed based on the HARs implication in human-specific variability and their role in neurodevelopmental regulation.

On the one hand, from the functional perspective, *KCNH2*, by modulating the neuronal firing, and *CACAN1C*, by regulating the calcium influx into the cell and mediating between neuronal excitation to transcription, are intimately related to excitability and synaptic plasticity mechanisms. On their side, *DISC1*, by mediating numerous signalling pathways involved in several cell homeostatic functions, DNA repair and its involvement in dendritic spine development, and ZNF804A, by its putative role as a transcription factor of genes related to neurite outgrowth and dendritic branching, both seem to have a predominant role in neurodevelopment (Kamiya et al. 2005; Pessia et al. 2008; Citri and Malenka 2008; Hill and Bray 2012; Girgenti et al. 2012; Hill et al. 2012; Bhat et al. 2012; Ji et al. 2012; Striessnig et al. 2014; Deans et al. 2017; Tomoda et al. 2017; Bauer and Schwarz 2018; Weng et al. 2018; Chapman et al. 2019; Zhou et al. 2020; Dong et al. 2021). On the other hand, as reported in the review, there is a considerable amount of evidence that points towards HARs' biological function as transcriptional regulators, most likely to function as transcriptional enhancers of genes involved in neural proliferation and differentiation active during prenatal stages of development (Doan et al. 2016; Won et al. 2019; Uebbing et al. 2021; Girskis et al. 2021).

The biological effects of the variants analysed: from the cellular level to animal models

Genetic and molecular studies also converge on the gene expression impact of some of these SNPs. First, the *KCNH2*-rs3800779 modulates the expression of a primate-specific isoform enriched in the brain, the KCNH2-3.1 (Huffaker et al. 2009). In turn, KCNH2-3.1 expression changes seem to result in immature neuronal architecture, cognitive deficits, abnormal synchrony and neural transmission between the hippocampus and the prefrontal cortex in mice models (Carr et al. 2016; Ren et al. 2020).

Second, in mutant mice experiments, the *DISC1*-rs3738401 SNP, together with other missense polymorphisms, seems to disrupt the Wnt signalling pathway, a pathway that regulates neural cells (Singh et al. 2011; Rosso and Inestrosa 2013). Moreover, this SNP also modulated the proliferation of neural-like mouse cells impacting the cell cycle and increasing the rates of premature proliferation, results that were later validated in human

lymphoblasts and that evidenced the reduced Wnt activity associated with the rs3738401-A allele (Singh et al. 2011).

Third, results based on human-induced neurons and post-mortem samples describe that *CACNA1C*-rs1006737 changes mRNA levels. These mRNA level changes have been reported to affect calcium current density (Bigos et al. 2010; Yoshimizu et al. 2015; Eckart et al. 2016). Convergently, CACNA1C functional alterations have been associated with alterations in hippocampal neurogenesis, including reductions in cell survival rate and proliferation, and rodent models with CACNA1C dysfunction present impairments in learning and memory (Moon et al. 2018).

Fourth, the *ZNF804A*-rs1344706 has been described to influence the expression of specific *ZNF804A* transcripts in the human foetal brain, such as *ZNF804A^{E3E4}*, which is highly expressed in the dorsolateral prefrontal cortex and diminished in the same region in adult brains from patients with schizophrenia (Hill and Bray 2012; Tao et al. 2014). Furthermore, murine models have evidenced that suppressing the Z*nf804a* gene reduced total dendritic spine density and that the overexpression of the *ZNF804A^{E3E4}* transcript produces an increase in total dendritic spine density in cortical rat neurons (Deans et al. 2017; Zhou et al. 2020).

Lastly, although sequence changes in HARs have been poorly investigated regarding their functional effects, several rare mutations associated with autism have been validated in mouse neural-like cells. Point mutations at HARs identified through whole-genome sequencing in individuals with autism spectrum disorder were found to affect the expression and enhancer activity of three neurodevelopmental gens: *CUX1*, a transcription factor involved in neuronal differentiation; *PTBP2*, an RNA-binding protein regulating the splicing in neural differentiation; and *GPC4*, a dosage-sensitive gene in the adult brain essential for excitatory synapse development in mice (Doan et al. 2016). In line, it has been recently described that changes in HARs' sequence are associated with variability in their enhancer activity, affecting the expression of the target genes (Whalen et al. 2023).

To understand the described biological effects of the genetic variants analysed, it is compulsory to investigate the molecular mechanisms by which the genetic variants exert their effects. In this regard, the common denominator seems to be transcriptional regulatory mechanisms altering mRNA production or stability, which are paramount mechanisms in psychiatric disorders (Schork et al. 2013; Roussos et al. 2014; Flint and Ideker 2019).

In this sense, based on data from the Epigenomics Consortium Roadmap (Roadmap Epigenomics Consortium et al. 2015) compiled on the Haploreg platform (Ward and Kellis 2012), a database to obtain information on the impact of variants on chromatin states, protein binding sites, regulatory motifs and expression

(https://pubs.broadinstitute.org/mammals/haploreg/, accessed on February 14th 2023), there is evidence that *KCNH2*-rs3800779, both *DISC1*-HEP3 SNPs (rs3738401 and rs751229), and *CACNA1C*-rs1006737 could modify histone marks associated with enhancers and promoters at several adult brain regions. Moreover, data retrieved from Haploreg indicates that *KCNH2*-rs3800779, *DISC1*-rs3738401 and *CACNA1C*-rs1006737 could also modify foetal gene expression regulation. Convergently, the mechanisms by which HARs would modulate neurodevelopmental expression machinery would be through their transcription factor binding affinity (Doan et al. 2016; Girskis et al. 2021). It has been described that 70% of HARs overlapped with open chromatin regions and/or regions with marks of active transcription in the human brain (Whalen et al. 2023), and more than 45% of HARs present marks of active chromatin states in neural and foetal brain tissue (Doan et al. 2016; Won et al. 2019; Girskis et al. 2021). These results add to previous expression analyses of GWAS data on schizophrenia and other psychiatric illnesses, which have revealed that the expression of genes associated with the disorder is enriched in human brain tissue, as well as the importance of gene-expression regulatory mechanisms of these brain-expressed genes in the pathophysiology of neurodevelopmental psychiatric disorders (Ripke et al. 2014; Gusev et al. 2018; Gandal et al. 2018; Huo et al. 2019; Trubetskoy et al. 2022).

The neurobiological consequences: brain-based, cognitive and clinical phenotypes

The influence that gene variants have on gene expression may be mediating the detected effects on brain function and structure. These expression differences would result in neurodevelopmental impairments leading to structural and excitability alterations.

Investigations on the involvement of candidate plasticity in relevant brain functions and phenotypes are numerous. First, *KCNH2* has been associated with cognitive affectations, brain structural changes, such as reduced hippocampal grey matter volumes, and functional alterations, including the hippocampus and prefrontal cortex connectivity changes and whole-brain electrophysiological differences (Huffaker et al. 2009; Hashimoto et al. 2013; Henningsson et al. 2015; Lubeiro et al. 2020).

Common *DISC1* variants modulate cognitive performance in domains such as working memory and attention (Carless et al. 2011; Vázquez-Bourgon et al. 2015), and numerous studies provide evidence on DISC1 protein effect on brain structural changes and functional alterations (Johnstone et al. 2011; Duff et al. 2013). In addition, changes in *DISC1* mRNA levels have been related to schizophrenia symptom severity and the risk for the disorder itself (Chen et al. 2022).

On its side, *CACNA1C* has been extensively studied regarding schizophrenia intermediate phenotypes in humans. Genetic variants in the gene region have been described to modulate cognitive function in patients with psychiatric disorders and relatives (Novaes de Oliveira Roldan et al. 2022). In line, analyses in patients and controls have described grey and white matter alterations (Wolf et al. 2014; Gurung and Prata 2015; Huang et al. 2016; Lancaster et al. 2016). Moreover, there is extensive data on the modulatory effect of *CACNA1C* genetic variability in brain function and connectivity (Paulus et al. 2014; Gurung and Prata 2015; Cosgrove et al. 2017; Zhang et al. 2019).

Since the identification of *ZNF804A* in schizophrenia GWAS data, its sequence variability has been broadly studied concerning personality traits associated with psychosis, such as schizotypy and multiple cognitive dimensions (Walters et al. 2010; Hargreaves et al. 2012; Meller et al. 2019), and in line with our findings, common genetic variants have been reported to modulate brain structural and functional measures (Paulus et al. 2013; Gurung and Prata 2015; Zhang et al. 2016b; Tecelão et al. 2018; Zhao et al. 2020).

Lastly, brain phenotypes have also been investigated in the context of HARs variability. It has been observed that the expression patterns of genes associated with HARs correlate with brain functional connectivity and with brain information processing (Li et al., 2021; Luppi et al., 2022). These differences in brain network configuration and processing could be related to the observation of the effect of HARs genetic variability on intelligence and sociability (Wei et al. 2019; Cheung et al. 2022).

