UNIVERSIDAD DE CANTABRIA

PROGRAMA DE DOCTORADO

EN MEDICINA Y CIENCIAS DE LA SALUD

TESIS DOCTORAL

MECANISMOS QUE CONTROLAN LOS GENES CON RESPUESTA CLÍNICA A LOS ANTIPSICÓTICOS: PAPEL DE LA SEÑALIZACIÓN RECEPTOR D_1-cAMP/CREB EN LA REGULACIÓN DE *ADAMTS2*.

PhD THESIS

MECHANISMS CONTROLLING THE CLINICAL RESPONSE GENES TO ANTIPSYCHOTICS: ROLE OF D_1 RECEPTOR-cAMP/CREB SIGNALLING IN THE REGULATION OF *ADAMTS2*.

FULGENCIO RUSO JULVE

Dirigida por:

Dr. BENEDICTO CRESPO FACORRO

Dr. JOSÉ P. VAQUÉ DÍEZ

Escuela de Doctorado de la Universidad de Cantabria

Santander 2019.

Dr. Benedicto Crespo Facorro, director U.G.C. Salud Mental Hospital Universitario Virgen del Rocío, catedrático de psiquiatría de la Universidad de Sevilla e investigador responsible en el Instituto de Biomedicina de Sevilla (IBiS),

Dr. José P. Vaqué Díez, investigador Ramón y Cajal en el Departamento de Biología Molecular de la Universidad de Cantabria,

EXPONEN:

Que han llevado a cabo las funciones de CO-DIRECTORES DE TESIS del Trabajo de TESIS DOCTORAL realizado por el Licenciado Fulgencio Ruso Julve en el Instituto de Investigación Marqués de Valdecilla (IDIVAL) y el Departamento de Biología Molecular de la Universidad de Cantabria, títulado "MECANISMOS QUE CONTROLAN LOS GENES CON RESPUESTA CLÍNICA A LOS ANTIPSICÓTICOS: PAPEL DE LA SEÑALIZACIÓN RECEPTOR D_1-cAMP/CREB EN LA REGULACIÓN DE *ADAMTS2*".

Consideramos que los objetivos planteados y los resultados obtenidos en dicho trabajo, fundamentan las conclusiones a las que se llegan. Por lo tanto, el trabajo reúne los requisitos necesarios para su presentación como memoria de Doctorado por el interesado, al objeto de poder optar al título de DOCTOR por la Universidad de Cantabria.

En Santander, a veintidós de octubre de dos mil diecinueve.

Benedicto Crespo Facorro. José P. Vaqué Díez.

Esta Tesis Doctoral ha sido realizada conjuntamente en el Instituto de Investigación Marqués de Valdecilla (IDIVAL) y el Departamento de Biología Molecular de la Facultad de Medicina de la Universidad de Cantabria (Santander).

La financiación necesaria para la realización de esta Tesis doctoral ha sido aportada por el MINECO mediante los proyectos SAF2013-46292-R y SAF2016-76046-R, y por el ISCII mediante el Proyecto PI16/00156 y la financiación específica al grupo de investigación CIBERSAM-G26.

El autor de esta tesis ha disfrutado de contrato predoctoral de Formación de Personal Investigador (FPI), referencia BES-2014-070615, concedida por el MINECO, y de un contrato de 6 meses mediante la ayuda de Luchamos por la Vida 2019 en el Instituto de Investigación Marqués de Valdecilla (IDIVAL).

El dibujo de la portada de esta Tesis Doctoral fue realizado por Guillaume Lemarié.

A mis padres,
por darlo todo.

"Some people never go crazy.
What truly horrible lives they must lead."
Charles Bukowski.

AGRADECIMIENTOS

Ha costado su esfuerzo pero ya ha llegado el día. Siento que tengo mucho que agradecer a mucha gente y me viene a la cabeza una frase que me dijo un colega hace un tiempo: "somos los que nos preceden". Creo que no hay frase más acertada para empezar, porque sin toda la gente que ha trabajado durante tanto tiempo en este proyecto antes que yo y toda la gente que me ha ayudado, esta tesis no habría sido posible.

En primer lugar, quiero dar mi más sincero agradecimiento a mis directores de Tesis: Bene y Chepe. Por un lado, agradecerle a Bene por la oportunidad y confianza depositada en mi desde el principio, gracias por traerme a Santander y darme la oportunidad de trabajar en un proyecto tan fascinante. Gracias por sacar siempre algún hueco para mí, siempre me sorprenderá tu capacidad para organizar tantos frentes en tu día a día. Todo un ejemplo a seguir y un gran mentor. Por otro lado, y no menos importante, agradecerle a Chepe por su tremenda entrega durante todo este tiempo. Aunque nos costó salir de la "travesía por el desierto" del principio, la "ciencia siempre vence" y creo que hemos llegado al final enteros. Gracias por creer también en mí, darme un hueco en tu lab, tener siempre tu puerta abierta para cualquier cosa y ayudarme a madurar como científico (¿no crees que cada día soy menos "ice-man"?). En definitiva, os agradezco a los dos por saber guiarme entre dos mundos a veces complicados. Creo que sois la clara demostración que la ciencia puede ser beneficiosa para la gente. Mil gracias por vuestro entusiasmo, por enseñarme todo lo que soy como científico y por ser tan grandes Directores de Tesis.

Gracias también a Fuen, Elena y Álvaro (Team Pazos) por su tremenda ayuda y paciencia conmigo. Os agradezco enormemente todo el tiempo y ayuda que me habéis prestado estos últimos años. Para mí sois la tercera pata que ha hecho que finalmente encajaran todas las piezas del puzzle. También un recuerdo especial a Elsa que, aunque coincidimos poco, puso las bases de este proyecto. Siempre recordaré su enorme energía e implicación hasta el final. Tampoco me quiero olvidar de todos los jefazos que me han podido ayudar tanto en Idival, en la facultad, en el Ibbtec o en Salamanca, especialmente a Carlos, Nacho, Magda, Alberto, Albert y Berta.

Mis compañeros de Psiquiatría no han sido menos. Cuando llegué a la Residencia aquel primer día, me recibieron con los brazos abiertos y, aunque haya sido el bicho raro y haya estado poco tiempo por allí, siempre me han ayudado en todo lo que han podido. Gracias a Rosa y a Javier por estar ahí siempre que les he necesitado. Gracias a María J. por ser

tan sumamente paciente con mis dudas estadísticas (te has ganado el cielo conmigo). Gracias a Paula y Esther por ser tan buenas compañeras. Gracias a Víctor por ayudarme a montar las tablas de pacientes. Gracias a Diana por ser tan mami y estar pendiente de cómo estaba "el desaparecido".

Y, especialmente, no me olvidó de los más importantes: mi trío de compañeros de laboratorio. Tantas mudanzas y tanto tiempo encerrados en el laboratorio dan para mucha tontería, morcillas y diversión. Sin estos "sogilis" no creo que hubiera sido posible haber llegado mentalmente apto a esta fecha. Os doy las gracias por vuestra ayuda en la bancada pero, sobretodo, porque habéis hecho que estos años pasen volando y los vaya a recordar como de los mejores de mi vida. La joven Helena: caótica y dicharachera a partes iguales ("¿por qué gritas? ¡Porque yo siempre grito!"). El Papa siempre ha estado cuando la necesitaba la fallera (ya fuera en la bancada o corriendo por la bahía). Espero que Batista sea famoso y algún día nos contrates de postdocs (ya te lo dije). Agus: la última incorporación pero la entrada más fuerte, aunque nunca pares quieto en el lab ni en la calle. Mejor compañero de lab pero aún mejor compañero de fiestas, festivales y turbios varios ("yo al año que viene solo un festi"). Gracias por haber sido tan tú y haberme acogido tan bien. Y Nuri (aka Dj Tiësto): aunque al principio se pensara que venía de estancia un mes ("¿para qué iba a hablarte?") y ahora míranos, inseparables. Creo que, sin sus confesionarios y largas charlas sobre la vida, todo este tiempo habría sido muy diferente y menos fácil de lo que ha sido. La mejor compañera dentro y fuera del lab. Conocerte ha sido una suerte, pero más aún tenerte como amiga.

Tampoco me olvido del resto con quien he compartido bancada: la mami Carmencita, Rebe y su dedo corazón, la hater Rosó, las anécdotas surrealistas de Ruth, Rufino y sus chistes malos (pero malos, malos), la no-invitación de boda de Cris, Soraya y su banderita… y de los que pasaron brevemente como María G., MA, Maite y Ángelillo.

También quiero acordarme de todos los compañeros que he ido conociendo por los pasillos y que han acabado siendo compañeros de batalla dentro y fuera del lab. Gracias a la gente del Ibbtec por acoger tan bien a esos extraños del tercer piso, especialmente a Laurita, Vini, Eva… a todos. Gracias a Marta y Ana, porque siempre estabais para echar una mano y, como no, siempre estáis listas para uniros a cualquier planazo. Me llevo dos grandes amigas para siempre. Gracias a Emi (mi único apoyo neuro en todo este mar de biólogos del cáncer) por estar siempre interesado y por tu ayuda. Gracias a Ori, que aunque te conocí al final, ha sido una suerte descubrirte. Aunque sé que me dejo a mucha gente en el tintero, os agradezco que también hayáis formado parte de esta historia.

Y algún *rara avis* no científico también te encuentras por Cantabria. Gracias a Pablo por hablarme en aquella cola del bus. Conocerte ha sido una sorpresa maravillosa y tenerte ha sido el más grande apoyo. Y qué decir de mis queridos "Valderrávilos". Me acogisteis al llegar aquí y me hicisteis sentir que tenía una familia (disfuncional pero familia). Gracias Esther, José y Andrés. Gracias a Itzi, mi Pepa particular y guarda de aduanas que no me pasaba ni una. Sin tu "trisqueza" el Norte no sería igual, la mejor compañera de piso y la mejor esposa que se pueda tener.

Y gracias especialmente a Leiretxu: ¿Qué habría hecho yo sin tu "vamos a organizarnos" y tu enorme apoyo todos estos últimos años? Aunque la vida nos lleve por caminos diferentes, haber llegado hasta aquí también ha sido gracias a ti. No hay suficientes gracias para agradecerte haber sido de un equipo.

De mi gente de Valencia tampoco me quiero olvidar. Especialmente de mis tetes, Toni y Jorge, que me empujaron a cumplir un sueño. También de Mar, Tam y, sobretodo, de Guille: desde pequeños pensando en ser científicos y ahora, por fin, ya podemos ir los dos en Ryanair con el título de "Doctor".

Finalmente, gracias a toda mi familia. Os quiero agradecer todo lo que habéis hecho, por darlo todo. Por aguantarme, creer siempre en mí y soportar la distancia. Gracias a mi madre y a mi padre, todo este trabajo también os pertenece. Sé que no ha sido fácil para vosotros. Gracias a Cande y Alex, por estar siempre ahí, por seguir siempre unidos. La verdad: no hay hermanos más molones.

Supongo que ahora tocaría decir eso de "nueva etapa", "vida nueva" y esas cosas. Pero solo me sale decir: ¡Pues ya estaría!

Gracias.

UNIVERSIDAD DE CANTABRIA

PROGRAMA DE DOCTORADO

EN MEDICINA Y CIENCIAS DE LA SALUD

MECHANISMS CONTROLLING THE CLINICAL RESPONSE GENES TO ANTIPSYCHOTICS: ROLE OF D_1 RECEPTOR-cAMP/CREB SIGNALLING IN THE REGULATION OF *ADAMTS2*.

PhD THESIS

FULGENCIO RUSO JULVE

Escuela de Doctorado de la Universidad de Cantabria

Santander, octubre de 2019.

INDEX

LIST OF FIGURES

INTRODUCTION

RESULTS

DISCUSSION

LIST OF TABLES

INTRODUCTION

MATERIAL AND METHODS

RESULTS

ABBREVIATIONS

5-HT_{1A}	Serotonin 5-HT_{1A} receptor.
5-HT_{2A}	Serotonin 5-HT_{2A} receptor.
AAP	Atypical antipsychotic.
ABCA13	ATP-binding cassette sub-family A member 13.
AC	Adenylate cyclase.
ADAM	A disintegrin and metalloprotease family.
ADAMTS	A disintegrin and metalloprotease with thrombospondin motifs family.
ADAMTS2	ADAM Metallopeptidase with Thrombospondin Type 1 Motif 2.
ADHD	Attention-Deficit Hyperactivity Disorder.
AIDS	Acquired immune deficiency syndrome/Human immunodeficiency virus infection.
AKT	Protein kinase B, PKB.
ALPL	Tissue Non-Specific Alkaline Phosphatase.
AP	Antipsychotic.
AP-1	AP-1 transcription factor.
APD	Antipsychotic Drug.
ATF-1	Activating transcription factor-1.
BBB	Blood-brain barrier.
bcl-2	B-cell lymphoma 2 protein
BD	Bipolar disorder.
BDNF	Brain-derived neurotrophic factor.
BPRS	Brief Psychiatric Rating Scale.
bZIP	Basic region/leucine zipper domain.
C57BL/6	Mice strain.
CaMK	Ca^{2+}/calmodulin-dependent protein kinase.
CaMKII	Calcium/calmodulin-dependent protein kinase II.
CaMKIV	Calcium/calmodulin-dependent protein kinase IV.
cAMP	Cyclic adenosine monophosphate.
Cb	Cerebellum.

CBP	CREB-binding protein.
CCx	Cingular cortex.
CD1 mice	CD1 strain from Swiss mice.
CD177	CD177 antigen gene.
CDSS	Calgary Depression Rating Scale for Schizophrenia.
c-Fos	AP-1 transcription factor subunit protein.
CGI	Clinical Global Impression test.
ChIP	Chromatin immunoprecipitation.
CNS	Central Nervous System.
CNTNAP3	Contactin Associated Protein Like 3 gene.
CNV	Copy Number Variants.
CRE	cAMP response element.
CREB	cAMP response element-binding protein.
CRE-luc	CRE-luciferase reporter gene.
CREM	cAMP response element modulator.
CRGA	Clinical Response Gene to Antipsychotics.
CRISP3	Cysteine Rich Secretory Protein 3.
CSMD1	CUB And Sushi Multiple Domains 1.
CSPG	Chondroitin sulfate proteoglycan.
CTX	Cholera toxin.
Cx	Cortex.
D_1	Dopamine D1 receptor.
D_2	Dopamine D2 receptor.
D_3	Dopamine D3 receptor.
D_4	Dopamine D4 receptor.
D_5	Dopamine D5 receptor.
DALY	Disability-Adjusted Life Year.
DARPP-32	Dopamine- and cAMP-regulated neuronal phosphoprotein.
DG	Dentate gyrus.
DGip	Infrapyramidal blade of dentate gyrus.
DGN	Dorsal giant neuron.

Di	Diencephalon.
DMS	Dentate migration stream.
DNA	Deoxyribonucleic acid.
DOI	5-HT_{2A} and 5-HT_{2C} agonist.
DSM-IV	Diagnostic and Statistical Manual of Mental Disorders, IV edition.
DUP	Duration of untreated psychosis.
ECM	Extracellular matrix.
EGL	External granular layer.
EHF	ETS Domain-Containing Transcription Factor.
ELISA	Enzyme-linked immunosorbent assay.
ENTPD2	Ectonucleoside triphosphate diphosphohydrolase 2 enzyme.
EP	Endopiriform nucleus.
EPS	Extrapyramidal effects.
Erk	Extracellular signal–regulated kinases.
FatiGO	Significant associations gene Ontology terms web tool.
FEP	First Episodes of non-affective Psychosis.
FGF6	Fibroblast growth factor 6.
FI	Fimbria.
GABA	Gamma-Aminobutyric acid.
GDP	Gross Domestic Product.
GeneRIF	Gene Reference into Function.
$G_{i/o}$	$G_{i/o}$ alpha protein subunit.
GPCR	G protein-coupled receptors.
GPER	G Protein-Coupled Estrogen Receptor 1.
GRIK3	Glutamate Ionotropic Receptor Kainate Type Subunit 3.
$G_{s/olf}$	$G_{s/olf}$ alpha protein subunit.
GSK3	Glycogen synthase kinase 3.
GWAS	Genome-Wide Association Studies.
$G_{\alpha i}$	G Protein Subunit Alpha I1.
$G_{\alpha q}$	G Protein Subunit Alpha Q.
$G_{\alpha s}$	G Protein Subunit Alpha S.

ICx	Insular cortex.
INSC	Inscuteable Spindle Orientation Adaptor Protein.
ISO	Isthmic organizer region.
KID	Kinase inducible domain.
L-DOPA	3,4-dihydroxyphenylanine.
LPL	Lipoprotein Lipase.
Ls	Lateral septum.
LTF	Lactotransferrin.
MAPK	Mitogen-activated protein kinase.
MDD	Major Depression Disorder.
MES	Mesencephalon.
mGlu2	Metabotropic glutamate receptor 2.
MHC	Major Histocompatibility Complex.
ML	Molecular layer.
MMP8	Matrix metalloproteinase-8 or neutrophil collagenase.
MMP9	Matrix metallopeptidase 9 or gelatinase B.
MMPs	Matrix metallopeptidases.
mPFC	Medial prefrontal cortex.
mRNA	Messenger ribonucleic acid.
mRNA-seq	mRNA sequencing or 'whole Transcriptome Shotgun Sequencing'.
NAc	Nucleus accumbens.
NCBI	National Center for Biotechnology Information.
NFAT	Nuclear factor of activated T-cells.
NMDA	N-methyl-D-aspartate.
OLFM4	Olfactomedin 4.
OLR1	Oxidized Low Density Lipoprotein Receptor 1.
OMIM	Online Mendelian Inheritance in Man.
p38	p38 mitogen-activated protein kinases (MAPK).
p90RSK	p90 ribosomal S6 kinase.
Padj-value	Adjusted P-value.
PAFIP	'Programa Asistencial de las Fases Iniciales de Psicosis'.