Novel therapeutic strategies

The roles of these genes and regions in brain development, excitability and neural plasticity mechanisms, together with the apparent functional consequences of the polymorphisms, emphasise the idea of how multiple genetic variants of small effect may influence the deviances in the neurodevelopmental trajectories related to schizophrenia, but also accentuate the need to target and remediate the altered molecular process in adulthood.

In this regard, genetic and molecular studies provide clues on these genes' role in drug response and, therefore, on their potential druggability. Pharmacological studies have described the KCNH2-3.1 isoform as a binding site for antipsychotics, and its expression and trafficking can be modified by drugs (Kongsamut et al. 2002; Calcaterra et al. 2016). Furthermore, the A allele at *DISC1*-rs3738401 has also been shown as a modulator of the response to antipsychotics (Mouaffak et al. 2011). This effect could be related to the described interaction between the DISC1 protein and dopamine D2 receptors, the main antipsychotic target (Su et al. 2014). This DISC1-D2 protein complex

was increased in post-mortem brain samples of patients with schizophrenia, and disrupting it reduced schizophrenia-like behaviours in mice (Su et al. 2014). These findings and studies highlighting the interaction between DISC1 and dopamine system proteins suggest that DISC1 is a promising pharmacologic target for mental disorders (Dahoun et al. 2017; Tomoda et al. 2017). Convergently, CACNA1C and other voltage-gated calcium channels seem to be of interest for pharmacological interventions since individuals with severe mental illness, including schizophrenia, show decreased rates of psychiatric hospitalisation during periods of exposure to calcium channel blockers (Harrison et al. 2022). Furthermore, pharmacological treatment reverted behavioural and sociability impairments in *CACNA1C* mutant mice (Kabir et al. 2017; Hayes et al. 2019). Concerning *ZNF804A*, the GWAS-associated allele has been repeatedly related to poorer positive symptom improvement after antipsychotic treatment (Mössner et al. 2012; Zhang et al. 2012; Maity et al. 2021). This allele has also been associated with more prolonged hospitalizations in first-episode psychosis patients (Wickramasinghe et al. 2015), underlining the *ZNF804A* modulating role on clinical outcome. Finally, research on pharmacological interventions involving HAR sequences is scarce. Still, a study analysing the association between these evolutionary regions and drugs described that more than 30% of the approved drugs targeted at least one gene associated with HARs, especially drugs for neurological diseases (Chu et al. 2020).

In all, data from the different biological levels, together with our specific findings, point out the importance of synaptic plasticity and gene expression regulatory mechanisms in the biological basis of schizophrenia. Likewise, recently developed cellular model analyses have contributed to this view. Approaches using neurons derived from human induced pluripotent stem cells have validated the effect of schizophrenia-risk variants. They have revealed synergistic effects on gene expression and synaptic function, emphasising the importance of studying the combination of risk variants associated with these processes to understand the biological basis of schizophrenia (Schrode et al. 2019).

Additionally, this thesis also emphasises the complementarity of a dual candidate and whole-genome approach to uncover the biological mechanisms sustaining schizophrenia. On the one hand, candidate gene approaches, which typically follow previous evidence on the pathophysiology of the disorder, provide valuable information about specific genes and biological mechanisms that may be involved in schizophrenia, as well as identify potential drug targets. On the other hand, whole-genome approaches, involving the analysis of multiple genetic data (both at common and rare frequency levels) help to identify novel genetic variants that were not previously suspected to be involved in the pathogenesis (thanks to the hypothesis-free approach). In addition, genome-wide variability studies focusing on specific sets of biologically informative genes can uncover relevant pathways involved in the disorder and provide insight into

how the polygenic risk translates into the neurobiology of schizophrenia (Pergola et al. 2022). By combining these two approaches, it is possible to validate the findings from each other, whole-genome findings can help to prioritise different biological contexts, and candidate gene approaches may narrow the whole-genome targets. Accordingly, both approaches are valuable and valid in searching for the elusive genetic architecture of complex phenotypes (Moore 2017). Together, these approaches offer clues on the relevant functions and provide a more comprehensive understanding of the genetic basis of schizophrenia. In the end, the findings of this thesis bring to the forefront the importance of integrating the neurodevelopmental and synaptic plasticity disruption in an evolutionary framework to understand the brain functional and cognitive variability found in today's human populations and human brain disorders.

A look into the future

Although the specific methodological intricacies of the studies included in this thesis have already been outlined in each publication, a few caveats implicit in this work merit further discussion and may pave the way for future research.

The main limitation of all the presented works implicates the sample size and the lack of replication samples. However, apart from studies developed at international consortia such as ENIGMA and using larger samples such as the UK Biobank, most neuroimaging genetic studies include samples similar to ours of hundreds of individuals (Marek et al. 2022). In counterpart to the power and robustness of international studies, an in-house collected sample allows a better phenotypic screening, characterisation and homogeneity between cases and controls, valuable characteristics for association analyses on intermediate brain phenotypes. Indeed, small, or negligible genetic effects at the population-wide level may become stronger in smaller homogenous subgroups, especially in phenotypes influenced by different sets of causal variants (Robinson et al. 2014). Thus, while international efforts are of paramount importance to foster genetic discoveries, considering the heterogeneity of schizophrenia and the brain itself, the results and replication studies of well-characterised and homogeneous samples have to be also valued and encouraged.

Despite the biological plausibility of the reported findings, results exposed herein should be interpreted considering, first, the small effect of the genetic variants on the phenotypes analysed. Accordingly, while our results add knowledge on the role of synaptic plasticity-related genes in brain activity in schizophrenia, they must be considered under the prism of the complex polygenic architecture of the disorder. Second, compared to the global conclusions that can be derived from studies assessing the impact of thousands of genes in much larger samples, our results must be carefully extrapolated. Third, the genetic findings described in this thesis, although relevant to

schizophrenia, are not exclusive to this psychiatric disorder. Instead, these genetic signals are shared and implicated in a spectrum of psychiatric disorders, which also includes schizophrenia: *CACNA1C* is also among the top GWAS findings in bipolar disorder and major depression (Sullivan et al. 2009; Green et al. 2010a; Sklar et al. 2011; Shang et al. 2023) and, together with *ZNF804A*, was among the shared loci across five major psychiatric disorders (Smoller et al. 2013; Zhang et al. 2016a). In its turn, the HARs have been consistently associated with autism and clinical syndromes such as delirium (Doan et al. 2016; Wei et al. 2019; Won et al. 2019; Takahashi et al. 2020), which can be placed along the neurodevelopmental continuum of psychiatric disorders (Owen 2015; Owen and O'Donovan 2017). This aligns with analyses showing overlapping genetic influences across psychiatric disorders (Anttila et al. 2018; Smeland et al. 2020b; Hindley et al. 2022) and brain-related traits and conditions, including cognitive traits (Smeland et al. 2017, 2020a; Savage et al. 2018). These commonlatities are probably related with the pleiotropic pathways underpinning the pathological and clinical manifestations across brain disorders.

As well, it has to be emphasised that based on the main methodological approaches used in this thesis, rather than directly linking the analysed genetic background with the susceptibility of the disorder, we have assessed the capacity of the synaptic plasticity genes and HARs to shape brain functional response and brain cortical architecture. Since neuroimaging measures are believed to be closer to the genetic background than the diagnosis, the strategy used has helped to link the genetic variants underlying brain alterations (Rose and Donohoe 2013). Nonetheless, the ability to establish causality is hampered, and we cannot assume a clear directionality between the genetic variants studies and the neurobiological alterations leading to psychosis.

Considering that most of the genetic approaches in this thesis have been focused on testing the interaction between genetics and diagnosis, the modulation effect that medication may have on the functional and cognitive phenotypes has not been investigated. Notwithstanding, in all the studies assessing genetic and diagnosis interaction (*KCNH2*, *DISC1* and *CACNA1C* x *ZNF804A*), the effect of antipsychotics was at least partially dismissed based on the non-significant correlations between chlorpromazine equivalents and brain functional and behavioural responses. On the other hand, the analysis of HARs' PRSs effects on brain cortical structure within patients included antipsychotic therapy as a covariate. Still, while chlorpromazine equivalents are widely used as a proxy for the pharmacological status of patients with psychosis (Woods 2003), these complementary analyses do not enable us to completely rule out the effect of patients' pharmacologic profile, as several studies have pointed out that different antipsychotic treatments impact to a different extent on cognition, brain structure and function (Scherk and Falkai 2006; Ibi et al. 2017; Yang et al. 2021).

It is also worth mentioning that, despite the unequivocal link between HARs and neurodevelopmental process, brain architecture, functioning and cognitive and psychiatric phenotypes, these should not be the only evolutionary regions of interest to assess in the context of neuropsychiatric disorders. Recent findings suggest that some neurodevelopmental modifications could have also arisen from more recent genomic changes after the Human and Neanderthal split (Pinson et al. 2022). These findings could explain why regions accumulating human-specific variability after the divergence from Neanderthals seem to harbour schizophrenia susceptibility variants and modulate cognitive abilities (Srinivasan et al. 2016, 2018). Moreover, sequence changes would not be the only features to look at since regions with variable methylation marks between Humans and Neanderthal and Denisova are also enriched in genetic variability associated with schizophrenia (Banerjee et al. 2018). In line, human-specific epigenetic changes embraced by Human Gained Enhancers (HGEs), which are genomic regions that exhibit increased enhancer activity in the human lineage compared to primates, have also been involved in neurodevelopmental processes. Enrichment analyses show that genes associated with HGEs harbour copy number variation associated with schizophrenia (Won et al. 2019). Thus, future studies on human-specific genetic heritage acquired along the evolution would contribute to understanding the complex biological roots of brain function, capacities, and dysfunction leading to psychiatric disorders.

Lastly, while we are beginning to understand the complex picture of the biological determinants of human brain configuration and the pathophysiological foundations of neurodevelopmental psychiatric disorders, the difficulty in accessing the tissue that sustains all these alterations has largely hampered the advances.

New methodological advances may clearly open new venues in the field. For instance, induced neural pluripotent stem cells and brain organoids represent a step forward in generating models that are closer to the biological reality and can be used to recapitulate the neurodevelopmental process (Amin and Paşca 2018; Yoon et al. 2019; Arlotta and Paşca 2019; Pellegrini et al. 2020) offering an unprecedented vision on human-specific neurodevelopmental signatures (Mora-Bermúdez et al. 2016; Otani et al. 2016; Heide et al. 2018; Trujillo et al. 2021). While studies on brain organoids derived from patients with schizophrenia are starting, results describe altered neural progenitor survival and disrupted neurogenesis and show proteomic expression alterations in previously undescribed mechanisms such as spliceosomes and amino acid metabolisms, together with well-known disruptions in pathways related to axonal guidance and synaptogenesis (Notaras et al. 2022; Nascimento et al. 2022). Also, brain organoids with disruptions in DISC1 protein complexes present affectations in neural proliferation due to delays in cell-cycle progression, alterations similar to those observed in organoids from patients (Ye et al. 2017). Similarly, mouse-derived brain organoids containing the human ortholog sequence of a HAR evidenced greater forebrain neuroepithelium proliferation and

pattern, presumably through the HAR enhancer role on neurodevelopmental genes (Acosta et al. 2019).

Final remarks

The brain is the underpinning organ of the cognitive and behavioural repertories found in human populations. It has endowed humans with distinctive traits as a species but also represents the Achilles heel for neuropsychiatric disorders. A precise neurodevelopmental plan with unique human evolutionary traces is necessary for an adaptive neurobiological function. This process relies on a tightly orchestrated spatiotemporal transcriptome regulation, which varies substantially from brain to brain, underlying the variability in cognition, behaviour, and personality traits. However, alterations in this plan, leading to neuronal dysfunctions and neurotransmission alterations, pave the way for the emergence of neurodevelopmental-related psychiatric disorders such as schizophrenia.

The results presented in this thesis and the functional consequences of the DNA sequence changes analysed (both from the candidate gene approaches and the HAR-based approaches) emphasise the importance of genetic regulation of brain-expressed genes in the molecular signatures of psychiatric disorders such as schizophrenia. Then, the findings accentuate the importance of expression changes and encourage future studies on the biology of mental disorders to consider the impact of gene expression trajectories of schizophrenia.

Globally, the dual approach, shifting from candidate to genome-wide variability analyses, evidence that these two methodological views are entirely complementary and necessary for the complete understanding of the biological basis of the disorder. Moreover, the results point to the fact that the underlying aetiological foundations of schizophrenia are, on the one hand, related to the individual genetic differences altering neurodevelopment and synaptic expression trajectories and, on the other, to the genetic differences that define us as a species. Moreover,

Overall, this thesis contributes to comprehending the role of the analysed genes and sequences in the mechanisms underlying disorders inherently linked to the human condition and unique characteristics of our brain and ultimately help unravel what it means to be human.

Conclusions

The specific conclusions derived from the present thesis are:

1. Our data deepen into the role of synaptic plasticity as a key biological mechanism involved in brain activity and cognitive performance in schizophrenia. In particular, we show that common genetic variants at genes implicated in synaptic plasticity, excitatory and neurodevelopmental mechanisms participate, to some extent, in the functional differences observed between healthy controls and patients with schizophrenia during the performance of a task engaging attentional and working memory cognitive domains.

I. The *KCNH2* gene sequence variability modulates the brain activity associated with the execution of the attentional component of the N-back task. Healthy controls and subjects with schizophrenia present opposite activation patterns at the medial superior frontal cortex in response to attention depending on the rs3800779 genotype.

II. Three haplotypes at the *DISC1* gene, HEP1-CTG, HEP3-GA and HEP3-AA, are associated with the liability towards schizophrenia. In turn, HEP3-GA and HEP3-AA modulate the brain's functional response to attention and working memory conditional on the diagnostic status.

III. The epistasis between the *CACNA1C* and the *ZNF804A* genes is associated with the functional response to working memory load increase independently of the diagnosis status. While there was no three-way interaction with diagnosis, patients with both alleles of risk were the only ones showing an opposite difficulty modulatory response.

2. The Human Accelerated Regions (HARs) are part of the neurodevelopmental gene expression regulatory machinery and are involved in human-specific brain architecture, function, and susceptibility to psychiatric disorders.

I. The neurodevelopmental enhancer role of HARs underlies individual differences in brain structural and functional organization and sustains the inherent variability in cognition, behaviour, and susceptibility towards neurodevelopmental psychiatric disorders such as schizophrenia and autism.

II. The schizophrenia's polygenic load of HARs specifically associated with foetal active transcriptional regulatory elements influences the cortical surface area, but not the cortical thickness, in patients with schizophrenia.

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Curriculum Vitae

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Biologist and predoctoral researcher.
PFIS Predoctoral Grant (ISCIII, FI19/0352).
Neurobiology and Genetics of Psychotic disorders (NeuroBioGen), FIDMAG Germanes Hospitalàries Research Foundation.

ACADEMIC TRAINING

2017 – present	**Doctoral thesis in Biodiversity.** University of Barcelona
2014 – 2015	**Master's Degree in Biological Anthropology: specialization in Human Biodiversity and Biomedical Applications.** University of Barcelona and Autonomous University of Barcelona. (Qualification: 9.5)
2009 – 2014	**Bachelor's Degree in Biology.** Autonomous University of Barcelona. (Qualification: 6.9)

STAYS AND RESEARCH COLLABORATIONS

March 2022 – July 2022	**PhD research stay.** Precision Psychiatry Research Group, NORMENT Institute of Clinical Medicine. University of Oslo
June 2016 – December 2016	**Extracurricular collaboration.** Neurobiology and Genetics of Psychotic disorders (NeuroBioGen) research line, FIDMAG Germanes Hospitalàries Research Foundation
September 2015 – May 2016	**Extracurricular collaboration.** Population Genetics research line, Biological Anthropology Unit, Department OF Animal Biology, Vegetal Biology and Ecology. Autonomous University of Barcelona.

PARTICIPATION IN COMPETITIVE PROJECTS

2023 – 2026	**Researcher**. *Targeting mRNAs condensates in neurites for a better understanding of synaptic plasticity dysfunction in schizophrenia* (**41/C/2022**). Fundació La Marató de TV3, Barcelona, Spain. PI: Mar Fatjó-Vilas. 299.906,25€.
2023 – 2025	**Researcher** (Grupo CIBERSAM). *Medicina Personalizada (MedPer) en la detección precoz del deterioro cognitivo (DC) preclínico. Desarrollo de un modelo predictivo de riesgo* (**PMP22/00084**). Ministerio de Ciencia e Innovación, Madrid, Spain. PI: Ángeles Almeida Parra, PI Coordinated Project: Mar Fatjó-Vilas. 1.650.550€.
2022 – 2024	**Researcher**. *Grup de recerca consolidat FIDMAG Germanes Hospitalàries Research Foundation* (**2022SGR1475**). Comissionat per a Universitat i Recerca del DIUE (Agència de Gestió d'Aguts Universitaris i de Recerca – AGAUR). PI: Edith Pomarol-Clotet. 40.000€.
2021 – 2023	**Researcher.** *Neurodevelopment markers and schizophrenia: analysis of their shared genetic underpinnings and the modulation effect of prenatal stress* (**PI20/01002**). Instituto de Salud Carlos III, Madrid, Spain. PI: Mar Fatjó-Vilas. 63.500€.

2019 – 2021   **Researcher.** *Reguladores de la expresión génica como predictores de diagnóstico y biomarcadores de déficits cognitivos y cambios en el grosor cortical en psicosis de inicio reciente* (**PI18/01535**). Instituto de Salud Carlos III, Madrid, Spain. PI: Joan Soler. 76.230€.