PBMC	Peripheral blood mononuclear cell.
PFC	Prefrontal cortex.
PI3K	Phosphoinositide 3-kinase.
PKA	Protein kinase A.
PKC	Protein kinase C.
PNN	Perineuronal net.
PP1	Protein phosphatase 1.
PP2A	Protein phosphatase 2.
PP2B	Protein phosphatase 2B.
PP2C	Protein phosphatase 2C.
PTX	Pertussis toxin.
qPCR	Quantitative polymerase chain reaction.
Ras	Ras GTPase.
RFX2	Regulatory Factor X2.
RNA	Ribonucleic acid.
RNA	Ribonucleic acid.
Rsk	Ribosomal s6 kinase.
RT	Room temperature.
RT-qPCR	Reverse transcription qPCR.
S100B	S100 Calcium Binding Protein B.
SANS	Scale for the Assessment of Negative.
SAPS	Scale for the Assessment of Positive Symptoms.
SCZ	Schizophrenia.
SELENBP1	Selenium Binding Protein 1
Ser133	Phosphorylation site at serine 133 of CREB protein.
shRNA	Short/small hairpin RNA.
SK-N-SH	Human Neuroblastoma Cell Line.
Sl-M	Stratum lacunosum and moleculare of CA1.
SIPS	Structured interview for prodromal syndromes.
SN	Substantia Nigra.
SNc	Substantia Nigra pars compacta.

SNCA	Synuclein Alpha.
SNP	Single Nucleotide Polymorphism.
SNr	Substantia Nigra pars reticulata.
St	Striatal shell region.
STAT3	Signal transducer and activator of transcription 3.
SYN2	Synapsin II.
TAP	Typical antipsychotic.
TATA	TATA box, Goldberg-Hogness box.
TD	Tardive dyskinesia.
TGF-β	Transforming growth factor beta.
TPA	Tetradecanoylphorbol acetate.
TUBB2A	Tubulin Beta 2A Class IIa.
UNC45B	Unc-45 Myosin Chaperone B gene.
VTA	Ventral Tegmental Area.
WNT	Wnt signaling pathways.
YLD	Years Lost due to Disability.
YMRS	Young Mania Rating Scale.

INTRODUCTION

1. INTRODUCTION

1.1. Clinical and mechanistic insights of schizophrenia as a mental disorder.

Schizophrenia (SCZ) is a severe neuropsychiatric disorder that may have a profound effect on the affected individuals and in society, if optimal treatment is not implemented since early phases of the illness. More than 50% of individuals who receive a diagnosis have intermittent but long-term psychiatric alterations, and around 20% of them develop chronic symptoms and disability (Owen et al. 2016). The definition of SCZ has evolved through the last 100 years due to its increasing complexity (Insel 2010) as can be reflected by continuous changes in its diagnosis criteria in all editions of the Diagnostic and Statistical Manual of Mental Disorders (DSM-IV; **table 1.1.**) (Tandon et al. 2013; Tandon et al. 2008a; 2008b). Currently, no diagnostic tests nor biomarkers are available. Generally, diagnosis has been based on clinical observation, specifically: the identification of symptoms that tend to cluster together, the 'timing of the symptoms' appearance, and their tendency to resolve, recur or become chronic (Hyman 2007).

Currently, schizophrenia is one of the most studied pathologies in neuroscience and, possibly, still the most enigmatic in this discipline. The substantial gaps in our knowledge of the neurobiology that underlies mental disorders and SCZ largely derive from the difficulty of characterizing the mechanisms and circuitry that underlie higher brain functions, the complexity of the genetic and developmental underpinnings of normal and abnormal behavioural variation and the unsatisfactory utility of biomarkers and animal models of mental disorders (Hyman 2007). It is probably premature to bring neurobiology into the formal classification of the schizophrenia but is important incorporate neurobiology knowledge as tool to rethink the current approach to diagnoses this mental disorder.

Table 1.1. DSM-IV criteria diagnosis for schizophrenia. *Adapted from Tandom R et al., Schizophr. Res., 2013*

	DSM-IV
Disorder class	Schizophrenia and Other Psychotic Disorders.
A. Characteristic symptoms:	Two (or more) of the following, each present for a significant portion of time during a 1-month period (or less if successfully treated): 1. delusions 2. hallucinations 3. disorganized speech (e.g., frequent derailment or incoherence) 4. grossly disorganized or catatonic behavior 5. negative symptoms (i.e., affective flattering, alogia, or avolition). Note: Only one Criterion A symptom is required if delusions are bizarre or hallucinations consist of a voice keeping up a running commentary on the person's behavior or thoughts, or two or more voices conversing with each other.
B. Social/occupational dysfunction:	For a significant portion of the time since the onset of the disturbance, one or more major areas of functioning such as work, interpersonal relations, or self-care are markedly below the level achieved prior to the onset (or when the onset is in childhood or adolescence, failure to achieve expected level of interpersonal, academic, or occupational achievement).
C. Duration:	Continuous signs of the disturbance persist for at least 6 months. This 6-month period must include at least 1 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 and Mood Disorder exclusion:	Schizoaffective Disorder and Mood Disorder With Psychotic Features have been ruled out because either (1) no Major Depressive, Manic, or Mixed Episodes have occurred concurrently with the active-phase symptoms; or (2) if mood episodes have occurred during active-phase symptoms, their total duration has been brief relative to the duration of the active and residual periods.
E. Substance/general medical condition exclusion:	The disturbance is not due to the direct physiological effects of a substance (e.g., a drug of abuse, a medication) or a general medical condition.
F. Relationship to a Pervasive Developmental Disorder:	If there is a history of Autistic Disorder or another Pervasive Developmental Disorder, the additional diagnosis of Schizophrenia is made only if prominent delusions or hallucinations are also present for at least a month (or less if successfully treated).
Classification of longitudinal course (can be applied only after at least 1 year has elapsed since the initial onset of active-phase symptoms):	Episodic With Interepisode Residual Symptoms (episodes are defined by the reemergence of prominent psychotic symptoms); also specify if: With Prominent Negative Symptoms. Episodic With No Interepisode Residual Symptoms. Continuous (prominent psychotic symptoms are present throughout the period of observation); also specify if: With Prominent Negative Symptoms. Single Episode In Partial Remission; also specify if: With Prominent Negative Symptoms. Single Episode In Full Remission. Other or Unspecified Pattern.
Paranoid Type (295.30):	A type of Schizophrenia in which the following criteria are met: A. Preoccupation with one or more delusions or frequent auditory hallucinations. B. None of the following is prominent: disorganized speech, disorganized or catatonic behavior, or flat or inappropriate affect.
Disorganized Type (295.10):	A type of Schizophrenia in which the following criteria are met: A. All of the following are prominent: disorganized speech disorganized behavior flat or inappropriate affect B. The criteria are not met for Catatonic Type.
Catatonic Type (295.20):	A type of Schizophrenia in which the clinical picture is dominated by at least two of the following: 1. motoric immobility as evidenced by catalepsy (including waxy flexibility) or stupor. 2. excessive motor activity (that is apparently purposeless and not influenced by external stimuli). 3. extreme negativism (an apparently motiveless resistance to all instructions or maintenance of a rigid posture against attempts to be moved) or mutism. 4. peculiarities of voluntary movement as evidenced by posturing (voluntary assumption of inappropriate or bizarre postures), stereotyped movements, prominent mannerisms, or prominent grimacing. 5. echolalia or echopraxia.
Undifferentiated Type (295.90):	A type of Schizophrenia in which symptoms that meet Criterion A are present, but the criteria are not met for the Paranoid, Disorganized, or Catatonic Type.
Residual Type (295.60):	A type of Schizophrenia in which the following criteria are met: A. Absence of prominent delusions, hallucinations, disorganized speech, and grossly disorganized or catatonic behavior. B. There is continuing evidence of the disturbance, as indicated by the presence of negative symptoms or two or more symptoms listed in Criterion A for Schizophrenia, present in an attenuated form (e.g., odd beliefs, unusual perceptual experiences).

1.1.1. Clinical presentation, signs and symptoms of schizophrenia.

Highly variable psychopathology is characteristic of this mental disorder affecting cognition, emotion, perception of reality and other behaviour aspects (Saddock. 2008). For these reasons, SCZ is a complex syndrome with a heterogeneous combination of symptoms that also vary over time. The symptomatology has been traditionally classified into "positive", "negative" and "cognitive" categories (**Table 1.1.**). Positive symptoms reflect behaviour and thoughts that are not normally present, such as recurrent psychosis, which is the "loss of contact with reality" situation that consists of delusions, hallucinations and disorganized speech and behaviour. The amotivational syndrome is characterized by negative symptoms, which includes social withdrawal, affective flattening, alogia, anhedonia (the inability to feel emotions) and avolition (diminished initiative and energy). Finally, cognitive symptoms are expressed as a broad set of cognitive dysfunctions (Kahn et al. 2015).

Some authors questioned the current emphasis on psychosis symptoms in the diagnosis of the schizophrenia, which could be involuntarily hiding the true causes of the disease. Indeed, prognosis of SCZ has not changed substantially since the introduction of chlorpromazine as antipsychotic more than 50 years ago, and some argue it has not meaningfully improved since the illness was first described. In this way, old and new evidences focus on the cognitive underperformance as the possible origin of the disorder and the relevance that should be the main object of the treatment of the schizophrenia (Kahn and Keefe 2013).

1.1.2. Epidemiology of schizophrenia.

The risk of schizophrenia diagnosis throughout life affects about 1% of the population (Kahn et al. 2015). Furthermore, the annual incidence of psychosis varies between 7 and 20.1 per 100.000 inhabitants depending on we measure the first episodes of SCZ in the strict sense, or more broadly the first episodes of non-affective psychosis (Baldwin et al.

2005). But this incidence might be associated with clinical and social-demographic factors, male gender, urban environments, socioeconomic status variables and drug consumption, especially cannabis. In this way, a number of studies carried out by our group, established an annual incidence of 13.8 per 100.000 of first episodes of non-affective psychosis (FEP) for risk-age population of Cantabria (Spain) (Pelayo-Teran et al. 2008). In this line of evidence, several meta-analyses (Aleman et al. 2003; McGrath et al. 2004), detected that men have a higher probability of developing schizophrenia with a relative risk of 1.4 when compared to women. This, observation was confirmed by our group in Cantabria where a relative risk of 1.6 was found (Pelayo-Teran et al. 2008). Finally, data from literature reveal that there is a very significant association between a series of social variables, like for example residence in urban areas, that are potentially linked to negative psychological, biological and social factors which generate stress and social disintegration, and thus increase the risk of developing schizophrenia (McGrath et al. 2004; Selten and Cantor-Graae 2005; van Os et al. 2010).

1.1.3. Course of the illness.

First symptoms usually appear in early adulthood, between 15 and 35 years (50% before 25 years) (Saha et al. 2005), usually coinciding with an important stage in the complete individual development (**Table 1.2.**). Our studies carried out in Cantabria, revealed that SCZ appears between 3 and 5 years earlier in men than in women (Pelayo-Teran et al. 2008).

Schizophrenia and psychosis are classically presented as chronic course disorders with frequent relapses and tendency to develop resistance to treatment and social deficits. In this way, a number of studies demonstrated that a discrete proportion of patients (50%) experienced a recovery or significant clinical improvement: family history of psychosis, lengthy duration of untreated psychosis (DUP), poor adjustment during late adolescence and lower severity of positive symptoms at baseline may compromise the potential for

clinical response after a FEP in the short-term (Crespo-Facorro et al. 2013). This was confirmed in two meta-analyses that were performed by including all the data in literature of the 20th century: 40% of the patients had clinical improvement after 5-6 years of follow-up with 27.1% of these experiencing a "bad course" (Hegarty et al. 1994; Menezes et al. 2006). Studies from our group revealed that non-adherence to medication is the best predictor of relapse following a FEP (Caseiro et al. 2012). In this way, enhancing adherence to treatment and improving the timing in the clinical interventions is essential for the future of the clinical management of psychosis (Ayesa-Arriola et al. 2019).

Table 1.2. Stages of schizophrenia. Stage I, pre-symptomatic risk; stage II, pre-psychotic prodrome; stage III, acute psychosis; stage IV, chronic illness. *Adapted from Insel R, Nature (2010).*

	Stage I	Stage II	Stage III	Stage IV
Features	Genetic vulnerability Environmental exposure	Cognitive, behavioural and social deficits Help-seeking	Abnormal thought and behaviour Relapsing-remitting course	Loss of function Medical complications Incarceration
Diagnosis	Genetic sequence Family history	SIPS Cognitive assessment Imaging	Clinical interview Loss of insight	Clinical interview Loss of function
Disability	None/mild cognitive deficit	Change in school and social function	Acute loss of function Acute family distress	Chronic disability Unemployment Homelessmess
Intervention	Unknown	Cognitive training? Polyunsaturated fatty acids? Family support?	Medication Psychosocial interventions	Medication Psychosocial interventions Rehabilitations services

However, despite a good disorder "outcome", there is still a tendency to present a chronic evolution with deficits in psycho-social functioning. Therefore, this disorder causes a high degree of disability (Ertugrul and Ulug 2002), being considered as the third cause of disability in the world (WHO World Health Report 2010). Thus, schizophrenia is responsible for 1.1% of Disability-Adjusted Life Year (DALY) and 2.8% of Years Lost due to Disability (YLD) (Murray and Lopez 1996; Rossler et al. 2005). Previous work from our group support the notion that premorbid social adjustment is an important aspect in

functional outcome over the course of the illness, and neurocognition at baseline is not a predictive factor for long-term functional disability (Ayesa-Arriola et al. 2013). In addition, it is well known the people with affected families also suffer considerable overload due to the symptoms of the disorder (Schulze and Rossler 2005). Considering all the data depicted above, schizophrenia generates a significant cost to the community (Mayoral-van Son et al. 2018). Considering direct and indirect cost, although latter are mainly responsible for the high expense, it has been estimated to reach up to 2% of GDP in western countries (Black and Andreasen 1999). In the same way, this disorder produces a high cost in Spain, which factors that determine its high expenses are similar to other countries (Mayoral-van Son et al. 2018; Negrin and Vazquez-Polo 2006; Vazquez-Polo et al. 2005).

1.1.4. Cognitive functioning.

Almost 100 years ago when schizophrenia was first defined in its current form it was called 'dementia praecox', the focus being on the intellectual deterioration that accompanied the syndrome. In the following years, the focus shifted to the psychosis, the delusions, and the hallucinations as the cardinal features of the illness (Guloksuz and van Os 2018). However, in the last decades, there has been a resurgence of interest in the cognitive alterations of SCZ (attention, speed of processing, working and long-term memory, executive function, and social cognition) (Fioravanti et al. 2005). Although these symptoms have received poor clinical attention, they are important contributors to the patient's inability to regain function and vocation. These alterations are well established by the time of the first episode, show scarce relation to psychotic symptoms, are not much influenced by the currently medications, and often show no improvement despite complete resolution of psychotic symptoms (van Os and Kapur 2009). Consequently, development of new medications targeting cognitive dysfunctions and cognitive remediations approaches are essential in the therapeutic progress of schizophrenia.

1.1.5. Mechanisms and pathophysiology of schizophrenia.

Schizophrenia is hypothesized to be the result of a complex interplay between genetic and environmental risk factors that influence early brain development and the trajectory of biological adaptation of life experiences (Howes and Murray 2014; Weinberger and Levitt 2011). Recent studies have focused on the molecular signatures of a more-subtle pathology that primarily involves the functional state of specific cell populations and the architecture of cell-cell communication. Results revealed abnormalities in cellular and neurotransmitter mechanisms in SCZ patients, but it is difficult to disambiguate what is related to the state of illness and the epiphenomena of illness (effects of treatment, disease chronicity and co-morbidity) from basic mechanisms that lead to illness (Kahn et al. 2015).

1.1.5.1. Gene and environment interplay.

Many genetic epidemiological studies have shown, for more than 50 years, that genetic factors contribute substantially, but not exclusively, to the underlying cause of schizophrenia (Owen et al. 2016). Twin studies suggest that the syndrome has heritability estimates of around 80% (Cardno and Gottesman 2000; Polderman et al. 2015). Recent findings have suggested that a small proportion of SCZ incidence could be explained by rare structural variations (copy number variants, CNVs: large regions of the genome that have been deleted or duplicated), that taken individually, can confer a relatively high risk of schizophrenia (Malhotra and Sebat 2012). Although the net contribution of mutations of this type is unknown, and much larger sequencing studies are pending (Owen et al. 2016). Nonetheless, these alleles conferring high individual risk are rare in the general population without relatives with schizophrenia, maybe due to effects of natural selection (reduced fecundity in patients) (Rees et al. 2014) (**Figure 1.1.**).

Relevant studies have demonstrated significant sharing of common risk variants between schizophrenia and other mental disorders, such as major depression disorder (MDD),

bipolar disorder (BD), anxiety disorder and attention deficit hyperactivity disorder (ADHD), whereas neurological disorders are more genetically distinct (Anttila et al. 2018). Across categories, psychiatry and neurologic disorders share relatively little common genetic risks, suggesting that multiple different and largely independently regulated aetiological pathways may give rise to similar clinical manifestations (e.g., psychosis, which manifests in both SCZ and Alzheimer's disease) (Buckley et al. 2009; Lyketsos et al. 2000). These evidences suggest that existing diagnostic categories might not be best for stratification of cases for research into disease cause and pathogenesis.

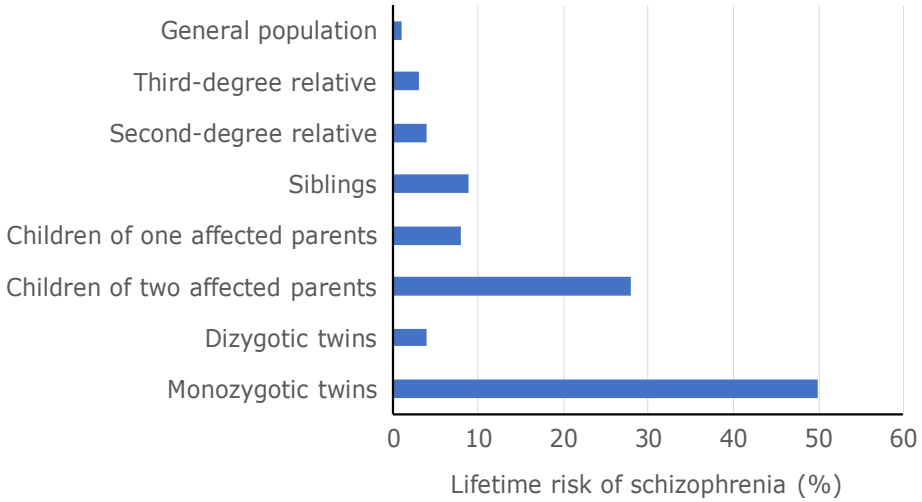


Figure 1.1. Lifetime risk of schizophrenia in relatives of people with schizophrenia. *Adapted from Kahn RS et al., Nature reviews (2015).*

Rare mutations, CNVs, and SNPs have been reported in genes encoding a range of synaptic proteins, such as glutamate receptors, voltage dependent calcium channel family of proteins, and D_2 receptor which is the principal target of antipsychotic drugs (APDs) (Hall et al. 2015; Ripke et al. 2014). The relation between glutamatergic dysfunction and abnormalities of dopamine (DA) signalling might provide a clue as to how psychosis and cognitive deficits arise in SCZ and related disorders (Owen et al. 2016). These are very unlikely to be the only mechanisms involved, and more possible processes are expected

to emerge as we move into the next phase of genomic studies. Of note, the most significant association from genome-wide association studies (GWAS) of schizophrenia is with multiple highly correlated variants in major histocompatibility complex (MHC) (Ripke et al. 2014; Ripke et al. 2013), findings that are in accord with epidemiological and clinical studies implicating immune and inflammatory processes in psychiatric disorders (Smyth and Lawrie 2013).

It is noteworthy that most genetic discoveries in schizophrenia do not yet have direct clinical application, but a positive test (such as a chromosomal microarray analysis) would have implications for genetic counselling and medical management because many CNVs are associated with specific patterns of physical morbidities (Baker et al. 2014). A genetic diagnosis might also have psychological benefits for patients and their families by reducing internalised stigma and self-blame.

1.1.5.2. Neurodevelopmental factors.

A large number of evidences suggest that schizophrenia has its origins in early life (Owen et al. 2011) (**Figure 1.2.**). This "neurodevelopmental hypothesis" is consistent with epidemiological data revealing a link between infections during pregnancy (Kneeland and Fatemi 2013), obstetric complications and increased risk of developing the disorder (Mittal et al. 2009). Interestingly, many of the genes that have been associated with SCZ show preferential expression during fetal development (Gulsuner et al. 2013). These evidences suggest that individuals who manifest schizophrenia as adults have compromised early neurodevelopmental milestones (Sorensen et al. 2010). Neurodegeneration is not found in individuals with schizophrenia and cognitive development is compromised in patients long before they manifest the conditions in early adult life (Kahn and Keefe 2013).

Several investigations are required to clarify why the early development antecedents would manifest as cognitive and social difficulties for the first two decades of life and then

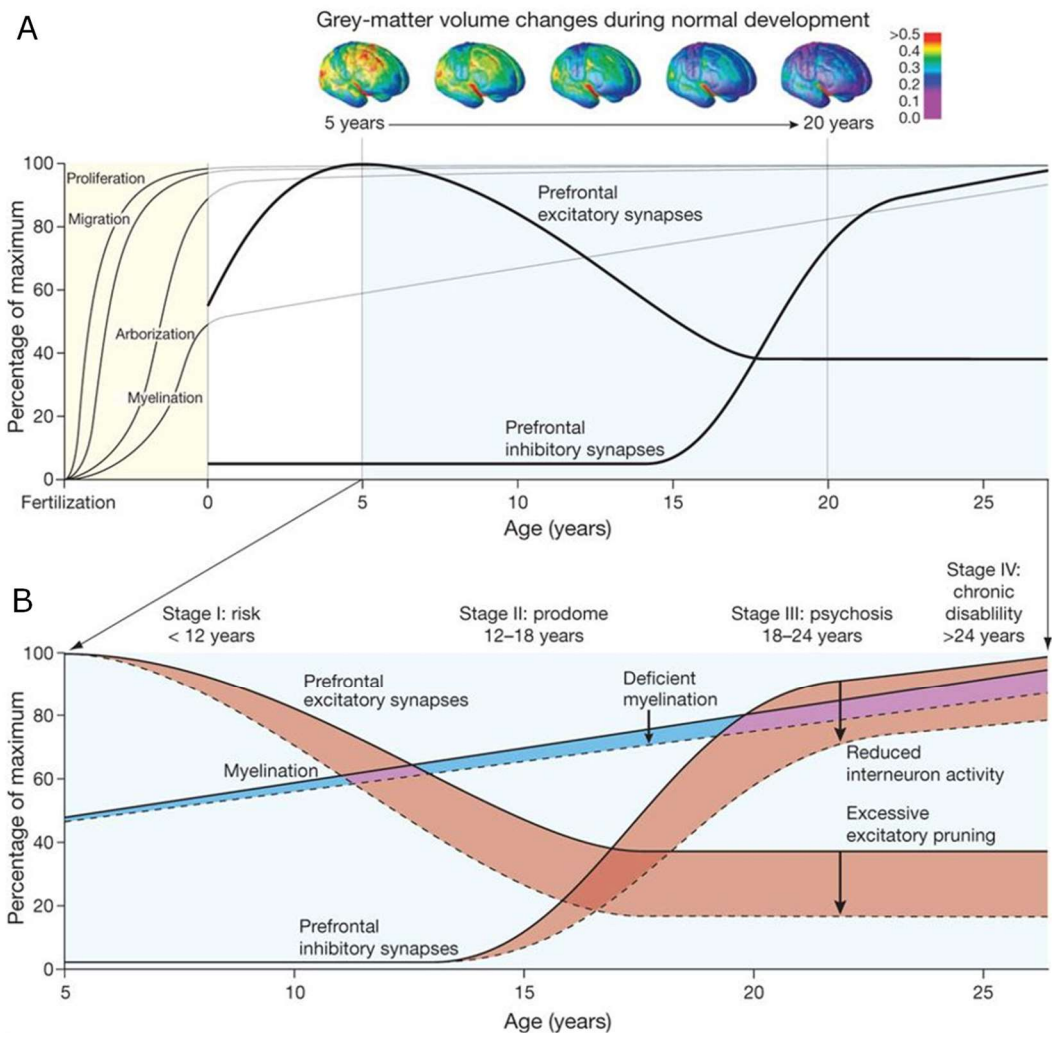


Figure 1.2. Neurodevelopmental model of schizophrenia. (A) Normal cortical development involves proliferation, migration, arborization (circuit formation) and myelination, with the first two processes occurring mostly during prenatal life and the latter two continuing through the first two post-natal decades. The combined effects of pruning of the neuronal arbor and myelin deposition are thought to account for the progressive reduction of grey-matter volume observed with longitudinal neuroimaging. Beneath this observed overall reduction, local changes are far more complex. Data from human and nonhuman primate brain indicate increases in inhibitory and decreases in excitatory synaptic strength occurring in prefrontal cortex throughout adolescence and early adulthood, during the period of prodrome and emergence of psychosis. (B) The trajectory in children developing schizophrenia could include reduced elaboration of inhibitory pathways and excessive pruning of excitatory pathways leading to altered excitatory–inhibitory balance in the prefrontal cortex. Reduced myelination would alter connectivity. Although some data support each of these possible neurodevelopmental mechanisms for schizophrenia, none has been proven to cause the syndrome. Detection of prodromal neurodevelopmental changes could permit early intervention with potential prevention or preemption of psychosis. *Adapted from Insel R, Nature (2010).*

emerge as a profound psychotic illness in early adulthood. An important factor might be the dramatic alterations in cortical synaptic organization, area with a key role in cognition and memory, observed by neuroimaging in patients (Paus et al. 2008). For these reasons, increasingly, it is conceivable that SCZ is not a disease *per se* but a state of brain maturation with a particular pattern of emergent response to experience, which, for various diverse and complex genetic and environmental reasons, 1 % of the population manifest (Weinberger 1987).

1.2. Dopamine system and pharmacology of psychosis.

1.2.1. The dopaminergic system.

Dopamine receptors (D_1, D_2, D_3, D_4, and D_5) are G protein-coupled receptors (GPCRs) that mediate all of the physiological functions of the catecholaminergic neurotransmitter dopamine (DA), ranging from voluntary movement and reward to hormonal regulation and hypertension (Meneses 2014). Dopamine, like other neurotransmitters, is stored in synaptic vesicles and is typically released in an activity-dependent manner. These receptors are conventionally classified in mammals in two general categories: (1) D_1-class family (D_1 and D_5 receptors) coupled with a $G_{\alpha s/olf}$ subunit that increase cAMP levels and activate protein kinase A (PKA); and (2) D_2-class family (D_2, D_3, and D_4 receptors) coupled with a $G_{\alpha i/o}$ subunit that decrease cAMP levels (Beaulieu and Gainetdinov 2011). Moreover, evidence suggest that dopamine receptors directly interact with various ion channels, including subunits of the NMDA receptor, within different brain regions (LaLumiere 2014). D_1-class receptors (hereafter referred to D_1) are typically found on the postsynaptic cell but can be located presynaptically, functioning to increase transmitter release, whereas D_2-class receptors (hereafter D_2) are located both presynaptically, functioning to inhibit neurotransmitter release, and postsynaptically (Beaulieu and Gainetdinov 2011).

Dopamine-producing neurons are present within only a few cell groups in the central nervous system (CNS) (**Figure 1.3.**). The major groups are located in the A8, A9 and A10 cell groups within the midbrain and collectively form the ventral tegmental area (VTA)-substantia nigra (SN) (Bjorklund and Dunnett 2007). The SN is divided into two components: the pars compacta (SNc), which comprise the A9 dopamine neurons; and the pars reticulata (SNr), which contains GABAergic cells. In contrast, the A12 and A13 cell groups, which also produce dopamine, are found in the hypothalamus, whereas a third group, the A11 cells, is located in the subparafasicular thalamic nucleus (Bjorklund and Dunnett 2007). The VTA and SNc project throughout the forebrain and are responsible for most of the dopaminergic innervation of the forebrain (**Figure 1.3.**). The SNc provides a highly dense innervation of the dorsal striatum with little dopamine innervation of other structures. In contrast, the VTA projects to regions of the limbic system, including the hippocampus, the amygdala, and the bed nucleus of the stria terminalis, as well as to a variety of cortical regions, including the prefrontal cortex (PFC), and thus, the entire VTA innervation of the forebrain is known as the "mesocorticolimbic system" (LaLumiere 2014). D_1 receptors can be found at moderate-to-dense levels

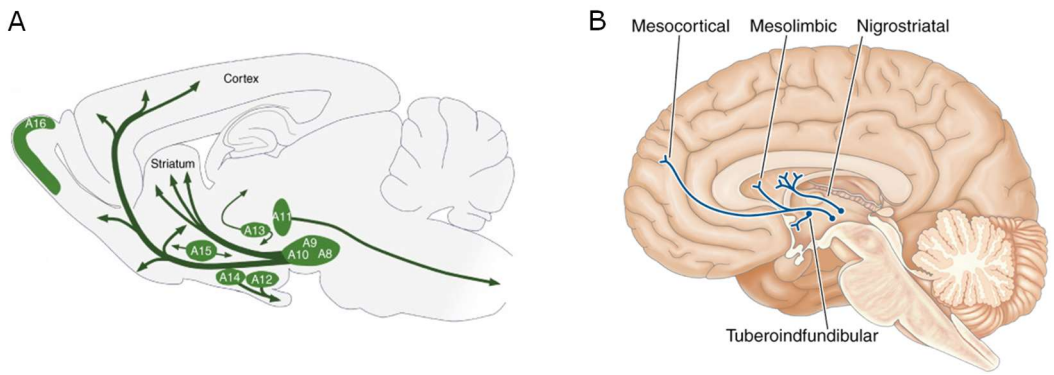


Figure 1.3. Distribution of dopamine neuron cell groups in rodent and human brain. The dopamine neurons in the mammalian brain are localized in nine distinctive cell groups, distributed from the mesencephalon to the olfactory bulb, as illustrated schematically, in a sagittal view, in (A) the adult rodent and (B) the adult human brain. The principal projections of the DA cell groups are illustrated by arrows). *Adapted from Björklund A and Dunnett S, TRENDS in Neurosciences (2007).*

throughout the target regions of the mesocorticolimbic and nigrostrial pathways. D_5 receptors, in contrast, are expressed at considerably lower levels in cortical regions, the hippocampus, and the striatum. D_2-class receptors are found at dense levels throughout the striatum and are also located in cortical and limbic targets as well as within the midbrain (VTA-SN) (Beaulieu and Gainetdinov 2011).

1.2.2. Schizophrenia and dopaminergic system

Pharmacological agents targeting dopaminergic neurotransmission have been clinically used in the management of several neurological and psychiatric disorders (e.g., Parkinson's disease, schizophrenia, bipolar disorder, Huntington's disease, ADHD, and Tourette's syndrome) (Meneses 2014). There is a substantial evidence that the dopaminergic system is responsive in the pathogenesis of SCZ and related disorders (Howes and Kapur 2009). To explain its key role, the "dopaminergic hypothesis" of schizophrenia is based on two main observations: (1) all APDs in use today block dopamine D_2 at clinically effective doses (Kapur and Remington 2001); and (2) drugs that drive DA release or increase DA transmission (such as amphetamine and L-DOPA), will exacerbate psychosis in patients with SCZ and can induce schizophrenia-like symptoms in control individuals (Angrist et al. 1974; Janowsky et al. 1973). Nonetheless, there is a little evidence for dysfunction within the dopamine system itself in individuals with schizophrenia (Grace 1991; 2012), and the focus of much research has turned instead to the dysregulation of the dopamine system by afferent structures.

The role of D_1 in PFC function is well acknowledged (Takahashi et al. 2012). The PFC is implicated in cognitive processes such as reasoning, planning and spatial ability (Wood and Grafman 2003), and particularly for this reason the cognitive role of D_1 receptors in schizophrenia has been investigated in various studies (Floresco 2013). Older studies in working memory shown that PFC dopamine acting via D_1 receptors, regulates cognition in accordance to an "inverted-U"-shaped function, so that so little or too much activity

has detrimental effects on performance. However, recent studies have indicated that the receptor mechanism through which mesocortical dopamine regulates different aspects of behavioral flexibility can vary considerably across different dopamine receptors and cognitive operations (Meneses 2014).

1.2.3. Antipsychotic drugs and pharmacology of schizophrenia.

Antipsychotics (APs) are the mainstay drugs for treating schizophrenia, bipolar disorder and other brain diseases with psychotic features (Miyamoto et al. 2012). Historically, these drugs are divided into two categories: (1) typical antipsychotics (TAPs), referred to as first-generation drugs; and (2) atypical antipsychotics (AAPs), referred to as second-generation drugs. The distinction is based solely on the concept that AAPs have reduced extrapyramidal side effects (EPS) such as parkinsonism and tardive dyskinesia (TD), and eventually a better profile in terms of social and cognitive improvement (Meltzer 2013). However, this classification has been questioned by different authors, suggesting that each AP is unique (Grunder et al. 2009; Keefe et al. 2004).

The first antipsychotic drug was chlorpromazine, discovered serendipitously in the early 1950s. These new drugs shown antipsychotic actions as well as EPS, led to their being called "neuroleptic drugs", reducing hospitalization and deaths of SCZ and psychotic patients. After, in the 1960s, it started to be understood that these medications act by blocking receptors for the neurotransmitter dopamine (Miller 2009a). Then, in the 1970s, the AAPs were introduced through clozapine. These new drugs were demonstrated to be very effective not only for treating the positive symptoms but also to reduce the negative and cognitive associated problems, including a strong reduction of motor-related side effects (Wenthur and Lindsley 2013). Despite this, symptoms are less but not eliminated, and other problems such as weight gain and the "metabolic syndrome" have caused increasing concern (Miller 2009a). Particularly, clozapine proved cognitive improvement and effective in patients resistant to other APDs (Gillespie et al. 2017), focusing the

present introduction on this AAP drug. Unfortunately, clozapine benefits have been outweighed by its potential side effects, like for example the increased risk of severe haematological effects (i.e. neutropenia and agranulocytosis), or weight gain (Miller 2009a). For this reason, there has been a strong effort to find new drugs as effective as clozapine but devoid its relevant side effects.