2018 – 2020   **Researcher.** *Assessment of the impact of genetic variability in Human Accelerated Regions (HARs) on brain abnormalities in schizophrenia* (**NARSAD2017**). NARSAD Young Investigator Grant from the Brain & Behavior Research Foundation, New York, United States of America. PI: Mar Fatjó-Vilas. 70.000$.

SCIENTIFIC PUBLICATIONS

Articles in international scientific journals:

1. **Guardiola-Ripoll, M**; Sotero-Moreno, A; Kebir, O; Chaumett, B; Almodóvar-Payá,C; Hostalet, N; Moreira, M; Giralt, M; Salvador, R; Fatjó-Vilas, M. *Endocannabinoid system genetic variability role on dermatoglyphic patterns and schizophrenia-spectrum disorders (Submitted).*

2. Oscoz-Irurozqui, M and Almodóvar-Payá, C; **Guardiola-Ripoll, M**; Guerrero-Pedraza, A; Salgado-Pineda, P; Sarró, S; Pomarol-Clotet, E; Fatjó-Vilas, M. *Cannabis use and endocannabinoid receptor genes: effects on brain activity in first-episode psychosis (Submitted).*

3. **Guardiola-Ripoll, M**; Almodóvar-Payá, C; Arias-Magnasco, A; Latorre-Guardia, M; Papiol, S; Canales-Rodríguez, EJ; García-León, MA; Fuentes-Claramonte, P; Salavert, J; Tristany, J; Torres, L; Rodríguez-Cano, E; Salvador, R; Pomarol-Clotet, E; Fatjó-Vilas, M. *Human-specific evolutionary markers linked to foetal neurodevelopment modulate brain surface area in schizophrenia (Submitted).*

4. **Guardiola-Ripoll, M**; Fatjó-Vilas, M. *A systematic review of the Human Accelerated Regions in schizophrenia and related disorders: where the evolutionary and neurodevelopmental hypotheses converge.* **Int J Mol Sci**. 2023 Feb; 24(4):3597. doi: 10.3390/ijms24043597. (IF 2021=6.208, 69/297 (Q1) Biochemistry & Molecular Biology)

5. Salvador, R; García-León, MA; Feria-Raposo, I; Botillo-Martín, C; Martín-Lorenzo, C; Corte-Souto, C; Aguilar-Valero,T; Gil-Sanz,D; Porta-Pelayo, D; Martín-Carrasco, M; del Olmo-Romero, F; Santiago-Bautista, JM; Herrero-Muñecas, P; Castillo-Oramas, E; Larrubia-Romero, J; Rios-Alvarado, Z; Larraz-Romero, JA; **Guardiola-Ripoll,M**; Almodóvar-Payá, C; Fatjó-Vilas, M; Sarró, S; McKenna, PJ; HHFingerprints Group, Pomarol-Clotet, E. *Fingerprints as predictors of schizophrenia: a Deep learning study.* **Schizophrenia Bulletin**. 2022. sbac173. https://doi.org/10.1093/schbul/sbac173. (IF 2021=7.348, 30/155 (Q1) Psychiatry)

6. Almodóvar-Payá, C and **Guardiola-Ripoll, M**; Giralt-López, M; Gallego, C; Miret, S; Muñoz, MJ; Parellada, M; Lázaro, L; Cuesta, MJ; Fañanás, L; Arias, B; Pomarol-Clotet, E; Fatjó-Vilas, M. *NRN1 Gene as a Potential Marker of Early-Onset Schizophrenia: Evidence from Genetic and Neuroimaging Approaches.* **Int J Mol Sci**. 2022 Jul 5;23(13):7456. doi: 10.3390/ijms23137456. PMID: 35806464; PMCID: PMC9267632. (IF 2021=6.208, 69/297 (Q1) Biochemistry & Molecular Biology)

7. **Guardiola-Ripoll, M** and Sotero, A; Almodóvar-Payá, C; Hostalet, N; Salgado-Pineda, P; Salvador, R; Ortiz-Gil, J; Sarró, S; McKenna, PJ; Madre, M; Pomarol-Clotet, E; Fatjó, Vilas, M. *Combining fMRI and DISC1 gene haplotypes to understand working memory-related brain activity in schizophrenia.* **Sci Rep**. 2022 May 5;12(1):7351. doi: 10.1038/s41598-022-10660-8. PMID: 35513527; PMCID: PMC9072540. (IF 2021=4.997, 19/74 (Q2) Multidisciplinary Sciences)

8. **Guardiola-Ripoll, M** and Almodóvar-Payá, C; Lubeiro, A; Salgado-Pineda, P; Salvador, R; Gomar, JJ; Guerrero-Pedraza, A; Sarró, S; Maristany, T; Fernendez-Linsenbarth, I; Hernández-García, M; Papiol, S; Molina, V; Pomarol-Clotet, E; Fatjó-Vilas, M. *New insights of the role of the KCNH2 gene in schizophrenia: An fMRI case-control study.* **Eur Neuropsychopharmacol**. 2022 Jul;60:38-47. doi: 10.1016/j.euroneuro.2022.04.012. Epub 2022 May 26. PMID: 35635995. (IF 2021=5.415, 48/212 (Q1) Clinical Neurology)

9. **Guardiola-Ripoll M** and Almodóvar-Payá, C; Lubeiro, A; Sotero, A; Salgado-Pineda, P; Salvador, R; Papiol, S; Ortiz-Gil, J; Sarró, S; McKenna, PJ; Maristany, T; Molina, V; Pomarol-Clotet, E; Fatjó, Vilas, M. *A functional neuroimaging association study on the interplay between two schizophrenia genome-wide associated genes (CACNA1C and ZNF804A).* **Eur Arch Psychiatry Clin Neurosci**. 2022 Oct;272(7):1229-1239. doi: 10.1007/s00406-022-01447-z. Epub 2022 Jul 7. PMID: 35796825. (IF 2021=5.760, 41/212 (Q1) Clinical Neurology)

10. Mitjans, M and Papiol, S; Barrot, C; **Guardiola-Ripoll, M**; Giménez, A; Gavín, P; Acosta, M; Gutiérrez, B; Schulze, T; Fatjó-Vilas, M; Arias, B and Benabarre, A. *Completed suicide is associated with a higher polygenic burden for psychiatric disorders*. **Eur Arch Psychiatry Clin Neurosci**. 2022 Apr;272(3):355-358. doi: 10.1007/s00406-022-01398-5. PMID: 35284949. (IF 2021=5.760, 41/212 (Q1) Clinical Neurology)

11. Santo-Angles, A; Fuentes-Claramonte, P; Argila, I; **Guardiola-Ripoll, M**; Almodóvar-Payá, C; Munuera, J; McKenna, PJ; Pomarol-Clotet, E; Radua, J. *Reward and fictive prediction error signals in ventral striatum: asymmetry between factual and counterfactual processing.* **Brain Structure and Function.** 2021 Apr 11. doi: 10.1007/s00429-021-02270-3. Epub ahead of print. PMID: 33839955. (IF 2021=3.748, 1/21 (D1) Anatomy & Morphology)

12. Fuentes-Claramonte, P; Santo-Angles, A; Argila-Plaza, I; Lechón, M; **Guardiola-Ripoll, M**; Almodóvar-Payá, C; Cullen, B; Evans, JJ; Manly, T; Gee, A; Maristany, T; Sarró, S; Pomarol-Clotet, E; McKenna, PJ; Salvador, R. *Brain imaging of executive function with the computerised multiple elements test.* **Brain Imaging Behav.** 2021 Jan 26. doi: 10.1007/s11682-020-00425-0. Epub ahead of print. PMID: 33501628. (IF 2021=3.224, 7/14 (Q2) Neuroimaging)

13. Fatjó-Vilas, M; Soler, J; Ibáñez, MI; Moya-Higueras, J; Ortet, G; **Guardiola-Ripoll, M**; Fañanás, L; Arias, B. *The effect of the AKT1 gene and cannabis use on cognitive performance in healthy subjects.* **J Psychopharmacol**. 2020 Sep;34(9):990-998. doi: 10.1177/0269881120928179. Epub 2020 Jun 13. PMID: 32536252. (IF 2021=4.562, 70/212 (Q2) Clinical Neurology)

14. Fullana, M A; Tortella-Feliu, M; Fernández de la Cruz, L; Chamorro, J; Pérez-Vigil, A; Ioannidis, J P A; Solanes, A; **Guardiola, M**; Almodóvar, C; Miranda-Olivos, R; Ramella-Cravaro, V; Vilar, A; Reichenberg, A; Mataix-Cols, D; Vieta, E; Fusar-Poli, P; Fatjó-Vilas, M; Radua, J. *Risk and protective factors for anxiety and obsessive-compulsive disorders: an umbrella review of systematic reviews and meta-analyses.* **Psychol Med**. 2020 Jun;50(8):1300-1315. doi: 10.1017/S0033291719001247. Epub 2019 Jun 7. PMID: 31172897. (IF 2021=10.592, 11/143 (D1) Psychiatry)

15. Lubeiro, A; Fatjó-Vilas, M; **Guardiola, M**; Almodóvar, C; Gomez-Pilar, J; Cea-Cañas, B; Poza, J; Palomino, A; Gómez-García, M; Zugasti, J; Molina, V. *Analysis of KCNH2 and CACNA1C schizophrenia risk genes on EEG functional network modulation during an auditory odd-ball task.* **Eur Arch Psychiatry Clin Neurosci**. 2020 Jun;270(4):433-442. doi: 10.1007/s00406-018-0977-0. Epub 2019 Jan 3. PMID: 30607529. (IF 2021=5.760, 41/212 (Q1) Clinical Neurology)

Articles in national scientific journals:

1. Fatjó-Vilas, M; Almodóvar-Payá, C; **Guardiola-Ripoll, M**; Oscoz-Irurozqui, M; Sotero, A; Salgado-Pineda, P; Salvador, R; McKenna, PJ; Pomarol-Clotet, E. Factors genètics i salut mental: estudis aplicats a la recerca de les causes i de la millora diagnòstica. The Business, Research, Ageing, Innovation & Neuroscience journal (Brain), 2020 September; 4:22-24.