The discovery of the AAPs introduced a new concept in relation to the mechanism of action, classically assigned to the dopamine D_2 receptor. These drugs shown low affinity for the D_2, been an effective APD through the involvement of other receptors, such as serotonin 5-HT_{2A} receptors (Aringhieri et al. 2018). From the involvement of other receptors in the pharmacology of the SCZ and psychosis, a number of hypothesis have been tried to explain unambiguously and unsatisfactory the mechanism of "atypia" (Miyamoto et al. 2005) (**Figure 1.4.**). Indeed, many evidences have pointed out the importance of other GPCRs beyond D_2 and 5-HT_{2A}, such as other dopamine, serotonin,

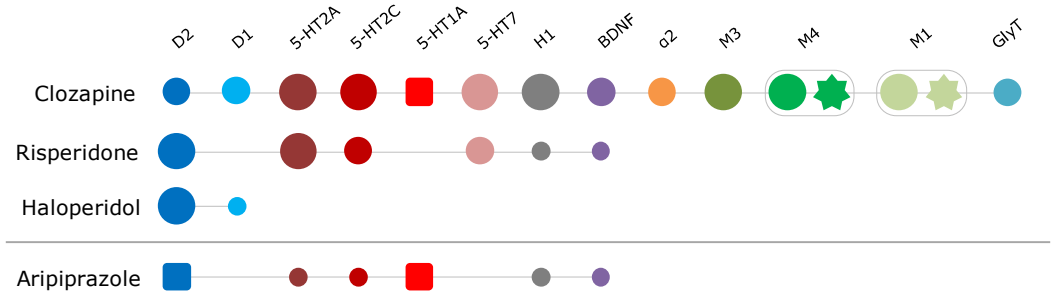


Figure 1.4. Molecular targets of antipsychotic drugs. List of the most relevant targets involved in the mechanism of action of APDs based on receptor occupancy. Values are reported as high (●), medium (●) and low (●). ●, ■ and ✶ represent receptor antagonism, partial agonism and positive allosterism, respectively. ● represents BDNF production, while * represents positive allosterism by the clozapine metabolite, norclozapine, at M1 and M4 receptors. Aripiprazole is shown at the bottom for its different mechanism of action. Clozapine covers a wide range of molecular targets among all APDs, while risperidone and haloperidol are mostly limited to just a few, and this might explain clozapine's superiority among APDs. *Adapted from Aringhieri S et al., Pharmacol Ther (2018).*

muscarinic, adrenergic, glutamatergic and histamine receptors (Meltzer and Massey 2011). Besides GPCRs, other targets have been considered, such as ion channels (e.g. NMDA), transporters (e.g. glycine transporters) and enzymes (e.g. GSK3).

Although acute events, such as psychoses, are probably controlled by short-term effects of AAPs that are mostly mediated by their receptors affinities, it is evident that these drugs have more complex effects, particularly in the long term, involving intracellular mechanism that may regulate neuronal functionality, neuroplasticity and neurogenesis through the activation of signalling pathways like MAPK, PKA and PI3K/AKT (Aringhieri et al. 2018).

1.2.3.1. Neuroleptic-resistant psychosis and clinical differences among antipsychotic drugs.

In clinical practice, the question as to which APD should be preferred to ensure the highest therapeutic success for treating schizophrenia or other psychoses is a complex subject. It has long been suspected that some psychotic patients do not respond to standard APDs (Miller 2009a; 2009b). It is particularly relevant for psychiatric disorders where more than 1/3rd of the patients do not receive any benefit from the pharmacological treatment (refractory to treatment), and where 20-60% of the patients, in the long-term, suspend drug usage either due to side effects or for non-adherence (poor compliance), leading to increased morbidity and mortality (Aringhieri et al. 2018).

Many studies demonstrated a better outcome with AAPs compared to TAPs in several aspects, such as efficacy, quality of life, tolerability, drop out and side effects (Leucht et al. 2013; Leucht et al. 2009; Leucht et al. 2017). However, not all AAPs have achieved the same results in the treatment of positive and negative symptoms of SCZ, and some authors have proposed a continuum spectrum of atypia that ranges from risperidone, the least atypical (with similar effects than lower doses of haloperidol), to clozapine, the most atypical (Aringhieri et al. 2018). In this context, clozapine is probably the most effective

therapeutic medication as compared to other APD (Leucht et al. 2009; Miller 2009a; 2009b). On treatment-resistant patients, clozapine is superior to all other AAPs, and since its discovery is still considered the "gold standard" for treatment-refractory schizophrenia (Siskind et al. 2016).

1.2.3.2. Antipsychotic side effects.

Regarding motor side effects, there is a continuum among AAPs starting from clozapine that practically never shows EPS and ending with risperidone that shows a notable rate of parkinsonism (Leucht et al. 2013). Also, clozapine very rarely causes tardive dyskinesia (TD) (Aringhieri et al. 2018). Though in terms of motor side effects and hyperprolactinemia the AAPs are superior to TAPs, unfortunately the AAPs cause weight gain and other metabolic problems. New AAPs seem to have a low likelihood to cause this side effects, but this advantage has to be balanced against therapeutic efficacy (Leucht et al. 2017). On negative symptoms, the data are consistently in favour of AAPs: TAPs have a negative impact on dopaminergic activity in the PFC mainly their strong D_2 antagonism (Aringhieri et al. 2018).

Regarding the cognitive deficits associated with schizophrenia, AAPs may produce a mild remediation with different effects on specific cognitive domains. Clozapine significantly improves verbal fluency more than other AAPs (Woodward et al. 2005), possibly related to increased release of dopamine and other neurotransmitters in the PFC and hippocampus (Ichikawa et al. 2002; Shirazi-Southall et al. 2002). Unfortunately, these favourable characteristics of clozapine have to be well balanced with its side effects, like agranulocytosis, weight gain and metabolic problems (Aringhieri et al. 2018). Thus, since the conception of personalized pharmacotherapy, strong efforts may be done to understand all interindividual variables that influence the therapeutic response of schizophrenia patients.

1.3. CREB role in psychosis.

1.3.1. Molecular structure of CREB.

In human and mouse, the CREB (cAMP response element-binding protein) genes are located on chromosome 2q33.3 and consist of 11 exons and 3 isoforms (α, β and Δ) produced through alternative splicing with identical function (Ichiki 2006). The full-length sequence of CREB can be divided in four functional domains (**Figure 1.5.**): (1) Q1 basal transcriptional activity domain, localized at N-terminus, interacts with TATA binding protein and promotes gene transcription (Felinski and Quinn 2001); (2) kinase inducible domain (KID), localized in the middle region that contains Ser133 which phosphorylation by multiple protein kinases is necessary for the activation of CREB (Sun et al. 2016); (3) a glutamine-rich Q2 domain for constitutive activation, responsible for binding with RNA polymerase II initiation complex through the recognition and binding to the canonical CRE (5'-TGACGTCA-3') (Altarejos and Montminy 2011); and (4) a basic region/leucine zipper domain (bZIP), at carboxy terminal, required for the dimerization of CREB (Schumacher et al. 2000).

The upstream protein kinases activating CREB include protein kinase A (PKA), protein kinase B (PKB, also known as Akt), protein kinase C (PKC), calcium/calmodulin-dependent protein kinase II (CaMKII), p90 ribosomal S6 kinase (p90RSK), casein kinase I and casein kinase II (Trinh et al. 2013; Wen et al. 2010) (**Figures 1.5 and 1.6.**). PKA phosphorylation does not alter the secondary structure of CREB, and therefore, has no effect on the binding of CREB to DNA (Richards et al. 1996). Other CREB family members are cAMP response element modulator (CREM) and activating transcription factor-1 (ATF-1), which structure and biological functions are similar to CREB (Wu et al. 1998). Both members, forms heterodimers with CREB and these interactions in the pathophysiology of schizophrenia remain unknown.

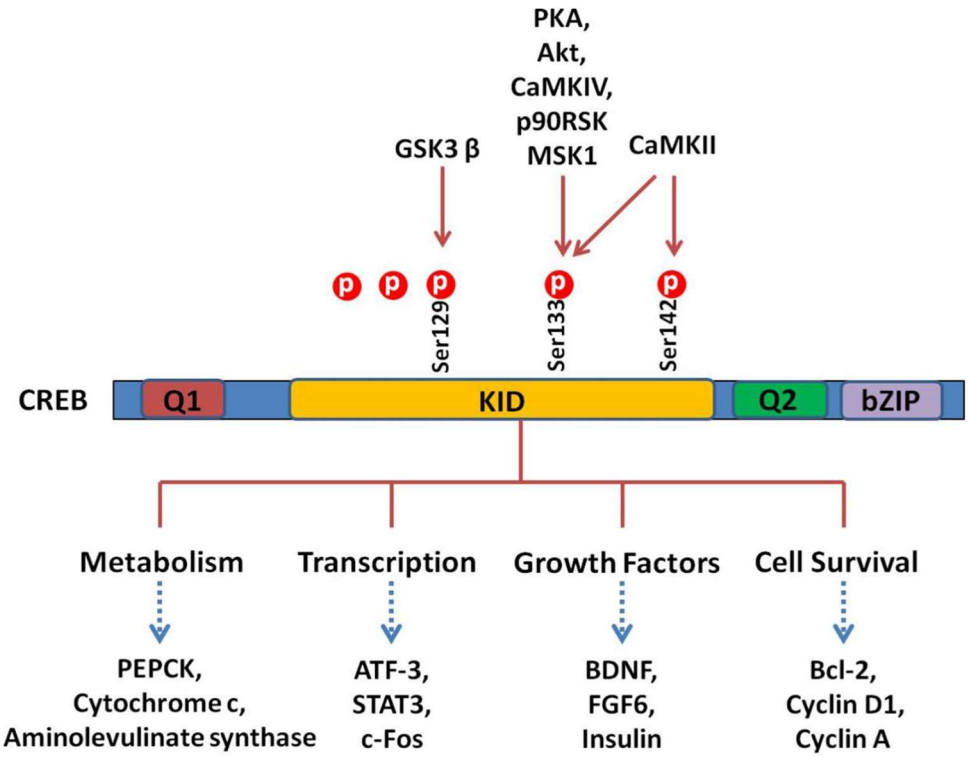


Figure 1.5. CREB and its downstream substrates. The CREB contains Q1, kinase-inducible domain (KID), Q2, and bZIP domains. The crucial event in the activation of CREB is the phosphorylation of Ser133 in KID. This domain could be phoshphorylated by multiple protein kinases such as PKA, Akt, CaMKs, p90RSK, and MSK1. The CREB as a nuclear transcription factor binds to CRE (cAMP response element), regulating transcription activity of its downstream substrates, which regulate neuronal processes, including metabolism and survival and expression of different transcription factors and growth factors. *Adapted from Wang et al., Front Mol Neurosci (2018).*

1.3.2. Dopamine-mediated signalling affects the activity of CREB.

Dopamine binding to its receptors enhances the phosphorylation of CREB through different pathways, been CREB critical in the signal transduction of the dopamine receptors (**Figure 1.6.**): (1) binding of dopamine to D_1 receptor elevates intracellular cAMP levels and activates PKA, followed by the phosphorylation of CREB (Boyd and Mailman 2012; Chartoff et al. 2003); (2) binding of dopamine to D_2 receptor reduces cAMP production and adenylate cyclase (AC) activity followed by reduced phosphorylation of CREB (Boyd and Mailman 2012). However, repeated treatment with the selective D_2

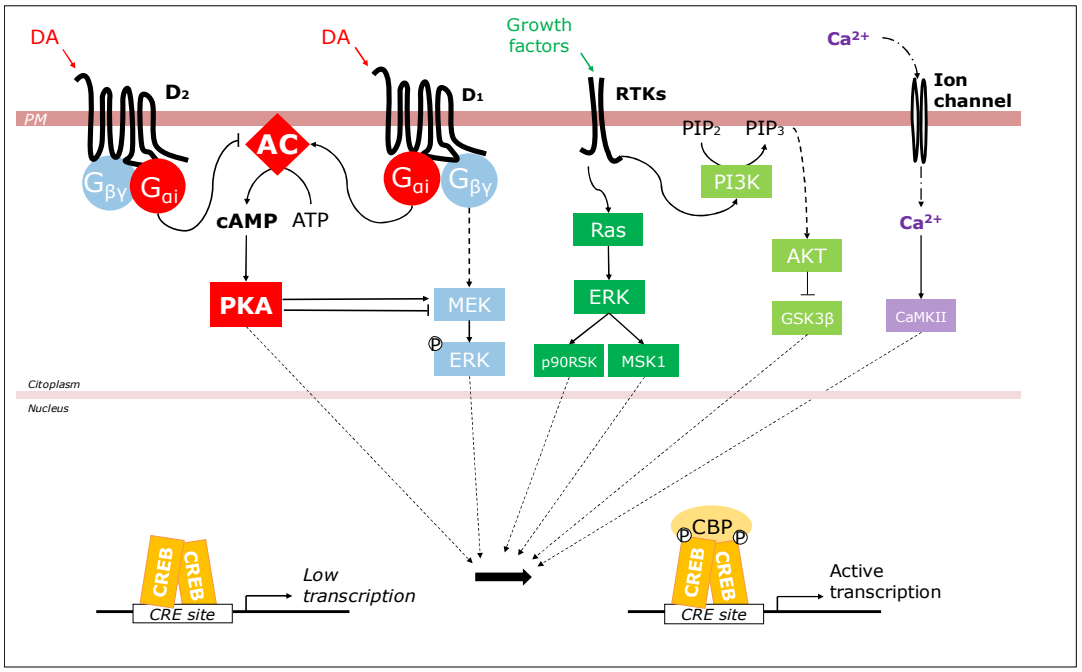


Figure 1.6. CREB upstream signaling cascades in neurons. CREB can be regulated by different pathways: (1) AC activated upon stimulation of dopamine D1-class receptors (Gαs) by dopamine neurotransmitter increases cAMP levels, which, in turn, activate PKA. The catalytic subunits of PKA translocate into the nucleus and phosphorylate CREB at Ser133. AC can be inhibited upon stimulation of dopamine D2-class receptors (Gαi) by dopamine (coloured in red). (2) Gβγ subunit can activate MAPKs (MEK/ERK1/2) signaling (coloured in blue). (3) Binding of growth factors to receptor tyrosine kinases (RTK) stimulate the activation of PI3K/Akt/GSK3b (coloured in light green) and Ras/Raf/MEK/ERK/p90/RSK and ERK/MSK1 (coloured in dark green) signaling pathways, which subsequently enhance the phosphorylation of CREB at different sites. (4) Activation of excitatory NMDA receptors will increase the phosphorylation of CREB through Ca2+/CaMK-dependent pathways (coloured in purple).

receptor agonist (quinpirole) enhances PKA activity and increases phospho-CREB expression in the nucleus accumbens (NAc) (Culm et al. 2004); (3) activation of D_2 receptor by its agonist quinpirole causes an elevation of intracellular Ca^{2+} and activation of PKC, phosphorylating CREB (Yan et al. 1999). DARPP-32 is possibly a molecule that links D_2-mediated signalling and CREB (Yan et al. 1999); (4) stimulation of D_1 and D_2 receptors activate Akt kinase that directly phosphorylates CREB at Ser133 in striatal neurons (Brami-Cherrier et al. 2002); and (5) CREB stimulates the expression of a number of genes containing CREBs in their promoter region (Montminy 1997). It has been

also reported that D_1/D_2 receptors play a synergic role in inducing CREB-DNA binding activities (Kashihara et al. 1999).

As mentioner early, different protein kinases can phosphorylate CREB at the KID domain (Guo et al. 2017) (**Figure 1.5.**). This domain contains several multiple phosphorylation sites for many canonical signalling pathways, such as Ras/MAPK/p90RSK, Ca2+/CaMK, PI3K/Akt/GSK3β and cAMP/PKA pathways (**Figures 1.5 and 1.6.**). Specifically, in the cAMP/PKA/CREB signalling pathway, the activated PKA enters into the nucleus and promotes the binding of Ser133 phosphorylated CREB to a CRE region and then, the CBP (CREB-binding protein) is recruited and bound to CREB, which co-activates CREB. It has been described that mutation of Ser133 destroys the transcription activity of this protein (Karin and Smeal 1992). CREB activation stimulates or inhibits the expression of its downstream target genes, involved in metabolism (such as cytochrome c), transcription (such as STAT3 or c-Fos), cells survival/cell cycle (such as bcl-2 or cyclin D1) and growth factors (such as BDNF or FGF6) (Sakamoto and Frank 2009). Also, the CREB can be phosphorylated at other residues, including Ser129, Ser142 and Ser143 (**Figure 1.5.**), involving different kinases such as CaMKII (**Figure 1.6.**).

Serine/threonine protein phosphatase enzymes are essential to many neuronal functions. Specifically, in mammals, include PP1, PP2A, PP2B and PP2C (Shi 2009) that act on upstream CREB signalling (Wadzinski et al. 1993; Yang et al. 2015) (**Figure 1.6.**). It well known that CREB phosphorylation intensity and durability are parallel with the transcriptional regulation of target genes containing the CRE sequence (Hagiwara et al. 1992) and the balance between CREB-target kinases and phosphatases determines the degree of CRE-dependent gene transcription (Hagiwara et al. 1992).

1.3.3. Biological functions of CREB in mood disorders and CNS.

CREB is expressed in different brain areas, including the NAc (Mantamadiotis et al. 2002). Experimental changes in CREB activity in the dopaminergic neurons in this after a

stimulus, have been related with changes in animal behavior. Elevations in CREB activity in NAc reduces sensitivity to emotional and stress stimuli (Barrot et al. 2002) or anhedonia-like and depression-like symptoms (Newton et al. 2002; Pliakas et al. 2001). In animal models of schizophrenia, PKA activity and CREB phosphorylation in the NAc are decreased, and treatment with antipsychotics increases CREB activity in the NAc, and this neuro-adaptative response facilitates the recovery of sensorimotor gating (Culm et al. 2004).

As discussed above, according to the "neurodevelopmental hypothesis", abnormalities of early brain development increase the risk for the subsequent emergence of symptoms such as psychosis (Schmidt and Mirnics 2015). In this sense, dysfunction of BDNF signalling, an important neurotrophin that promotes development of certain populations of neuronal cells (Nieto et al. 2013), leads to deficits in neuronal growth and synaptic transmission, leading to disorganized brain function, which contributes to the development of schizophrenia (Palomino et al. 2006). It is well documented that BDNF promotes phosphorylation of CREB, which, in turn, promotes the transcription of BDNF gene by increasing intracellular Ca^{2+} that leads to the activation of CaMKIV. Also, neuronal exposure to BDNF activates the Ras/Erk/Rsk pathway (Finkbeiner et al. 1997; Pizzorusso et al. 2000) (**Figure 1.6.**).

CREB expression and transcriptional activity are regulated in both embryonic and mature brain, and it is implicated in neuronal survival as well as in neurogenesis, processes associated with the pathology of schizophrenia (Wang et al. 2018). A number of studies indicate that the pathological consequences resulting from CREB alteration are mediated through different mechanistic processes (Sakamoto et al. 2011). CREB ablation triggers non-specific neuronal cell death by pro-apoptotic signals (Zeng et al. 2016), and progressive degeneration in brain hippocampus and dorsolateral striatum (Dawson and Ginty 2002). Otherwise, it is noteworthy that chronic CREB activation is related with loss

of hippocampal neurons through excitotoxicity mechanism and sporadic epileptic seizures (Lopez de Armentia et al. 2007). These results suggest that the timing of CREB regulation may be a key for the associate changes in the cellular neuronal responses.

1.3.4. CREB in schizophrenia treatment

The role of CREB in treatment with APDs has been studied in animal models and it has been observed that various antipsychotic drugs could have different roles on CREB phosphorylation in neurons. In this regard, it was reported that haloperidol and eticlopride, selective D_2 receptor antagonist, stimulated the phosphorylation of CREB in the dorsal striatum in rats. In contrast, clozapine reduced CREB phosphorylation, indicating that haloperidol and clozapine induce distinct patterns of CREB phosphorylation in dorsal striatum (Pozzi et al. 2003). Furthermore, neurons in different maturation stages may have distinct phenotypes regarding the phosphorylation of CREB in response to the same antipsychotic drug. For example, low concentrations of haloperidol and risperidone stimulate the phosphorylation of Erk and CREB in hippocampal neurons cultures after 25 days in vitro, but not at 10 days (Yang et al. 2004).

Hence, phosphorylation level of CREB in the brain sections has been highly related with mood disorders and its regulation could be a suitable strategy that could be applied for the treatment of this disorder (Wang et al. 2018). Even though, its effectiveness will depend upon further research characterizing the role of CREB in the brain and the pathological processes of schizophrenia.

1.4. Biomarkers for schizophrenia diagnosis and antipsychotic response: more than a tool for diagnosis?

1.4.1. Peripheral blood-based biomarkers by transcriptome sequencing (mRNA-seq).

Identifying biomarkers that can be used as diagnostics or predictors of treatment response (theranostics) in people with SCZ will be an important step towards being able to provide personalized treatment and would support efforts to develop new drug treatments. Current neurological findings of the study of the pathology have not yet been translated into biomarkers that are practical in clinical use because brain biopsies are not acceptable and neuroimaging techniques are expensive and the results are inconclusive (Lai et al. 2016). Relative to organ tissue (e.g., brain), body fluids such as blood, urine, and cerebral spinal fluid (CSF) represent more easily accessible sources for detection of systemic biomarkers (Chan et al. 2011). The blood, for example, can be sampled using standardized and routine clinical procedures without significant patient discomfort. The main advantages associated with using blood as a source of biomarkers include the fact that it is possible to design standardized sample collection procedures, it is available in sufficient quantities, and it can be sampled on multiple occasions with relative ease (Lakhan and Kramer 2009). Thus, in recent years, there has been search for blood-based biomarkers for SCZ as a valid alternative because the dysregulation of gene expression, epigenetic patterns, protein quantities, metabolic and inflammatory molecules in peripheral blood have been shown to have distinct patterns in people with SCZ (Chan et al. 2015; Sainz et al. 2013).

Modern sequencing allows the investigation of levels of thousands of mRNAs (transcriptome) at the same time. In psychiatry, such studies have involved the use of whole blood, lymphocytes or PBMC samples (Lai et al. 2016; Sainz et al. 2013) to identify potential biomarkers. This approach has typically led to a large set of mRNAs or proteins

being proposed as possible biomarkers in SCZ, but most findings await replication. Briefly, these studies identified a number of potential biomarkers in peripheral blood with a cluster of reports suggesting that markers associated with immune function/inflammation might be of interest (Lai et al. 2016).

1.4.2. Schizophrenia gene expression profile reverted to normal levels by antipsychotics: Clinical Response Genes to Antipsychotics (CRGAs).

To identify candidate genes altered at the onset of the pathology and which were controlled by AAPs, our group, in the last years, have been focused in the investigation of the expression profiles from peripheral blood of SCZ patients. In this way, our group sequenced total mRNA from blood's PBMCs of a cohort of drug-naïve schizophrenia patients and the same patients after treatment with atypical antipsychotic, where all patients showed improved clinical symptoms (Crespo-Facorro et al. 2015; Sainz et al. 2013).

1.4.2.1. Inflammatory and immune response genes have significantly altered expression in schizophrenia.

Initially, our group analysed the blood transcriptome of 36 drug-naïve schizophrenia patients at FEP (First Episode of Psychosis) and 40 healthy matched controls (without a history of neuropsychiatric disorders) by next-generation sequencing. Results shown 200 genes with significant differential expression (p-value adjusted <0.05) in the 22278 human genes analysed (**Annexed table 1**) (Sainz et al. 2013), where a total of 37 genes out of them were identify in the GWAS catalogue (https://www.genome.gov/gwastudies/). Among these 200 genes, *ADAMTS2* has the lowest P_{adj} value (1.3236E-69) and has been associated with ADHD (time to onset) in a GWAS (Lasky-Su et al. 2008). Three differentially expressed genes (*CSMD1*, *EHF* and *RFX2*) are associated to schizophrenia in GWAS. *CSMD1* associated with schizophrenia among other traits (Ripke et al. 2011), *EHF* associated with response to antipsychotic

treatment and volumetric brain magnetic resonance imaging in schizophrenia patients (McClay et al. 2011; Seshadri et al. 2007), and *RFX2*, which is not reported but has an intronic SNP with a p-value = 3.5 x 10^{-6} in a schizophrenia and bipolar disorder GWAS meta-analysis (Wang et al. 2010).

According to the scientific literature deposited in the GeneRIF repository (NCBI), 8 out of the 200 differentially expressed genes have been related to schizophrenia: *GRIK3*, *LPL*, *S100B*, *SNCA*, *SYN2*, *TUBB2A*, *SELENBP1*, *CSMD1*; increased levels of S100B have been reported in schizophrenia, which is consistent with our observation of significantly higher expression of the gene in schizophrenia patients. Also, lower copy number of *SELENBP1* in schizophrenia patients has been reported, which is consistent with the significantly lower expression levels we observe in our study (Begni et al. 2002; Liu et al. 2005; Martins-de-Souza et al. 2010; Steiner et al. 2006; Xie et al. 2011).

Functional analyses indicated a significant enrichment in seven gene ontology biological processes (P_{adj} values < 0.05) (**Table 1.3.**), related to protein processing and inflammatory and immune response. There are 20 differential expression genes involved in the immune, inflammatory response and the response to wounding, 15 of these genes are upregulated and 5 downregulated in schizophrenics.

Table 1.3. Significantly enriched gene ontology. *Adapted from Sainz J et al., 2013.*

Biological process	Differential expression genes	Observed % of all	Expected % of all
Protein processing (GO:0016485)	8	4.00	0.53
Protein maturation (GO:0051604)	8	4.00	0.57
Protein maturation by peptide bond cleavage (GO:0051605)	7	3.50	0.42
Acute inflammatory response (GO:0002526)	9	4.50	0.81
Activation of plasma proteins involved in acute inflammatory response (GO:0002541)	6	3.00	0.24
Response to wounding (GO:0009611)	18	9.00	3.24
Innate immune response (GO:0045087)	10	5.00	0.80

1.4.2.2. Schizophrenia gene expression profile reverted to normal levels by antipsychotics.

In a later work, to characterize candidate genes targeted by AAPs, our group investigated the expression profiles altered by these drugs (Sainz et al. 2013). We sequenced total mRNA of a cohort of 22 individuals before treatment and the same individuals 3 months after treatment started. These patients were part of a larger cohort used before (Sainz et al. 2013), showing improved symptoms after treatment. Results showed 17 genes with significant differential expression (**Table 1.4.**) after adjusting for multiple testing (P_{adj} Value <0.05) in the 21495 human genes analysed (**Annexed table 2.**). Interestingly, six of the differentially expressed genes between untreated and treated patients (*ADAMTS2*, *CD177*, *CNTNAP3*, *ENTPD2*, *RFX2*, and *UNC45B*) also have differential expression between drug-naïve patients and healthy controls. These 6 schizophrenia-related genes that we found in our previous study case/control (Sainz et al. 2013) have their expression levels significantly higher in patients than in controls and, after medication with atypical

Table 1.4. Differential expression in schizophrenia patients before and after treatment. *Adapted from Crespo-Facorro et al., 2015.*

geneID	Gene Symbol	Base Mean	Base Mean Not Medicated	Base Mean Medicated	Fold Change	Log2 Fold Change	Pval	Padj
9509	**ADAMTS2**	23.86339982	43.58273814	4.144061509	0.095084928	-3.39463952	1.93223E-45	3.77076E-41
57126	**CD177**	125.2142026	182.6429658	67.7854394	0.371136327	-1.42997888	4.15587E-15	4.05509E-11
146862	**UNC45B**	228.318709	355.5905317	101.0468862	0.284166414	-1.81519204	9.35727E-13	6.08691E-09
10321	**CRISP3**	115.7986838	67.59205817	164.0053094	2.426399104	1.27881687	4.87135E-09	2.37661E-05
85495	**RPPH1**	26.04702513	39.64589219	12.44815807	0.313983552	-1.67123911	3.214E-07	0.001254426
249	**ALPL**	4431.746109	5880.320968	2983.171249	0.507314357	-0.97904811	1.4913E-06	0.003449261
4317	**MMP8**	183.6663537	123.5348176	243.7978898	1.973515601	0.980767924	1.80178E-06	0.003449261
4973	**OLR1**	33.1319214	20.85963419	45.40420861	2.176654116	1.122112172	1.40581E-06	0.003449261
10562	**OLFM4**	137.50003	93.07893836	181.9211215	1.954482128	0.966786393	1.94424E-06	0.003449261
1088	**CEACAM8**	187.2128837	127.3498437	247.0759236	1.940135271	0.956157244	1.59811E-06	0.003449261
154664	**ABCA13**	92.17901569	61.88536784	122.4726635	1.979024571	0.984789524	1.36575E-06	0.003449261
5990	**RFX2**	520.3558229	681.1696202	359.5420255	0.527830389	-0.92185368	7.00393E-06	0.010513971
4057	**LTF**	1632.349968	1170.323611	2094.376324	1.789570257	0.839613184	6.5125E-06	0.010513971
2852	**GPER1**	28.33693561	36.77092153	19.90294968	0.541268721	-0.88558307	1.00986E-05	0.014076694
79937	**CNTNAP3**	195.2279073	249.0334206	141.422394	0.5678852	-0.81632878	1.68285E-05	0.021893882
954	**ENTPD2**	29.04177998	36.51237117	21.57118878	0.590791233	-0.75927968	2.51645E-05	0.030692793
387755	**INSC**	31.73879717	41.6047807	21.87281365	0.525728373	-0.9276105	3.82829E-05	0.043946467

antipsychotics, their expression reverted to control levels (**Figure 1.7.**). These results support the theory that the expression of these six genes could be modulated by the drugs and the possibility that they are implicated in the positive symptoms of the disease (hallucinations, delusions, positive formal thought disorders, bizarre behavior) that are improved by the antipsychotics.

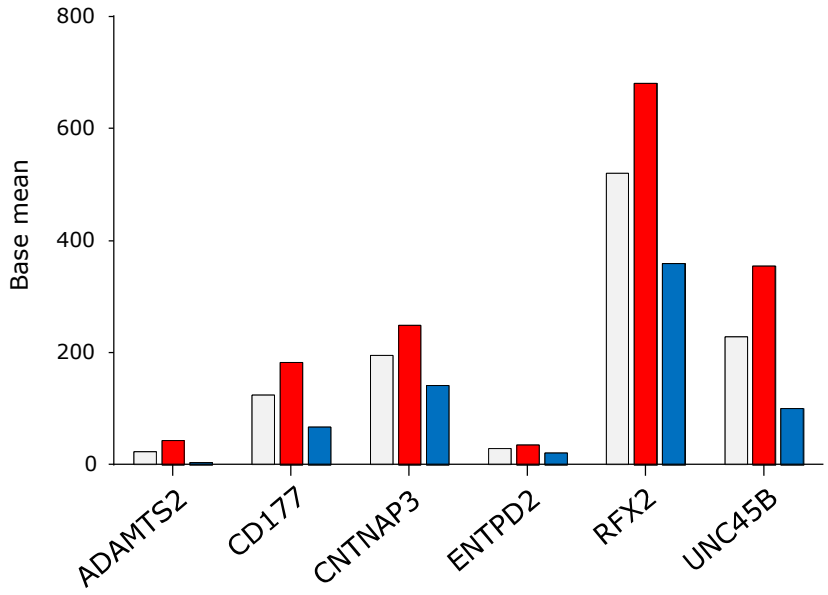


Figure 1.7. Schizophrenia differential expression genes reverted by antipsychotic medications. Base mean: mean of normalized counts, averaged over all samples for each condition; controls: normalized expression level of controls; untreated cases: normalized expression level of untreated patients; treated cases: normalized expression level from the patients, obtained after 3 months of treatment. *Adapted from Crespo-Facorro B et al., Int J Neuropsychopharmacol. (2015).*

Results shown that these gene-set were enriched for the cellular components (extracellular matrix -ECM- and proteinaceous extracellular matrix) which are involved in many functions, such as segregating tissues from one another and regulating intercellular communication. The four genes included in these categories are *ENTPD2, MMP8*, *ADAMTS2*, and *CRISP3*. Also, eight genes out of the 17 (*LTF*, *OLFM4*, *ADAMTS2*, *RFX2*, *ALPL*, *MMP8*, *ABCA13*, and *INSC*) appear to associate, either as reported or as mapped

gene, with 24 diseases/traits in the GWAS. The association with diseases includes SCZ and bipolar disorder (*RFX2*) (Wang et al. 2010), time to onset of ADHD (*ADAMTS2*) (Lasky-Su et al. 2008), response to amphetamine (*ABCA13*) (Hart et al. 2012), AIDS progression (*LTF*) (Troyer et al. 2011), metabolic traits (*ALPL*) (Suhre et al. 2011), obesity (*OLFM4*) (Bradfield et al. 2012), obesity related traits (INSC) (Comuzzie et al. 2012), and visceral adipose tissue adjusted for body mass index (*ADAMTS2*) (Fox et al. 2012), among other disease-traits.

Furthermore, 15 out of the 17 differential expression genes are annotated for disease or functionality in 719 publications deposited in the GeneRIF repository. Among these genes, *ABCA13* has been implicated in schizophrenia, bipolar disorder, and depression (Knight et al. 2009). The 15 differential-expression genes annotated are significantly enriched for two known adverse effects caused by the atypical antipsychotics: obesity and diabetes. Five genes (*GPER*, *LTF*, *MMP8*, *OLR1*, and *OLFM4*) have been implicated with obesity (Belo et al. 2009; Bradfield et al. 2012; Brinkley et al. 2008; Haas et al. 2009; Moreno-Navarrete et al. 2013). Four of the genes (*ALPL*, *LTF*, *MMP8*, and *OLR1*) have been implicated with diabetes (Kanazawa et al. 2009; Tan et al. 2008; van der Zijl et al. 2010; Vengen et al. 2010). Moreover, it is known that all antipsychotics tend to block receptors in the brain's dopamine pathways, and that estrogen increases the concentration of neurotransmitters such as dopamine at neuronal synapses and their receptors, affecting their release, reuptake, and enzymatic inactivation (McEwen and Alves 1999). We found a significant enrichment of genes (*GPER*, *LTF*, and *OLFM4*) related to estrogen (Dassen et al. 2010; Revankar et al. 2005; Teng et al. 2002). Also, as we observed previously (Sainz et al. 2013), results revealed an enrichment of inflammatory genes: *ALPL*, *GPER*, *LTF*, *MMP8*, *OLR1*, and *CRISP3* (Chakrabarti and Davidge 2012; Cheung et al. 2008; Leppilahti et al. 2011; Plager et al. 2010; Takanabe-Mori et al. 2013; Weinberg 2001).

These works, despite of their limitations (small sample size), shown significant results which could help to predict the effects of the atypical antipsychotics used in clinics by monitoring the expression levels of the reported genes. Results showed six genes (*ADAMTS2*, *CD177*, *CNTNAP3*, *ENTPD2*, *RFX2*, and *UNC45B*) related with positive symptoms improved by APDs and a list of 17 genes that could be responsible for some adverse effects (such obesity and diabetes) related with APD treatment. Further information is needed to understand the real possibilities of this information.

1.5. Extracellular matrix abnormalities in central nervous system (CNS) and its potential role in psychotic disorders.

The extracellular matrix (ECM) occupies approximately a volume fraction of 10-20% of the normal adult brain (Dityatev and Schachner 2003; Sykova and Nicholson 2008). The ECM is produced intracellularly and secreted to form a dense network of proteins and glycans, occupying the parenchyma of virtually all cells. Structurally, the ECM provides neural cells with points of anchorage and facilitates the organization of these cells into distinct CNS regions (Lau et al. 2013). Chemically, it is a source of diverse molecular signals that guide cellular growth, activity and survival (Lau et al. 2013). During CNS development, specific ECM components are regulated in a temporal and spatial manner to facilitate neurogenesis, neural cell migration and differentiation, and axonal growth and pathfinding (Bandtlow and Zimmermann 2000). By adulthood, the ECM has changed in composition and acts to stabilize structures including synapses, to regulate synaptic plasticity and to prevent abnormal synaptic remodelling (Dityatev and Schachner 2003). The ECM also fills the synaptic cleft, although its biochemical composition within this specialized space is different, and less well known, with respect to the rest of the brain ECM (Zuber et al. 2005).