WORKS SUBMITTED TO NATIONAL AND INTERNATIONAL CONFERENCES

1. **Guardiola-Ripoll M**; Almodóvar-Payá, C; Arias, A; Latorre-Guardia, M; Canales-Rodríguez, EJ; García-León, MA; Fuentes-Claramonte, P; Salvador, R; Pomarol-Clotet, E; Papiol, S; Fatjó-Vilas, M. *Genetic markers of evolution and brain cortical surface area in schizophrenia: are they related?* (Poster communication). Organization for Human Brain Mapping OHBM 2023 Congress. July 22nd-26th 2023, Montréal, Canada.

2. Almodóvar-Payá, C; **Guardiola-Ripoll, M**; Gallego, C; Callado, LF; Giralt-Lopez, M; Latorre-Guardia, M; Salgado-Pineda, P; Canales-Rodríguez, E; Miret, S; Fañanás, L; Penadés, R; Fuentes-Claramonte, P; Salvador, R; Arias, B; Pomarol-Clotet, E; Fatjó-Vilas, M. *The effect of NRN1 on schizophrenia: an intersectional study integrating molecular and neuroimaging approaches* (Poster communication). 35th ECNP Congress. October 15th-18th 2022, Vienna, Austria.

3. Martínez, M; **Guardiola-Ripoll, M**; Oscoz-Irurozqui, M; Almodóvar-Payá, C; Guerrero-Pedraza, A; Hostalet, N; Salvador, R; Carrión, MI; Maristany, T; Pomarol-Clotet, E; Fatjó-Vilas, M. DRD2 and DRD3 genes, cannabis use and brain activity in first-episode psychosis (Oral and poster communication). 35th ECNP Congress. October 15th-18th 2022, Vienna, Austria

4. Martínez, M; **Guardiola-Ripoll, M**; Oscoz-Irurozqui, M; Allmodóvar-Payá, C; Guerrero-Pedraza, A; Hostalet, N; Salvador, R; Carrión, MI; Maristany, T; Pomarol-Clotet, E; Fatjó-Vilas, M. *Polymorphic variants at dopamine receptor genes (DRD2 and DRD3) and cannabis use: effects on brain activity in first-episode psychosis (FEP)* (Poster communication). 22nd World Congress of Psychiatry (WCP). August 3rd-6th 2022, Bangkok, Thailand.

5. Sotero-Moreno, A; **Guardiola-Ripoll, M**; Moreira, M; Giralt-López, M; Almodóvar-Payá, C; Hostalet, N; Miret, S; Campanera, S; Muñoz, MJ; Salvador, R; Fañanás, L; Pomarol-Clotet, E; Fatjó-Vilas, M. *Biomarcadores del neurodesarrollo en esquizofrenia: genética, neuroimagen y dermatoglifos* (Oral communication). I Congreso Multidisciplinario Internacional del Hospital Psiquiátrico Santa Rosita. Salud Mental a través del Tiempo, por un Hospital Psiquiátrico ¡SIN ESTIGMAS! May 18th-20th 2022, Virtual.

6. **Guardiola-Ripoll, M**; Sotero-Moreno, A; Kebir, O; Chaumette, B; Almodóvar-Payá, C; Hostalet, N; Moreira, M; Giralt, M; Salvador, R; Odile-Krebs, M; Fatjó-Vilas, M. *Markers in Schizophrenia-Spectrum disorders: Analysis of the Combined Role of the Cannabinoid Receptor 1 Gene (CNR1) and Dermatoglyphics* (Poster communication). 2022 Congress of the Schizophrenia International Research Society (SIRS). April 6th-10th, Florence, Italy.

7. Latorre-Guardia M; Almodóvar-Payá C; Arias B; Penadés B; **Guardiola-Ripoll M**; García-León MA; Fuentes-Claramonte P; Pomarol-Clotet E; Fatjó-Vilas M. *FKBP5 methylation patterns and brain structural correlates in schizophrenia* (Oral communication). 2022 Congress of the Schizophrenia International Research Society. April 6th-10th, Florence, Italy.

8. Almodóvar-Payá, C; Latorre-Guardia, M; Arias, B; Penadés, R; **Guardiola-Ripoll, M**; Fuentes-Claramonte, P; García-León, MA; Pomarol-Clotet, E; Fatjó-Vilas, M. *Methylation and brain structure in schizophrenia: analysis of the role of COMT and BDNF genes* (Poster communication). World Congress of Psychiatric Genetics (WCPG). 11th-15th October 2021, Virtual.

9. Almodóvar-Payá, C and **Guardiola-Ripoll, M**; Gallego, C; Hostalet, N; Sotero, A; Guerreo-Pedraza, A; Ramiro, N; Torres, L; Salvador, R; Pomarol-Clotet, E; Fatjó-Vilas, M. *The interplay between cannabinoid receptor genes and cannabis use: effects on brain activity in first-episode psychosis* (Poster communication). World Congress of Psychiatric Genetics (WCPG). 11th-15th October 2021, Virtual.

10. Moreno, M; Ortiz, R; **Almodóvar-Payá, C**; Guardiola-Ripoll, M; Callado, LF; Fatjó-Vilas, M; Gallego, C. *Study of NRN1 expression in brain: analysis in individuals diagnosed with schizophrenia and healthy subjects* (Poster communication). World Congress of Psychiatric Genetics (WCPG). 11th-15th October 2021, Virtual.

11. Oscoz-Irurozqui, M; Almodóvar-Payá, C; **Guardiola-Ripoll, M**; Guerreo-Pedraza; Hostalet, N; Pomarol-Clotet, E; Fatjó-Vilas, M. *Cannabis Use and CNR1 Gene: Effects on Psychotic Symptoms and Cognition in First-episode Psychosis* (Poster communication). World Congress of Psychiatric Genetics (WCPG). 11th-15th October 2021, Virtual.

12. Almodóvar-Payá, C; Latorre-Guardia, M; Arias, B; Penadés, R; **Guardiola-Ripoll, M**; Fuentes-Claramonte, P; García-León, M; Pomarol-Clotet, E; Fatjó-Vilas,M. *Candidate genes of schizophrenia methylation patterns and brain structural correlates* (Poster communication). 34th European College of Neuropsychopharmacology Congress (ECNP). October 2nd – 5th 2021, Lisbon, Portugal.

13. Oscoz Irurozqui, M; Almodóvar-Payá, C; **Guardiola-Ripoll, M**; Guerrero-Pedraza, A; Aquino, A; Salgado-Pineda, P; Sarró, S; Pomarol-Clotet, E; Fatjó-Vilas, M. *Cannabinoid receptor genes, cannabis use and brain activity in first-episode psychosis* (Poster communication). 34th European College of Neuropsychopharmacology Congress (ECNP). October 2nd – 5th 2021, Lisbon, Portugal.

14. **Guardiola-Ripoll, M** and Sotero, A; Almodóvar-Payá, C; Hostalet, N; Salgado-Pineda, P; Salvador, R; Ortiz-Gil, J; Sarró, S; McKenna, PJ; Madre, M; Pomarol-Clotet, E; Fatjó-Vilas, M. *DISC1 gene and brain activity: a genetic neuroimaging association study* (Oral communication). 2021 Congress of the Schizophrenia International Research Society (SIRS). 17th-21st April 2021, Virtual. *Co-chair at the Oral Session: Genomes, Brain and Symptoms.

15. **Guardiola-Ripoll, M**; Almodóvar-Payá, C; Lubeiro, A; Sotero, A; Salgado-Pineda, P; Salvador, R; Papiol, S; Ortiz-Gil, J; Sarró, S; McKenna, PJ; Maristany, T; Molina, V; Pomarol-Clotet, E; Fatjó-Vilas, M. *The interplay between two schizophrenia GWAS genes on fMRI working memory response: Evidence of CACNA1C x ZNF804A epistatic effect on brain function* (Poster communication). 2021 Congress of the Schizophrenia International Research Society (SIRS). 17th-21st April 2021, Virtual.

16. Almodóvar-Payá, C; **Guardiola-Ripoll, M**; Gallego, C; Hostalet, N; Sotero, A; Guerrero-Pedraza, A; Ramiro, N; Torres, Ll; Salvador, R; Pomarol-Clotet, E; Fatjó-Vilas, M. *Evidence of epistatic effect involving the NRN1, KCNH2 and CACNA1C genes on the risk for schizophrenia-spectrum disorders and its impact on psychopathology* (Poster communication). 2021 Congress of the Schizophrenia International Research Society (SIRS). 17th-21st April 2021, Virtual.

17. Almodóvar-Payá, C; García-Torrents, E; **Guardiola-Ripoll, M**; Canales-Rodríguez, E; Hostalet, N; Gallego, C; Martín-Subero, M; Madre, M; Pomarol-Clotet, E; Fatjó-Vilas, M. *The effect of NRN1 gene on cortical thickness in healthy subjects and subjects with schizophrenia* (Poster communication). 2021 Congress of the Schizophrenia International Research Society (SIRS). 17th-21st April 2021, Virtual.

18. Oscoz-Irurozqui, M; Almodóvar-Payá, C; **Guardiola-Ripoll, M**; Guerrero-Pedraza, A; Salgado-Pineda, P; Sarró, S; Pomarol-Clotet, E; Fatjó-Vilas, M. *The interplay between cannabinoid receptor genes and cannabis use: effects on brain activity in first-episode psychosis* (Poster communication). 2021 Congress of the Schizophrenia International Research Society (SIRS). 17th-21st April 2021, Virtual.

19. Almodóvar-Payá, C; **Guardiola-Ripoll, M**; Hostalet, N; Giralt-López, M; Arias, B; Prats, C; Gallego, C; Millet, S; Fañanás, L; Pomarol-Clotet, E; Fatjó-Vilas, M. *Common variants at plasticity gene NRN1 and its interaction with BDNF gene are associated with early-onset schizophrenia* (Poster communication). XX World Psychiatry Association (WPA) World Congress of Psychiatry. 10th-13th May 2021, Virtual.

20. Almodóvar-Payá, C; Garcia-Torrents, E; **Guardiola-Ripoll, M**; Canales-Rodríguez, EJ; Hostalet, N; Gallego, C; Martín-Subero, M; Madre, M; Pomarol-Clotet, E; Fatjó-Vilas, M. *The role of NRN1 gene in schizophrenia: analysis of its genetic variability in cortical thickness* (Oral communication). XX World Psychiatry Association (WPA) World Congress of Psychiatry. 10th-13th May 2021, Virtual.

21. **Guardiola-Ripoll, M**; Almodóvar-Payá, C; Lubeiro, A; Sotero, A; Salgado-Pineda, P; Salvador, R; Papiol, S; Ortiz-Gil, J; Sarró, S; McKenna, PJ; Maristany, T; Molina, V; Pomarol-Clotet, E; Fatjó-Vilas, M. *Epistatic effect of two schizophrenia genome-wide associated candidate genes (CACNA1C and ZNF804A) on working memory functional and behavioural response* (Oral communication). XX World Psychiatry Association (WPA) World Congress of Psychiatry. 10th-13th May 2021, Virtual.

22. **Guardiola-Ripoll, M** and Sotero, A; Almodóvar-Payá, C; Hostalet, N; Salgado-Pineda, P; Salvador, R; Ortiz-Gil, J; Sarró, S; McKenna, PJ; Madre, M; Pomarol-Clotet, E; Fatjó-Vilas, M. *Effect of schizophrenia risk haplotypes of DISC1 gene on working memory: a case-control neuroimaging association study response* (Oral communication). XX World Psychiatry Association (WPA) World Congress of Psychiatry. 10th-13th May 2021, Virtual.

23. Oscoz-Irurozqui, M; Almodóvar-Payá, C; **Guardiola-Ripoll, M**; Guerrero-Pedraza, A; Salgado-Pineda, P; Sarró, S; Pomarol-Clotet, E; Fatjó-Vilas, M. *Cannabis use and cannabinoid receptor genes interaction modulates brain activity in first-episode psychosis* (Poster communication). XX World Psychiatry Association (WPA) World Congress of Psychiatry. 10th-13th May 2021, Virtual.

24. Oscoz-Irurozqui, M and **Guardiola-Ripoll, M**; Almodóvar-Payá, C; Sarró, S; Guerrero-Pedraza, A; Pomarol-Clotet, E; Fatjó-Vilas, M. *Cannabis use and genes of the endocannabinoid system: their role in psychotic symptoms and cognition in first-episode psychosis* (Oral communication). XXII Congreso de Patología Dual. 16th-19th November 2020, Virtual.

25. Fatjó-Vilas M and **Guardiola-Ripoll, M**; Almodóvar- Payá, C; Salgado-Pineda, P; Lubeiro, A; Alonso-Lana, S; Ortiz-Gil, J; Guerrero-Pedraza, A; Sarro, S; Pomarol-Clotet, E. *The role of KCNH2 gene on cognitive deficits in schizophrenia: a functional MRI study* (Poster communication). ResDem 2019: II International Conference on Cognitive Reserve in Dementia and other Disorders. 15th-16th November 2019, Munich, Germany.

26. Oscoz-Irurozqui, M and **Guardiola-Ripoll, M**; Almodóvar-Payá, C; Sarró, S; Guerrero-Pedraza, A; Pomarol-Clotet, E; Fatjó-Vilas, M. *Cannabis use and genes of Dopaminergic and Endocannabinoid systems: their role in psychotic symptoms and cognition in First-episode Psychosis* (Poster communication). XIX World Psychiatric Association (WPA) World Congress. 21st-24th August 2019, Lisbon, Portugal.

27. Oscoz-Irurozqui, M; Almodóvar-Payá, C; **Guardiola-Ripoll, M**; Guerrero-Pedraza, A; Salgado-Pineda, P; Sarró, S; Pomarol-Clotet, E; Fatjó-Vilas, M. *Cannabis use and CNR2 gene interaction modulates brain activity in first episode psychosis* (Poster communication). III World Congress and VI International Congress on Dual Disorders from the Spanish Society of Dual Pathology (SEPD). 19th-22nd June 2019, Madrid, Spain.

28. Fuentes-Claramonte, P; Santo-Angles, A; Salvador, R; Argila, I; Albacete, A; **Guardiola-Ripoll, M;** Almodovar, C; Ramiro, N; Boix, E; Salgado-Pineda, P; Bosque, C; Torres, ML; Panicalli, F; Sarri, C; Portillo, F; McKenna, PJ; Pomarol-Clotet, E. *Negative symptoms in schizophrenia as a deficit in goal management: an fMRI study* (Poster communication). 2019 OHBM Annual Meeting of the Organization for Human Brain Mapping. 9th-13th June 2019, Rome, Italy.

29. Santo-Angles, A; Fuentes-Claramonte, P; Argila, I; **Guardiola-Ripoll, M**; Almodovar, C; McKenna, PJ; Pomarol-Clotet, E; Radua, J. *Reward and Fictive Prediction Error signals in the Ventral Striatum* (Poster communication). 2019 OHBM Annual Meeting of the Organization for Human Brain Mapping. 9th-13th June 2019, Rome, Italy.

30. Fatjó-Vilas, M; Soler, J; Ibáñez, MI; Moya, J; Ortet, G; **Guardiola, M**; Fañanás, L; Arias, B. *AKT1 Gene and Cannabis Use: Analysis of Moderation Effects on Cognitive Performance in Non-Clinical Subjects* (Poster communication). XXVI World Congress of Psychiatric Genetics. 11th-15th October 2018, Glasgow, United Kingdom.

31. **Guardiola, M**; Almodóvar, C; Salgado-Pineda, P; Lubeiro, A; Alonso-Lana, S; Ortiz-Gil, J; Guerrer-Pedraza, A; Sarró, S; Pomarol-Clotet, E, Fatjó-Vilas, M. *Effect of KCNH2 and CACNA1C on Cognitive Performance and Brain Activity: Genetic Association Study in Schizophrenia Patients and Healthy Subjects* (Poster communication). XXVI World Congress of Psychiatric Genetics. 11th-15th October 2018, Glasgow, United Kingdom.

32. Almodóvar, C; **Guardiola, M**; Salgado-Pineda, P; Gallego, C; Prats, C; Arias, B; Pomarol-Clotet, E; McKenna, PJ; Fatjó-Vilas, M. *Evidence of NRN1 Gene Effect on Schizophrenia Age at Onset and Brain Activity* (Poster communication). XXVI World Congress of Psychiatric Genetics. 11th-15th October 2018, Glasgow, United Kingdom.