The ECM in the adult CNS is localized to three principal compartments (**Figure 1.8.**): (1) the basement membrane (basal lamina) that is a continuous ECM structure that lines the parenchymal side of cerebral microvessels which main role is maintaining the integrity of the blood-brain barrier (BBB) and, predominantly, it is made up of collagen, laminin-nidogen complexes (entactin), fibronectin, dystroglycan and perlecan; (2) the perineuronal nets (PNNs) that are dense ECM aggregates composed principally of proteoglycans, tenascin R and link proteins that surrounds neuronal cell bodies and proximal dendrites, which main role is preserving neuronal health and maintaining synaptic plasticity; and (3) the neural interstitial matrix, composed of components dispersed in the parenchyma that comprises a dense network of proteoglycans, hyaluronan, tenascins and link proteins, as well as relatively small amounts of fibrous proteins (collagens and elastin) and adhesive glycoproteins (laminins and fibronectin) (Lau et al. 2013). Insults to the CNS during neuropathological conditions result in alterations to the brain's ECM and these changes could contribute to pathophysiology, even in mental disorders.

Emerging evidence suggest a key role for the ECM abnormalities in schizophrenia (Berretta 2012). Immunohistochemical studies indicated a marked decrease in the numbers of CSPG-labeled PNNs in the amygdala, entorhinal cortex and PFC of patients with SCZ compared with normal controls. Studies revealed that normal complements of these neurons and related PNNs are preserved in SCZ patients but its binding to proteoglycan is dysregulated (Pantazopoulos et al. 2010; Sethi and Zaia 2017). Reelin, tenascin X, fibronectin, and integrins have been extensively investigated in SCZ. These ECM glycoproteins perform diverse roles in the developing and adult brain, such as neuronal migration, synapse development, synaptic plasticity, and learning and memory (Sethi and Zaia 2017). Several studies revealed dysregulation expression of these glycoproteins related with the pathophysiology of schizophrenia, and it is well known the

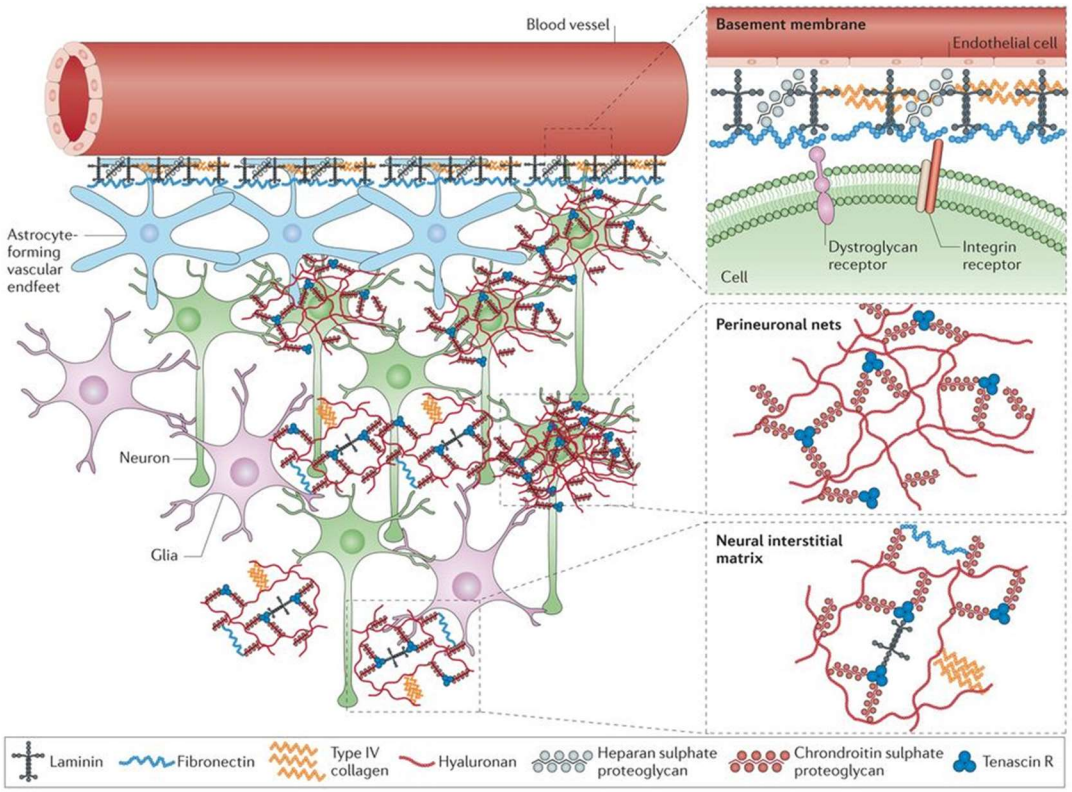


Figure 1.8. The three major compartments of the ECM in the CNS. Extracellular matrix (ECM) components are arranged into basement membranes that lie outside cerebral vessels, condensed as perineuronal nets around the cell bodies and dendrites of neurons or diffusely distributed as the neural interstitial matrix between cells of the CNS parenchyma. The pink glial cells depict astrocytes, oligodendrocytes or microglia. *Adapted from Lau LW et al., Nature reviews (2013).*

decreased expression of reelin in these patients (Dong et al. 2005; Impagnatiello et al. 1998; Sethi and Zaia 2017). Also, integrins, that assist in cell-cell and cell-ECM interactions and play a significant role in the modulation of neural synaptic activity, have been associated with the pathophysiology of SCZ (Walsh et al. 2002). Levels of proteolytic enzymes, including matrix metalloproteases (MMPs), members of the a disintegrin and metalloprotease (ADAM) family, and members of the ADAM with thrombospondin motifs (ADAMTS) family, have been associated with SCZ (Sethi and Zaia 2017). Elevated levels of MMP-9 have been reported in previous SCZ studies, including those of treatment-resistant schizophrenic patients (Domenici et al. 2010; Yamamori et al. 2013).

Additionally, altered mRNA expression MMPs (MMP-16, -24 and -25) and ADAMTS (ADAMTS-1 and -6) proteases has also been reported previously in SCZ (Pietersen et al. 2014a; 2014b).

1.5.1. A Disintegrin and Metalloproteinase with Thrombospondin motifs (ADAMTS) family.

The ADAMTS proteases belong to 'a disintegrin and metalloproteinase with thrombospondin motifs' family, composed of 19 members. This family are multi-domain proteins synthesized as pre-pro-enzymes (**Figure 1.9.**). Pre-pro-ADAMTS proteases can be cleaved by furin or furin-like proteases at the N- and C-terminal positions triggering, respectively, their activity and their future location within the ECM. When secreted, ADAMTS proteases are capable of binding ECM components via their thrombospondin motifs, which can be then cleaved by the metalloproteinase domain (Lemarchant et al. 2013). They are classified in three subfamilies based on their preference to cleave specific ECM macromolecules: proteoglycans (ADAMTS-1, -4, -5, -8, -9, -15 and -20), pro-collagens (ADAMTS-2, -3 and -14), and the von Willebrand factor (ADAMTS-13) (Kelwick et al. 2015). ADAMTS proteases are expressed throughout the CNS, including the spinal cord, brain stem, hippocampus, striatum and cortex (Gottschall and Howell 2015). *In vivo* data supports that most ADAMTS are produced by astrocytes, especially after injury, although ADAMTS are also produced by neurons and microglia (Lemarchant et al. 2013).

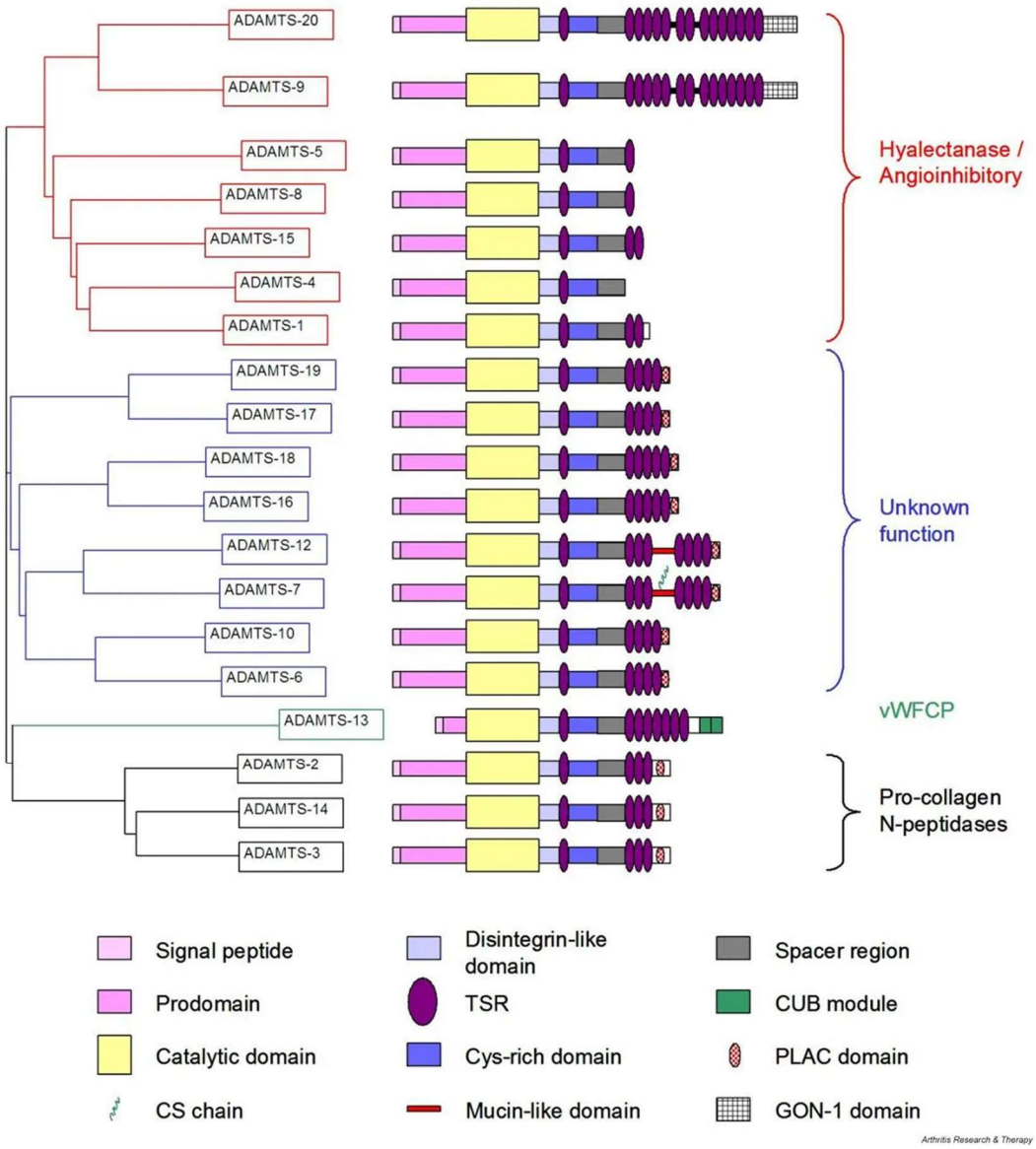


Figure 1.9. The ADAMTS (A Disintegrin And Metalloproteinase with Thrombospondin motifs) family. The basic domain organization of the 19 ADAMTS family members and their major functional groups. Structurally the ADAMTS members are broadly organized into a proteinase domain and an ancillary domain. The proteinase domain comprises the signal, pro, metalloproteinase and disintegrin-like domains. The greatest variability between ADAMTS members is found in the ancillary domain, which is composed of one or more thrombospondin type 1 sequence repeats (TSRs), a cysteine-rich domain and a spacer domain. Some family members also have one or more specialist domains as part of their ancillary domain. The diagram is drawn to scale. CS, chondroitin sulphate; CUB, complement C1r/C1s, Uegf (epidermal growth factor-related sea urchin protein) and BMP-1 (bone morphogenic protein-1); PLAC, protease and lacunin; TSR, thrombospondin I-like repeat; vWFCP, von Willebrand factor-cleaving protease. *Adapted from Jones GC and Riley GP, Arthritis Res Ther. (2005).*

1.5.2. ADAMTS family in the CNS and its possible role in psychotic disorders.

The specific role ADAMTS proteases play in physiological and pathological nervous system functions remains under investigation. Studies have predominantly focused on ADAMTS family members that can cleave lecticans. There is a substantive data that demonstrates that ADAMTS cleavage of brevican may facilitate the invasiveness of the glial tumors (Nakada et al. 2005). Several studies indicate that the ADAMTS are involved in recovery from spinal cord injury, where leads to a glial scar that is rich in growth inhibitory molecules, which diminishes functional recovery (Demircan et al. 2013). In studies both within and outside the CNS, ADAMTS proteases have been identified as having inflammatory and anti-angiogenic actions (Lemarchant et al. 2013). In addition, because several ADAMTS can cleave versican deposited in the vasculature, it is possible that these proteins, may contribute to the opening of the blood brain barrier after spinal cord injury or ischemic stroke (Lemarchant et al. 2013). Also, it was hypothesized that increased proteolytic activity of ADAMTS13 regulates blood-brain barrier (BBB) permeability to reduce secondary inflammation after spinal cord injury (SCI) (Gurses et al. 2016).

Indeed, increasing evidence suggests that ADAMTS may play a role in neuroplasticity. Alterations in the expression of these proteases could be involved in the overexpression of ECM proteins, such as versican which was associated with altered inhibitory neurotransmission in hippocampus. The majority of data suggest that the ADAMTS are involved in the activation of plasticity mechanism, possibly on neurites and synapses (Gottschall and Howell 2015). In fact, these proteins have a great deal of speculative potential toward their role in neural and synaptic plasticity, and potential for repair mechanism that involves turnover of the ECM proteins (Gottschall et al. 2005).

1.5.3. 'A disintegrin and metalloproteinase with thrombospondin motif 2' (*ADAMTS2)* gene

ADAMTS2 gene is located on chromosome 5q35.3 and encodes the enzyme 'A disintegrin and metalloproteinase with thrombospondin motif 2', also known as procollagen I N-proteinase (PC I-NP)(Tang and Hong 1999). It is a metalloproteinase of the ADAMTS family that contains, among other features, a Zn2þ-binding catalytic site required for its procollagen processing activity, and four thrombospondin type I repeats (TSPI) most probably crucial for the recognition of the substrate and the 3D-structure of the protein (**Figure 1.9.**) (Jones and Riley 2005; Kelwick et al. 2015). Alternative splicing results in multiple transcript variants, at least one of which encodes an isoform that is proteolytically processed. This enzyme is secreted to ECM and immobilized at the cells surface where is responsible for processing a number of types of procollagen proteins type I and II (precursor of collagen type I and II, does not act on type III collagen) (Lapiere et al. 1971). Its function consists to cleave a short chain of amino acids at the procollagen extreme, been essential to collagen molecules to normal function and assembly into fibrils outside cells (Bekhouche and Colige 2015). ADAMTS2 protein is synthesized as an inactive proenzyme which is activated by cleavage between the prodomain and the metalloproteinase domain (Colige et al. 2005) and its activity require a neutral to slightly basic pH and the presence of zinc and calcium ions (Colige et al. 1995). This activity can be also controlled by other mechanism such as clearing by internalization into cells or co-localization with their substrates (Bekhouche and Colige 2015).

Mutations in this gene cause 'Ehlers-Danlos syndrome type VIIC' (also known as Dermatosparaxis; OMIM: 225410), an autosomal recessive disorder of connective-tissue resulting from a defect in the processing of type I procollagen to collagen, with accumulation in most tissues of molecules that retain the amino-terminal propeptide (Colige et al. 2004). These precursor molecules self-assemble into abnormal ribbon-like fibrils that fail to provide normal tensile strength to tissues (severe skin fragility) (Colige

et al. 2004). Genome-wide association studies (GWAS) have been associated *ADAMTS2* polymorphism in the predisposition to paediatric stroke (Arning et al. 2012), obesity (Fox et al. 2012) and attention deficit hyperactivity disorder (ADHD) (Lasky-Su et al. 2008).

ADAMTS2 gene is expressed in a number of healthy tissues in human and mouse organism (https://www.proteinatlas.org), mainly expressed by fibroblast and cells of mesenchymal origin. Its expression alterations have been related with some pathogenic conditions. *ADAMTS2* gene deficiency in mice has been shown to be associated with male infertility (Dubail and Apte 2015). However, the role of *ADAMTS2* in the heart has not yet been defined, up-regulated expression of *ADAMTS2* have been observed with acute myocardial infarction in human hearts and hypertrophic murine hearts (Lee et al. 2012; Wang et al. 2017). Also, several studies related ADAMTS2 with cancer. *ADAMTS2* has been shown to have antiangiogenic and anti-proliferative properties in endothelial cell cultures and in a choroidal neovascularization *in vivo* model (Dubail et al. 2010). Furthermore, it has been reported that transforming growth factor-beta (TGFβ) induces secretion of activated *ADAMTS2* (Wang et al. 2003). In contrast, some studies have been indicated that *ADAMTS2* up-regulation was relevant to poor prognosis in gastric cancer tumour (Jiang et al. 2019). A puzzling observation about *ADAMTS2* gene was the description of its overexpression by macrophages and peripheral blood monocytes stimulated by glucocorticoids (Hofer et al. 2008). As these cells have little chance to be implicated in procollagen processing, it means that other functions or substrates should be involved.

In CNS, ADAMTS2 protein and mRNA expression can be detected in "healthy" human and mouse brain samples (https://www.proteinatlas.org). There is scarce information regarding specific *ADAMTS2* expression and activity in the CNS, being the first time that it was related with a mental disorder the recent work by our group (Crespo-Facorro et al. 2015; Sainz et al. 2013). In this way, this thesis aims to explore the potential role of this gene and its regulation in the context of the schizophrenia disorder.