33. **Guardiola, M**; Almodóvar, C; Lubeiro, A; Alonso-Lana, S; Ortiz-Gil, J; Guerrero-Pedraza, A; Sarró, S; Pomarol-Clotet, E; Fatjó-Vilas, M. *Calcium and potassium voltage-gated channels genes association analysis: evidence on their role in cognitive performance of schizophrenia patients and healthy subjects* (Poster communication). 6th Biennial Schizophrenia International Research Society Conference 2018. 4th-8th April 2018, Florence, Italy. Schizophrenia Bulletin 44 (Supplement 1):s145-s146.

34. Almodóvar, C; **Guardiola, M**; Salgado-Pineda, P; Moreno, M; Gallego, C; Prats, C; Arias, B; Pomarol-Clotet, E; McKenna, PJ; Fatjó-Vilas, M. *NRN1 and functional MRI: association analysis in Schizophrenia Patients and Healthy Subjects* (Poster communication). 6th Biennial Schizophrenia International Research Society Conference 2018. 4th-8th April 2018, Florence, Italy. Schizophrenia Bulletin 44 (Supplement 1):s174-s175.

35. Lubeiro, A; Molina Rodriguez, V; **Guardiola, M**; Gómez Pilar, J; Fatjó-Vilas, M. *KCNH2 polymorphism associated to altered EEG functional network modulation in schizophrenia* (Poster communication). 6th Biennial Schizophrenia International Research Society Conference 2018. 4th-8th April 2018, Florence, Italy. Schizophrenia Bulletin 44 (Supplement 1): s397-s398.

36. **Guardiola, M**; Soler, J; Ibáñez, MI; Moya, J; Ortet, G; Fañanás, L; Arias, B; Fatjó-Vilas, M. *AKT1 gene and cannabis interaction effects on cognitive performance in healthy subjects* (Poster communication). XX Congreso de la Sociedad Española de Antropología Física. 12th-14th July 2017, Barcelona, Spain.

37. González, M.M; Ramos, A; Mateiu, L; **Guardiola, M**; Goios, A; Aluja, M.P; Santos, C. *Classification of nuclear insertions of mitochondrial origin: Candidates to the study of human variability* (Poster communication). 16th Portugaliae Genetica Congress. 20th-21st March 2014, Oporto, Portugal.

WORKS SUBMITTED TO NATIONAL WORSHOPS AND SEMINARS

1. **Guardiola-Ripoll M**; Almodóvar-Payá, C; Arias, A; Latorre-Guardia, M; Canales-Rodríguez, EJ; García-León, MA; Fuentes-Claramonte, P; Salvador, R; Pomarol-Clotet, E; Papiol, S; Fatjó-Vilas, M. *The evolutionary traces behind schizophrenia: Human Accelerated Regions (HARs) linked to foetal neurodevelopment modulate cortical surface area in patients with the disorder*. X Laboratorio de Ideas CIBERSAM 2023. April 20th-21st 2023, Reus, Spain.

2. Sotero-Moreno, A; **Guardiola-Ripoll, M**; Moreira, M; Giralt-López, M; Almodóvar-Payá, C; Hostalet, N; Miret, S; Campanera, S; Muñoz, MJ; Salvador, R; Fañanás, L; Pomarol-Clotet, E; Fatjó-Vilas, M. *Disrupted in Schizophrenia 1 gene (DISC1) as a potential mediator of dermatoglyphic neurodevelopmental markers in schizophrenia* (Oral communication). XII Simposi de Neurobiologia: Cap a la Medicina Traslacional. Societat Catalana de Biologia. 13th May 2022, Barcelona, Spain.

3. Latorre-Guardia, M & Almodóvar-Payá, C; Arias, B; Penadés, R; **Guardiola-Ripoll, M**; García-León, MA; Fuentes-Claramonte, P; Pomarol-Clotet, E; Fatjó-Vilas, M. *FKBP5 methylation patterns: cortical thickness and clinical correlates in schizophrenia* (Oral communication). XII Jornada de Cromatina i Epigenètica de la Societat Catalana de Biologia. 13th May 2022, Barcelona, Spain.

4. **Guardiola-Ripoll, M**; Sotero-Moreno, A; Kebir, O; Chaumette, B; Almodóvar-Payá, C; Hostalet, N; Moreira, M; Giralt, M; Salvador, R; Odile-Krebs, M; Fatjó-Vilas, M. *Endocannabinoid system genetic variability role on dermatoglyphic patterns and schizophrenia-spectrum disorders* (Oral communication). 5th Biomed PhD Day. 10th February 2022, Barcelona, Spain.

5. **Guardiola-Ripoll, M**; Sotero, A; Almodóvar-Payá C; Hostalet, N; Salvador, R; Pomarol-Clotet, E; Fatjó-Vilas, M. *Novel findings on the pathophysiological mechanisms in schizophrenia: studies on the brain functional effects of haplotypic variability and genetic epistasis* (Oral communication). VIII Laboratorio de Ideas CIBERSAM 2021. 25th-27th May 2021, Virtual.

6. Latorre-Guardia, M; Almodóvar-Payá, C; Penadés, R; Arias,B; **Guardiola-Ripoll, M**; Fuentes-Claramonte, P; García-León, MA; Pomarol-Clotet, E; Fatjó-Vilas, M. *Impact of COMT and BDNF epigenetic profiles on brain cortex in schizophrenia* (Oral communication). XI Jornada de Cromatina i Epigenética de la Societat Catalana de Biologia. 14th May 2021, Barcelona, Spain.

7. **Guardiola-Ripoll, M**; Almodóvar, C; Salgado-Pineda, P; Lubeiro, A; Alonso-Lana, S; Ortiz-Gil, J; Guerrer-Pedraza, A; Sarró, S; Pomarol-Clotet, E, Fatjó-Vilas, M. *Genetic variability in neural excitability genes modulates cognitive performance and brain activity a case-control study in schizophrenia* (Oral communication). XI Simposi de Neurobiologia de la Societat Catalana de Biologia. 12th-13th November 2018, Barcelona, Spain.

8. Almodóvar, C; **Guardiola-Ripoll, M**; Salgado-Pineda, P; Gallego, C; Prats, C; Arias, B; Pomarol-Clotet, E; McKenna, PJ; Fatjó-Vilas, M. *The role of Neuritin gene in modulating schizophrenia age at onset and brain activity during a working memory task* (Oral communication). XI Simposi de Neurobiologia de la Societat Catalana de Biologia. 12th-13th November 2018, Barcelona, Spain.

9. **Guardiola-Ripoll, M**. *Cóm la variabilitat genética modula l'activitat cerebral: anàlisi dels gens implicats en l'excitabilitat neuronal* (Oral communication). Clinical Session at Benito Menni CASM, Barcelona, Spain. 7th June 2018, Sant Boi de Llobregat, Spain.

10. **Guardiola-Ripoll, M**. *CACNA1C and KCNH2 effect on Cognitive Performance and Brain Activity in Schizophrenia patients and healthy subjects: Preliminary results* (Oral communication). VI Laboratorio de Ideas CIBERSAM 2018. 31st May- 1st June 2018, San Fernando, Spain.

11. Oscoz-Irurozqui, M; **Guardiola-Ripoll, M**; Almodóvar, C; Fatjó-Vilas, M; Sarró, S; Guerrero-Pedraza, A; Pomarol-Clotet, E; *Variabilidad en genes del sistema dopaminérgico y cannabinoide y su asociación con el consumo de cannabis en pacientes con un primer episodio psicótico: evidencias preliminares* (Poster communication). Jornada Cloenda 2017-2018 de la Societat Catalana de Psiquiatria i Salut Mental. 1st June 2018, Sitges, Spain.

12. **Guardiola, M**; Lubeiro, A; Gallego, C; Callado, JL; Pomarol-Clotet, E; Molina, V; Fatjó-Vilas, M. *Synaptic plasticity and schizophrenia: genetic association analysis of KIS kinase* (Poster communication). V Laboratorio de Ideas CIBERSAM 2017. 1st-2nd June 2017, Santander, Spain.

13. Soler, J; Ibáñez, I; Moya, J; Ortet, G; **Guardiola, M**; Fañanás, L; Arias, B; Fatjó-Vilas, M. *Interacción del gen ZNF804A y el consumo de cannabis: efecto sobre el riesgo para desarrollar psicosis en una muestra de población general* (Poster communication). V Laboratorio de Ideas CIBERSAM 2017. 1st-2nd June 2017, Santander, Spain.

14. Soler, J; Giralt, M; **Guardiola, M**; Miret, S; Pomarol-Clotet, E; Parellada, M; Fañanás, L; Fatjó-Vilas, M. *A study of the effect of the DISC1 gene in the vulnerability for Schizophrenia-spectrum disorders through its association with neurodevelopment markers* (Oral communication). X Simposi de Neurobiologia del Institut d'Estudis Catalans. 7th October 2016, Barcelona, Spain.

15. Perera, S; Ramos, A; Alvarez, L; **Guardiola, M**; Lima, M; Aluja, M.P.; Santos, C. *Reappraising the Human Mitochondrial DNA Recombination Dogma* (Oral communication). XXI Seminario de Genética de Poblaciones y Evolución. 5th October 2017, Sitges, Spain.

16. **Guardiola-Ripoll, M**. *Homo genus specific NUMTs: identification and variation in Human Populations* (Oral communication). II Jornada Científica del Departament de Biologia Animal, Biologia Vegetal i d'Ecologia. 7th June 2016, Cerdanyola del Vallès, Spain.

SPECIALIZED TRAINING

1. *XIX curso intensivo de introducción a la investigación en neurociencias: The prenatal, perinatal and infant environmental risk factors in mental disorders* (7h). Entity: Consorcio CIBER para el área temática de Salud Mental (CIBERSAM). Date: 09.09.2022. Location: Barcelona, Spain

2. *Curso REDCap* (20h). Entity: x7 Perseventh. Date: 10.01.2022-21.01.2022. Online

3. *X foro internacional CIBERSAM de investigación en psiquiatría* (11h). Entity: Consorcio CIBER para el área temática de Salud Mental (CIBERSAM). Date: 23.11.2021 - 25.11.2021. Online

4. *Jornada científica: Utilización de muestras cerebrales humanas en el estudio de enfermedades psiquiátricas* (5h). Entity: Consorcio CIBER para el área temática de Salud Mental (CIBERSAM). Date: 11.11.2021. Online

5. *XXIV Congreso Nacional de Psiquiatria* (13h). Entitiy: Sociedad Española de Psiquiatria. Date: 28-10-2021-30.10.2021. Location: Valencia, Spain

6. *XVIII curso intensivo de introducción a la investigación en neurociencias: actualización en la evaluación, intervención e investigación del maltrato infantil al principio de la vida* (9h). Entity: Consorcio CIBER para el área temática de Salud Mental (CIBERSAM). Date: 28.06.2021 - 29.06.2021. Location: Barcelona, Spain

7. *XI Jornada de Cromatina i Epigenètica* (6h). Entity: Societat Catalana de Biología. Date: 14.05.2021.

8. *Jornada Científica: Descodificant el DNA, avenços i nous reptes per al futur* (2,5h). Entity: Societat Catalana de Biología. Date: 11.01.2021. Online

9. *IX foro internacional CIBERSAM de investigación en psiquiatría* (11h). Entity: Consorcio CIBER para el área temática de Salud Mental (CIBERSAM). Date: 17.11.2020 - 19.11.2020. Online

10. *Introduction to GWAS (Genome-Wide Association Studies* (36h). Entity: Transmitting Science. Date: 25.11.2019-29.11.2019. Location: Barcelona, Spain

11. *V Curso de Técnicas de Neuroimagen Avanzada* (80h). Entity: FIDMAG Germanes Hospitalàries Research Foundation. Date: 02.11.2018-12.04.2018. Location: Sant Boi de Llobregat, Spain

12. *III Curso de Actualización en Psicosis de inicio en la Infancia y la Adolescencia* (6h). Entity: Servicio de Psiquiatría y Psicología (Hospital Sant Joan de Déu) y Consorcio CIBER para el área temática de Salud Mental (CIBERSAM). Date: 17.05.2019. Location: Barcelona, Spain

13. *XVII Curso Intensivo de Introducción a la Investigación en Neurociencias: "Neuroendocrinologia del Trastorno Mental Infanto-Juvenil* (6h). Entity: Consorcio CIBER para el área temática de Salud Mental (CIBERSAM). Date: 10.05.2019. Location: Barcelona, Spain

14. *XVI Curso Intensivo de Introducción a la Investigación en Neurociencias: Estrés Oxidativo e Inflamación en Enfermedad Mental: ¿Causa o Consecuencia?* (7h). Entity: Consorcio CIBER para el área temática de Salud Mental (CIBERSAM). Date: 27.04.2018. Location: Barcelona, Spain

15. *VIII Jornadas Unidad de Crisis de Adolescentes: Maltrato en la Infancia y Adolescencia* (6,5h). Entity: FIDMAG Germanes Hospitalaries Research Foundation. Date: 28.02.2018. Location: Sant Boi de Llobregat, Spain

16. *B·DEBATE. Natural Selection in Humans: Understanding our adaptations* (15h). Entity: Biocat, "la Caixa" Foundation y Insititute of Evolutionary Biology (IBE). Date: 17-18.07.2017. Location: Barcelona, Spain

17. *XV Curso Intensivo de Introducción a la Investigación en Neurociencias: The Early Origin of Mental Health* (7h). Entity: Consorcio CIBER para el área temática de Salud Mental (CIBERSAM). Date: 28.06.2017. Location: Barcelona, Spain

18. *V Curso de Estadística Básica para Ciencias de la Salud con Excel* (16h). Entity: FIDMAG Germanes Hospitalaries Research Foundation. Date: 26.05.2018-02/09/15.06.2018. Location: Barcelona, Spain

19. *II Curso de Actualización en Psicosis de inicio en la Infancia y la Adolescencia* (8h). Entity: Consorcio CIBER para el área temática de Salud Mental (CIBERSAM). Date: 21.04.2017. Location: Barcelona, Spain

20. *XIV Curso Intensivo de Introducción a la Investigación en Neurociencias: Cannabis y Enfermedad Mental* (8h). Entity: Consorcio CIBER para el área temática de Salud Mental (CIBERSAM). Date: 27.01.2017. Location: Barcelona, Spain

TEACHING EXPERIENCE

Supervision of final projects:

1. Master's thesis co-direction (8h). Master's Degree in Neurosciences - University of Barcelona. Student: Angelo Gabriel Arias. Period: January 2022 – July 2022.
2. Bachelor's degree final project mentoring (12h). Biomedicine Degree - University of Barcelona. Student: Marina Díaz. Period: February 2022 – July 2022.
3. Master's thesis co-direction (8h). Master's Degree in Mental Health Research Initiation – CIBERSAM - University of Barcelona. Student: Maria Martínez. Period: December 2021 – July 2022.
4. *Master's thesis co-direction (8h). Master's Degree in Biological Anthropology University of Barcelona and Autonomous University of Barcelona. Student: María De La Rosa. Period: January 2021 – September 2021.*

Teaching activities:

1. Practicum I and II (12h) Mentoring. Biomedicine Degree. University of Barcelona. Period: November 2021 – December 2021.
2. Genetic Epidemiology II: Research Projects (4h). Biological Anthropology Master's Degree. University of Barcelona. Period: April 2022.
3. Genetic Epidemiology I Seminar: Meta-Analysis (3h). Biological Anthropology Master's Degree. University of Barcelona. Period: March 2022.
4. Genetic Basis of Diseases: Experimental workshop (10h). Biomedicine Degree. University of Barcelona. Period: November 2021 – December 2021.
5. Epidemiology Seminar: Meta-Analysis (2h). Biomedicine Degree. University of Barcelona. Period: November 2021.
6. Genetic Epidemiology II: Research Projects (3h). Biological Anthropology Master's Degree. University of Barcelona. Period: April 2021.
7. Genetic Epidemiology I Seminar: The Genetic Component of Complex Disorders (2h). Biological Anthropology Master's Degree. University of Barcelona. Period: March 2021.
8. Epidemiology Seminar: Meta-Analysis (2h). Biomedicine Degree. University of Barcelona. Period: December 2020.
9. Seminar within the teaching program of Aprenentatge i Serveis (ApS): Cannabis, genetics and mental disorders (2h). University of Barcelona. Period: May 2018.
10. Biological Anthropology Seminar (1h). Biology Degree. Autonomous University of Barcelona. Period: April 2018.

Supervision of research stays

1. Research stay co-direction. Master's Degree in Mental Health Research Initiation – CIBERSAM. Student: Alejandro Sotero. Period: January 2019 – June de 2019.
2. Research stay co-direction. Master's Degree in Mental Health Research Initiation – CIBERSAM. Student: Pilar Mayor. Period: February 2019.

AWARDS

1. Oscoz-Irurozqui, M and **Guardiola-Ripoll, M**; Almodóvar-Payá, C; Sarró, S; Guerrero-Pedraza, A; Pomarol-Clotet, E; Fatjó-Vilas, M. Cannabis use and genes of the endocannabinoid system: their role in psychotic symptoms and cognition in first-episode psychosis (**BEST ORAL COMMUNICATION AWARD**). XXII Congreso de Patología Dual. 16th-19th November 2020, Virtual.
2. Oscoz-Irurozqui, M; Almodóvar-Payá, C; **Guardiola-Ripoll, M**; Guerrero-Pedraza, A; Salgado-Pineda, P; Sarró, S; Pomarol-Clotet, E; Fatjó-Vilas, M. Cannabis use and *CNR2* gene interaction modulates brain activity in first episode psychosis (**BEST POSTER COMMUNICATION AWARD**). III World Congress and VI International Congress on Dual Disorders from the Spanish Society of Dual Pathology (SEPD). 19th-22nd June 2019, Madrid, Spain.