HYPOTHESIS AND OBJECTIVES

2. HYPOTHESIS AND OBJECTIVES

2.1. Hypothesis.

Schizophrenia is a neuropsychiatric disorder whose origin can be explained by genomic and mechanistic alterations that regulate important neuronal networks and signalling pathways at CNS and peripheral cells. These alterations can be associated with different stages of the disease and the heterogeneity in clinical response to antipsychotic treatment.

We hypothesized that a number of genes altered at onset and regulated after treatment could be related with the response to antipsychotic drugs. Also, these gene expression alterations might be mediated by molecular and cellular mechanisms at CNS which could elucidate new treatment targets.

2.2. Objectives.

The aim of this thesis is to dissect essential mechanisms that participate in the biology of the schizophrenia with potential for diagnosis and treatment. Also, to understand novel physiological aspects of disease.

This thesis focuses on three specific objectives:

1. Study the CRGAs expression profile in multiple experimental settings/models:

    1.1. To validate of differential CRGAs expression associated with different stages of schizophrenia using an independent cohort of patients.

    1.2. To study the transcriptional regulation of CRGAs in response to selective receptor agonist/antagonist and antipsychotic drugs in neuronal-derived cells.

    1.3. To analyse the CRGAs expression at different development stages in healthy mice brains and in a schizophrenia-like behavioral mice model brain.

2. To elucidate the intracellular signalling and mechanisms involved in *ADAMTS2* gene expression downstream the receptors targeted by antipsychotic drugs.

3. To investigate the role of CREB and D_1-class signalling in the *ADAMTS2* gene expression regulation.

MATERIAL AND METHODS

3. MATERIAL AND METHODS

3.1. Human samples.

3.1.1. Ethical aspects in human samples.

Conforming to international standards for research ethics, the research in this thesis was included in two research projects funded by 'Ministerio de Economía e Innovación' (SAF2013-46292-R and SAF2016-76046-R) which were also evaluated and approved by the Cantabria Ethics Institutional Review Board. Patients and their families provided a written informed consent to be included in the study, which conformed to international standards for research ethics and was approved by the local institutional review board.

The Mental Health Services of Cantabria provided the funding for implementing the programme. No pharmaceutical company supplied any financial support for it. The programme was designed and directed by Dr. Benedicto Crespo-Facorro.

3.1.2. Study settings (PAFIP program).

3.1.2.1. Reference population.

The area investigated was the autonomous community of Cantabria, located in the Northern coast of Spain. Cantabria has a population of 580229 inhabitants (2018), of whom 172044 live in the capital city of Santander. The unemployment rate is 12.2% (11.3% for males and 13.3% for females in March 2019). Of the employed population, 60% work in services, 32% in industry, and 8% in the primary sector. Although the rate of immigration is rising very rapidly in Spain, in this community, the proportion of immigrants is still very low (1.28%). It is estimated that more than 95% of the population of Cantabria is white European.

Cantabria has an autonomous public health system administered by the autonomous government with a free and universal coverage for the whole population. The psychiatric

department, located at the University Hospital 'Marqués de Valdecilla', provides the only acute psychiatric inpatient and 24h emergency care units for the whole autonomous community. In addition, there are five community mental health outpatient clinics in Cantabria, which maintain a close relationship with the academic department of psychiatry at the university hospital.

3.1.2.2. Clinical Program on Early Phases of non-affective Psychosis of Cantabria (PAFIP).

The data and biological samples from patients for these studies were obtained from an ongoing epidemiological and longitudinal intervention program of FEP (PAFIP, its Spanish acronym for 'Programa Asistencial de las Fases Iniciales de Psicosis'), carried out at the University Hospital 'Marqués de Valdecilla', and biological samples were provided by the IDIVAL biobank. An information campaign within all mental health outpatient units in Cantabria and family physicians was thoroughly carried out during a period of 3 months prior to starting the programme in order to increase referrals. Referrals to the PAFIP came from the inpatient unit and the accident and emergency department at the University Hospital 'Marqués de Valdecilla', from mental health units, and from other health-care workers throughout the entire region of Cantabria. After the initial contact by a qualified psychiatric nurse, an experienced psychiatrist carries out a formal interview for a full assessment of the patient and to confirm the presence of a non-affective psychotic disorder. As a clinical programme, the PAFIP includes inpatient and outpatient care, and provides specific and personalized clinical attention, including psychotherapeutic and psychopharmacological treatment of patients and also family interventions, from the onset of the illness up to 3 years. To guarantee the inclusion of all first episodes, regular meetings with the mental health and primary health-care centres are maintained.

3.1.3. Patients.

All referrals to PAFIP were screened for patients who met the following criteria: 1) 15–60 years; 2) living in the catchment area (Cantabria); 3) experiencing their first episode of psychosis; 4) no prior treatment with antipsychotic medication or, if previously treated, a total lifetime of adequate antipsychotic treatment of less than 6 weeks; and 5) DSM-IV criteria for schizophrenia, schizophreniform disorder, schizoaffective disorder, or brief psychotic disorder.

Patients were excluded for any of the following reasons: 1) meeting DSM-IV criteria for drug dependence; 2) meeting DSM-IV criteria for mental retardation; or 3) having a history of neurological disease or head injury. The diagnoses were confirmed using the Structured Clinical Interview for DSM-IV, carried out by an experienced psychiatrist 6 months after the baseline visit.

Our operational definition for a first episode of psychosis included individuals with a non-affective psychosis (meeting the inclusion criteria defined above) who have not previously received antipsychotic treatment regardless of the duration of psychosis.

All patients included in the present investigation underwent a head-to-head risperidone and aripiprazole randomized (simple randomization procedure), flexible-dose, open-label study (EudraCT number 2013-005399-16). At the present work, we collect three different cohorts for different purposes: (1) a cohort #1 with an independent set of 30 drug-naïve first episode non-affective individuals and a set of 10 healthy individuals (without a history of neuropsychiatric disorders) was used as a control group; (2) a cohort #2 with a set of 9 drug-naïve first episode non-affective individuals and a set of 10 healthy individuals; and (3) finally, a cohort #3 with two drug-naïve first episode non-affective individuals and two controls (still uncharacterized at the publication of this Thesis).

3.1.4. Premorbid, sociodemographic variables and blood sample collection.

Premorbid and sociodemographic information was collected from patients, relatives and previous medical records (cohort #1: **table 3.1.**; cohort #2: **table 3.2.**). Age of onset of psychosis was defined as the age when the first continuous (present most of the time) psychotic symptom emerged. Duration of untreated illness was defined as the time from the first unspecific symptoms related to psychosis (for such a symptom to be considered, there could be no return to the previous, stable level of functioning) to initiation of adequate antipsychotic drug treatment. Duration of untreated psychosis was defined as the time from the first continuous (present most of the time) psychotic symptom to initiation of adequate antipsychotic drug treatment. Information about alcohol, cannabis, and tobacco consumption was converted into binary variables and coded for either the presence or absence of use.

3.1.5. Study design.

This is a prospective, randomized, flexible-dose, open-label study. We used a simple randomization procedure. At study intake, all patients were antipsychotic-naïve and were randomized to aripiprazole and risperidone. Dose ranges were 5–30 mg/day aripiprazole and 2–6 mg/day risperidone. A rapid titration schedule (5 days) was used until the optimal dose was reached, unless severe side effects occurred. At the treating physician´s discretion, the dose and type of antipsychotic medication could be changed based on clinical efficacy and the profile of side effects during the follow-up period. Antimuscarinic medication, lormetazepam, and clonazepam were permitted for clinical reasons. No antimuscarinic agents were administered prophylactically. Antidepressants and mood stabilizers were permitted if clinically needed.

At the 3-month follow-up, patients were also on aripiprazole and risperidone. The mean daily dose of antipsychotics during follow-up was 181.72 (93.97) mg/day of

Table 3.1. Demographic and clinical characteristics of the cohort 1 of drug-naïve patients with a first episode of psychosis.

Cohort #1	Patient Cohort N= 30		Controls N= 10		Total N= 40			
	Mean	SD	Mean	SD	Mean	SD	Statistic	p
Age at admission, y	33.71	11.11	32.95	6.31	33.52	10.05	t = 0.27	0.790
Age at psychosis onset, y	33.34	11.23			33.34	11.23		
	N	%	N	%	N	%	Statistic	p
Diagnosis								
Schizophrenia	12	40			12	40		
Brief psychotic disorder	4	13.3			4	13.3		
Unspecified psychotic disorder	3	10			3	10		
Schizophreniform disorder	11	36.7			11	36.7		
Sex (male)	17	56.7	5	50	22	55.0	Fisher = 0.14	0.731
Race (white)	26	86.7	10	100	36	90	Fisher = 0.40	0.062
Tobacco use (yes)	21	75	4	40	25	65.8	Fisher = 3.66	0.070
Cannabis use (yes)	13	43.3	1	10	14	35	Fisher = 1.48	0.556
Cocaine use (yes)	4	13.3	0	0	4	10	Fisher = 5.53	0.037
Alcohol use (yes)	11	39.3	0	0	11	28.9	Fisher = 1.48	0.556

Table 3.2. Demographic and clinical characteristics of the cohort 2 of drug-naïve patients with a first episode of psychosis. [a] Wilcoxon Singed-Rank test for independent data; [b] Chi-Square test; [c] Fisher's Exact test.

Cohort #2	Patient Cohort N= 12		Controls N= 10		Total N= 22			
	Mean	SD	Mean	SD	Mean	SD	Statistic	p
Age, y	31.38	9.08	32.95	6.31	32.08	7.81	z= 19.0[a]	0.937
Age at psychosis onset, y	30.82	8.92			30.82	8.92		
	N	%	N	%	N	%	Statistic	p
Diagnosis								
Schizophrenia	3	25			3	25		
Brief psychotic disorder	1	8.3			1	8.3		
Schizophreniform disorder	8	66.7			8	66.7		
Sex (male)	7	58.3	5	50	12	54.5	Fisher= 0.34[b]	1.0
Race (white)	11	91.7	10	100	21	95.5	Fisher= 1.09[b]	1.0
Tobacco use (yes)	6	50	4	40	10	45.5	Fisher= 0.0[c]	1.0
Cannabis use (yes)	3	25	1	10	4	18.2	Fisher= 0.44[c]	1.0
Cocaine use (yes)	1	8.3	0	0	1	4.5	Fisher= 1.09[c]	1.0
Alcohol use (yes)	6	50	0	0	6	27.3	Fisher= 1.33[c]	0.567

chlorpormazine-equivalent doses. The mean daily dose of antipsychotics at 3 months was 163.64 (86.47) mg/day of chlorpormazine-equivalent doses.

3.1.6. Clinical assessment.

The severity of the Clinical Global Impression (CGI; 3) scale, the Brief Psychiatric Rating Scale (BPRS; expanded version of 24 items), the Scale for the Assessment of Positive symptoms (SAPS), the Scale for the Assessment of Negative symptoms (SANS), the Calgary Depression Scale for Schizophrenia (CDSS) and the Young Mania Rating Scale (YMRS) were used to evaluate clinical symptomatology. The same trained psychiatrist (Dr Crespo-Facorro) completed all clinical assessments. The severity of symptoms at baseline and 3/12 months and the percentage of change are reported below in results section (a reduction in total scores means clinical improvement).

The analysis of clinical efficacy reveals that there was a significant improvement of general psychopathology (assessed by the BPRS total score change at 3/12 months) and positive symptoms (hallucinations, delusions, positive formal thought disorders, bizarre behaviour; assessed by SAPS total score changes at 3/12 months). The patients were defined as responders to the optimum dose of antipsychotic at 3 months if there was a >50% reduction of the BPRS total score compared to intake. The rate of responders at 3 months was 95.55%.

3.1.7. Laboratory Assessments.

Blood samples were assessed for biochemical and haematological parameters. In order to minimize the effects of diet and technique, blood samples were obtained from fasting subjects from 8:00 to 10:00 a.m. by the same medical staff, in the same setting. None of the patients had a chronic inflammation or infection or were taking medication that could directly influence the results of blood tests.

3.1.8. PBMCs isolation from human peripheral blood.

PBMCs from peripheral blood of SCZ patients and healthy controls were used for quantifying the expression of specific genes at the mRNA level (RT-qPCR) or chromatin immunoprecipitation (ChIP) (see methodology below).

Peripheral blood from patients and healthy controls were previously isolated using the Tempus™ Blood RNA Tube. For the PBMCs isolation, a Ficoll-based isolation was performed using Ficoll-Paque Premium reagent (Sigma, MO, USA), following the manufacturer's instructions. Briefly, in a 15 ml tube with ficoll solution at room temperature, blood was added carefully through walls of the tube preventing them from mixing (2 ml of ficoll per 4 ml of blood). Then, tube was centrifuged at 1800 rpm (without brake) at room temperature for 20 min. After centrifugation, four phases were observed: plasma (yellow upper phase), PBMCs (interphase), ficoll (transparent phase) and erythrocytes (downer phase). With a Pasteur pipette, PBMCs were collected in a new tube and washed twice with 1X PBS, centrifuged 1500 rpm for 10 min at 4 ºC and finally, cell pellet was resuspended in 10 ml of 1X PBS. Finally, cells were proceeded directly in function of the technique.

3.2. Mice samples for in vivo studies.

3.2.1. Ethical aspects in mice samples.

All experiments were performed in compliance with the Spanish/European Union and United States laws on animal care in experimentation. Animals were fed with food and water *ad libitum*, and maintained in laminar flow racks under pathogen-free conditions for 12 h light and 12 h darkness. The specific regional institutional Animal Use and Care committee approved all experimental procedures, in each approach showed in this thesis (Virginia Commonwealth University and Miguel Hernández University, respectively).

3.2.2. Schizophrenia-like mice for gene expression studies in mice prefrontal cortex (PFC).

In order to preclinically evaluate the CRGAs expression in brain, we proceeded to evaluate the expression of the orthologous version of these genes in PFC of adult mice born to mothers submitted to influenza viral infection during gestation. Human epidemiological studies highlight a direct association between infections during early pregnancy and schizophrenia: influenza virus (Brown et al. 2004), rubella virus (Brown et al. 2001), genital and reproductive infections (Babulas et al. 2006), and bacterial infection (Sorensen et al. 2009).

Pregnant CD1 mice were subjected to influenza viral infection in collaboration with Dr. Javier González-Maeso group (Virginia Commonwealth University, USA), according to the protocol previously described (Moreno et al. 2011) and adult offspring were assessed for behavioural and neurochemical traits corresponding to schizophrenia-like phenotype, essentially increased head-twitch response elicited by treatment with the hallucinogenic drug DOI and changes in the relative 5-HT_{2A} and mGlu2 receptor expression levels detected by *ex vivo* radioligand binding assay. Briefly, on day 9.5 of pregnancy (equates to the end of the first trimester of human pregnancy), CD1 mice were infected intranasal with 103 pfu of influenza A/WSN/33 (H1N1) virus and then, the presence of influenza virus-specific antibodies in mice sera was detected by ELISA (described in (Tumpey et al. 2005)). Offspring were separated from their mothers after 3 weeks. Subsequent experiments were performed in adult mice (10-12 weeks). To induce different behavioural response in mice, doses of DOI (0.5 mg/kg) (Sigma, MO, USA), LY379268 (5 mg/kg) (Sigma), and MK-801 (0.5 mg/mg) (Tocris, UK) were injected intraperitoneally in mice and 15 min after were Head-twitch behavioural response tested. Videotapes were scored for head twitches by an experienced observer.

A group of eight animals that statistically presented these alterations were pair matched with control mice. In both experimental groups, we tested animals originating from at least two different litters. Tissue samples from mouse frontal cortex were obtained and mRNA was extracted to conduct subsequent quantitative real-time PCR (RT-qPCR) experiments as is described below.

3.2.3. Mice for protein expression studies in brain.

In order to detect the expression and the localization of the orthologous version of Adamts2 protein in mice brains by immunostaining, we used C57BL/6 wild-type mice in collaboration with Dr. Salvador Martinez group (Miguel Hernández University, Spain).

3.3. Human cell culture for *in vitro* studies.

3.3.1. Cell culture and maintenance.

Human neuroblastoma SK-N-SH (HTB-11) and HEK-293T (CRL-3216) cells were obtained from the American Type Cell Collection (ATCC, Rockville, MD). Cells were cultured in Modified Eagle's medium (Corning, United States) supplemented with 10% dialyzed heat-inactivated fetal bovine serum (HyClone, United States) or heat-inactivated fetal bovine serum (Gibco, Germany), respectively. Also with glucose (4.5 g/L), L-glutamine (292 mg/L), streptomycin sulphate (10 mg/L) and potassium penicillin (10000 U/L) (Lonza, United States).

SK-N-SH cells transfected with shRNA constructs were selected and maintained with 1 µg/ml Puromycin (Sigma). GFP and shRNA expression in these cells were induced by incubation with 1 µg/ml doxycycline (Sigma) in water for 72 h.

All cells were maintained exponentially growing in a humidified atmosphere at 37 ºC and 5% CO_2.

3.3.2. Drugs and pharmacological agents.

SKF 83822 hydrobromide, 7-Hydroxy-DPAT hydrobromide, (R)-(+)-8-Hydroxy-DPAT hydrobromide and TCB-2 were used in different concentrations (1 and 10 μM) and conditions as selective agonist of D_1, D_2, 5-HT_{1A} and 5-HT_{2A} receptors. SCH 39166 hydrobromide, L-741,626, MDL 100907 and WAY 100635 maleate were used at 1 μM as selective antagonists of D_1, D_2, 5-HT_{1A} and 5-HT_{2A} receptors, respectively. Haloperidol hydrochloride (1 μM), Clozapine (1 μM), Paliperidone (1 μM), Risperidone (10 μM) and Aripiprazole (1 μM) antipsychotics were used in different concentrations and conditions in this study.

Selumetinib (AZD6244) was used at 1 and 10 μM in order to inhibit MEK/ERK signalling. H 89 dihydrochloride and PKI 14-22 amide were used at 10 μM as selective inhibitors of the PKA.

TPA was used at 10 ng/ml to induce MAPK/ERK signalling activation. Forskolin was used at 1 and 10 μM to induce PKA activation.

Cholera toxin (CTX) was used at 100 ng/ml in order to activate $G_{\alpha s}$ subunit, pertussis toxin (PTX) was used at 50 ng/ml to inhibit $G_{\alpha i}$ subunit and YM-254890 was used at 1 μM as specific inhibitor of $G_{\alpha q}$ subunit.

Stock preparation: all drug and reagents used were dissolved in DMSO (VWR, Germany) to a final concentration of 10 mM, except TPA that was dissolved to a final concentration of 10 μg/ml. Stock solutions for agonist and antagonist

Table 3.3. provides a list of the drugs used in this study and their related information.

Table 3.3. Molecules and drugs used in this thesis work.

Reagent	Reference	Company	PubChem ID	Concentration	Vehicle	MW
(R)-(+)-8-Hydroxy-DPAT hydrobromide	1080	TOCRIS	11957570	1 µM	DMSO/Water	328,29
7-Hydroxy-DPAT hydrobromide	0706	TOCRIS	11957566	1 µM	DMSO	328,28
Aripiprazole	5584	TOCRIS	60795	1 µM	DMSO	448,39
Cholera toxin	C8052	Sigma		100 ng/ml	Water	
Clozapine	0444	TOCRIS	2818	1 µM	DMSO/EtOH	326,83
Forskolin	S2449	Selleckchem		10 µM	DMSO	410.5
H 89 dihydrochloride	2910	TOCRIS	5702541	10 µM	DMSO	519.28
Haloperidol hydrochloride	0931	TOCRIS	11495267	1 µM	DMSO/EtOH	412,33
L-741,626	1003	TOCRIS	133633	1 µM	DMSO	340.85
MDL 100907	4173	TOCRIS	5311271	1 µM	DMSO	373.46
Paliperidone	4493	TOCRIS	115237	1 µM	DMSO	426,48
Pertussis toxin	P2980	Sigma		50 ng/ml	DMSO	
PKI 14-22 amide, myristoylated	2546	TOCRIS	71312213	10 µM	DMSO	1209.5
Risperidone	2865	TOCRIS	5073	1 µM	DMSO/EtOH	414.98
SCH 39166 hydrobromide	2299	TOCRIS	16759171	1 µM	DMSO	394.73
Selumetinib (AZD6244)	S1008	Selleckchem		1 µM	DMSO	457.68
SKF 83822 hydrobromide	2075	TOCRIS	12909789	1, 10 µM	DMSO/EtOH	424.76
TPA	P1585	Sigma	24898219	10 ng/ml	DMSO	616.83
WAY 100635 maleate	4380	TOCRIS	11957721	1 µM	Water	538.64
YM-254890	AG-CN2-0509	Adipogen life sciences		1 µM	DMSO	960.1

3.3.3. Transfections.

Lipofectamine LTX with PLUS reagents (Invitrogen, CA, USA) was used SK-N-SH cells transfection. The day before transfection, cells were seed at 20-30% of confluence in 6-wells culture plates. The day of transfection 0.5 µg of DNA was diluted in 300 µl of MEM medium with 0.5 µl of PLUS reagent, mixed by vortexing and incubated for 5 min at RT. Then, 1.5 µl of Lipofectamine reagent was added to the dilution, mixed by vortexing again and incubated for at least 30 min at RT. Meanwhile media was removed to the cells. Finally, dilution was added to the cells and incubated for 3 h. After that, new complete medium was added to the cells.

In case of select stably cell lines, 48 h post transfection, medium was removed, and new medium was added with 1 µg/ml puromycin.

The expression vectors used for transfection are shown in **Table 3.4.**

Table 3.4. DNA constructions used in this thesis work.

Plasmid	Construct	Resistance	Reference	Origin
pGFPt-LV-hEF1 shCREB1	Lentiviral SMARTvector Inducible Human CREB1 shRNA, hEF1 + turboGFP	Ampicillin/ Puromycin	V3SH11252	Dharmacon
pGFPt-LV-hEF1 NTC	Lentiviral SMARTvector Inducible Human Non-targeting Control, hEF1 + turboGFP	Ampicillin/ Puromycin	VSC11653	Dharmacon
pGFP-V-RS shCREB1	Retroviral GFP vector Human 29mer shRNA construct	Kanamycin/ Puromycin	TG313740	Origene
pGFP-V-RS shCREB1	Retroviral GFP vector Human Non-effective 29-mer scrambled shRNA cassette	Kanamycin/ Puromycin	TR30013	Origene
pGL4.29 [luc2P/CRE/Hygro]	Firefly sp. luciferase reporter gene regulated with cAMP response element (CRE) site	Ampicillin/ Hygromycin	E8471	Promega
pRL-Null	Renilla sp. Luciferase reporter gene regulated by the T7 promoter	Ampicillin/ Hygromycin	E2231	Promega

3.3.4. Lentivirus production and infection.

a. Lentivirus production:

Lentivirus particles were produced to transduce SK-N-SH cells with inducible short hairpin (sh) sequences constructs, used to produce siRNA against human *CREB1* mRNA. SMARTvector carrying tGFP and human inducible *CREB1* shRNA mCMV constructs or non-target control (NTC) shRNA were used in this study (Dharmacon, CO, USA). See **table 3.4.** for more information about DNA constructs.

Lentiviral particles were produced by cotransfection of 293T cells using Trans-Lentiviral shRNA Packaging System (Dharmacon), according to the manufacture's protocol. The day

before transfection, 293T cells were seeded at 70% of confluence in 10 cm^2 culture plates. The day of transfection, 42 µg of each DNA construct and 30 µl de Trans-lentiviral pack mix were diluted in 1 ml of water. After, the following reagents were added sequentially: 105 µl $CaCl_2$, 1050 µl HBSS 2X solution, in a drop wise manner After an incubation for 3 min at RT, the dilution was incorporated to the plate with cells. Cells were incubated maximum 16 h with this mixture. Then, medium was removed and replaced with new MEM medium with 5% FBS and 1% P/S.

After two days after transfection, medium containing lentivirus was collected, centrifuged for 10 min at 1600 g (4 ºC) and filtered through 0.45 µm pore size sterile syringe filters (Merck Millipore, Ireland). Finally, lentivirus solution was conserved at -80 ºC.

b. Cell transduction:

For cell transduction, cells were seeded at 20% of confluence in 6-well culture plates. Next day, culture medium was removed and added new mix with 8 µg/ml polybrene, 1 ml of medium containing lentiviral particles and 1 ml of complete MEM medium. 48 h post transduction, medium was removed, and new medium was added with 1 µg/ml puromycin to select stably cell lines. After 7 days post-selection, cells were harvested and checked for short hairpin sequence construct acquisition and functionality by RT-qPCR and western blot.

3.3.5. Cell viability assays

To study the effect of the APDs over the SK-N-SH cells viability, we took advantage of the CellTiter-Glo Luminescent Cell Viability Assay kit (Promega, WI, USA), following the manufacturer's instructions. This method enables the identification of viable cells in culture based on quantitation of the free-ATP present as indicator of metabolically active cells. Cells were seeded in T96 well plates in growing conditions (2000 cells/well), and then incubated with different concentration of the APD for 48 h. Colorimetric changes

were quantified using the Spark Multimode Microplate Reader (Tecan Trading AG, Switzerland).

3.4. DNA and RNA analysis.

3.4.1. Bacterial transformation and plasmid DNA purification.

Plasmid DNA was transformed using heat-shock method into E. coli DH5α competent cells (Invitrogen) for DNA amplification.

Thus, 50 µl E. coli DH5α cells were mixed with 1 µg of plasmid DNA. The mixture was incubated in ice for 5 min. After that, a heat shock of 40 sec at 42 ºC was performed followed by other 2 min incubation in ice. Transformed E. coli DH5α cells were then supplemented with 1 ml SOC medium (Invitrogen) containing no antibiotics and incubated in a shaking incubator at 37 ºC 200 rpm for 1 h, allowing the antibiotic resistant gene to be expressed. Then, E. coli cells were spread on LB-agar growth media plates containing the corresponding antibiotic selection (100 µg/ml ampicillin or 50 µg/ml kanamycin depending on the case) and incubated overnight at 37 ºC.

The following day, three selected single colonies were both seeded again on LB-agar growth media "master" plates containing the corresponding antibiotic and incubated overnight at 37 ºC. Then, some cells from these separated single colonies were inoculated in 5 ml LB growth media bottles containing the corresponding antibiotic selection in an orbital shaking incubator at 37 ºC 150 rpm to let them grow separately overnight. Next day, the bacterial culture was harvested by centrifugation at 3000 rpm for 15 min (4 ºC) and plasmid DNA was purified with the QIAamp DNA Mini kit (Qiagen, Germany) following manufacturer's instructions.

For preparation of glycerol stocks of the verified DNA plasmid-transformed bacteria, positive single colonies that were seeded on LB-agar "master" plates were inoculated and let them grow overnight in 5 ml LB growth media containing the corresponding antibiotic selection in an orbital shaking incubator at 37 ºC 150 rpm. Next day, LB media grown cells were centrifuged at 3000 rpm for 10 min, the supernatant was discarded. Bacteria pellet was resuspended 1ml of LB-glycerol 1:1 (v/v), added to a cryotube and conserved at -80 ºC.

To prepare higher amounts of plasmids DNA for transfections, a little amount of the glycerol stock of the desire plasmid was inoculated into 5 ml LB growth medium containing the appropriate antibiotic and incubated for 8 h in an orbital shaking incubator at 37 ºC and 150 rpm. Then, the starter culture was used to inoculate 200 ml LB growth media containing the same selection antibiotic and grown overnight in the same culture conditions (37 ºC and 150 rpm). The following day, bacterial culture was centrifuged at 6000 g for 15 min and plasmid DNA was purified using the EndoFree Plasmid Maxi kit (Qiagen) following manufacturer's instructions. Plasmid DNA concentration was determined by measuring A_{260nm} using a microvolume espectrophotometer (NanoDrop 2000, Thermo Scientific, IL, USA).

3.4.2. RNA extraction and purification.

Extraction of human samples:

Total RNA from patients was extracted using the Tempus™ Spin RNA Isolation Kit (Applied Biosystems, UK) from PBMCs previously isolated from blood (see above), following the manufacturer's protocols. To define expression profiles, a key factor is that the RNA is intact. To select only RNA with good quality, the RNA Integrity Number (RIN) was characterized with a Bioanalyzer (Agilent Technologies, CA, USA) and samples with a RIN of at least 7.2 were selected. The selected samples have RINs that range from 7.2 to 9.8

with an average of 8.5. Overall, the rate of samples from patients with a RIN equal to or higher than 7.2 was slightly higher than 89%.

Extraction of tissue and cell line samples:

Total RNA extraction from human cell line and PFC-mice brains were performed using TRIzol reagent (Invitrogen). Adherent cells were lysed in the plate: growing media was removed by aspiration and 0.5 ml TRIzol was directly added to the plate (It is recommended 1 ml of TRIzol for a total amount of $5 \cdot 10^6$ cells). After 5 min of incubation at room temperature, cells were scratched from the plate and the homogenized sample was collected in a 1.5 ml tube. Otherwise, PFC-mice brains were lysed in 1 ml TRIzol and homogenize using a homogenizer (VWR) (It is recommended 1 mL of TRIzol reagent per 50–100 mg of tissue). Lysate can be stored at 20 ºC at this point.

Next, 0.2 ml chloroform per 1 mL TRIzol was added and vigorously mixed for 15 s. TRIzol-chloroform mixture was incubated 2-3 min at room temperature and then centrifuged 12000 g for 15 min at 4 ºC. The upper aqueous phase was collected and transferred to a new 1.5 mL tube. Then, 0.3 mL of 100% 2-propanol was added, mixed by inversion and incubated for 10 min at room temperature in order to precipitate the RNA. After this, it was centrifuged 12000 g for 10 min at 4 ºC. Supernatant was discarded and the RNA pellet was washed once with 0.5 mL of 75% ice-cold ethanol by vortexing and then centrifuged at 7500 g 5 min at 4 ºC. The ethanol was discarded, and the RNA pellet was resuspended in a final volume from 20-50 µl of RNAse-free water (depending on the RNA pellet observed). Finally, RNA resuspended was incubated in a heat block set at 55 ºC for 10 min to homogenise and then stored at -80 ºC.

RNA concentration was determined by measuring A_{260nm} using a microvolume spectrophotometer (Nanodrop 2000, Thermo Scientific). RNA integrity was checked by subjecting samples to electrophoresis separation in a conventional agarose gel. Low-

Agarose (Conda, Spain) was melting in 1X TAE buffer[1] at 1% (w/v) and, prior to gel casting, "Real Safe Dye" (Real laboratory, Spain) was added (dilution 1:20000). RNA samples were prepared as following: 0.2-1 µg of RNA was diluted in 5 µl RNAse-free water and 5 µl of RNA loading buffer (Thermo Scientific), heated for 10 min at 65 ºC, loaded into a gel and run for 20 min at 100 V in 1X TAE buffer. Finally, resulting separation was visualized in an UV-transilluminator and recorded using a Gel-Doc EZImager (Bio-Rad, CA, USA). Two bands corresponding to the 28S and 18S rRNA subunits should be observed.

3.4.3. Reverse transcription and quantitative polymerase chain reaction (RT-qPCR).

In order to analyse the expression of specific genes at the mRNA level, quantitative reverse transcription PCR (RT-qPCR) was performed.

First, reverse transcription (RT) was performed using total extracted RNA from mammalian cells (human SCZ patients, SK-N-SH cells and PFC-mice brains). Complementary DNA (cDNA) was obtained from 1 µg of total RNA as template using the SuperScript IV reverse transcriptase and random primers approach (Invitrogen), following the manufacturer's instruction in a total volume of 20 µl. Reaction was divided in a preincubation at 65 ºC for 5 min, and an incubation with RTase as follows: 10 min, 23 ºC//10 min, 55 ºC//10 min, 80 ºC. The obtained cDNA was stored at -20 ºC until used.

To quantify the levels of a specific mRNA in an experimental condition, cDNA was amplified by SYBR Green dye-based quantitative PCR using specific primers for the gene of interest. Primers were designed using the online PrimerBlast (NCBI; https://www.ncbi.nlm.nih.gov/tools/primer-blast/) software tool, according to PCR standard guidelines: length 18 to 25 bp, GC content 40 to 65%, no G at the 5' end, no

[1] *TAE buffer: 400 mM Tris, 200 mM acetic acid, 10 mM EDTA ph 8; stock concentration 10X and pH 8.3; stored at room temperature.*

secondary structures, Tm= 50-65 ºC. To check correct design and theorical conditions of PCR, an in-silico assay was performed using the online UCSC In-Silico PCR tool (UCSC, https://genome.ucsc.edu/cgi-bin/hgPcr). PCR conditions and primers efficiency were experimentally determined depending on the nature and complexity of each sequence (all primers used in this thesis had an efficiency between 80-120%, following the recommendations described in (Pfaffl 2004)). Primer sequences and amplicon sizes used in RT-qPCR assays are shown in **table 3.5**.

The Power SYBR™ Green PCR Master Mix (Applied Biosystems) supplied in a 2X concentration premix (containing SYBR GreenER dye, AmpliTaq DNA polymerase, UP (Ultra-Pure) with a proprietary hot start mechanism, heat-labile Uracil-DNA glycosylase (UDG), ROX dye passive reference, dNTP blend containing dUTP/dTTP and optimized buffer components) was used to amplify the cDNA in a 7300 Fast Real-Time PCR System (Applied Biosystems). The qPCR reaction was prepared as follows: 5 µl of Power SYBR™ Green PCR Master Mix were mixed with 0.3 µM of each primer (forward and reverse; stock concentration 10 µM), 1 µl of cDNA and DEPC-water until 10 µl of final volume. Then, reaction mix was added to a 384-well PCR plate in triplicates. Reaction mix without cDNA was used as no template control (NTC) to detect possible amplification signals from contaminant DNA or primer dimers. The protocol used for amplification and real time melting curve was the following:

Time	Temperature	Cycle
10 min	95 ºC	Hold
15 s	95 ºC	x40 cycles
10 min	60 ºC	
15 s	95 ºC	Melt curve
15 s	60 ºC	(+0.5 ºC x80 cycles)
15 s	95 ºC	

Table 3.5. Primer sequences used for RT-qPCR in this thesis work.

Gene target	Gene ID	Ref Seq		Primer sequences	Amplicon size (bp)	Efficience	Origin/ Publication	
ACTB	60	NM_001101.4	Fw	AGTGTGACGTGGACATCCGCAAAG	163	N/D	Donated by N. Varela group	Human (*H. sapiens*)
			Rv	ATCCACATCTGCTGGAAGGTGGAC				
ADAMTS2	9509	NM_014244.4	Fw	CCTGACATCCTCAAACGGGA	114	1.98 (99.27%)	This thesis/ Ruso-Julve et al., 2019	
			Rv	GTGTGGGTTGTCACACTGGC				
CD177	57126	NM_020406.3	Fw	CATGTGTGGAAGGTGTCCGA	94	2.22 (111.29%)	This thesis/ Ruso-Julve et al., 2019	
			Rv	TGAGCATCAACGTGTCCTGG				
CNTNAP3	79937	NM_033655.3	Fw	CACACTTCCTGGGGAGGTTC	185	2.35 (117.28%)	This thesis/ Ruso-Julve et al., 2019	
			Rv	CCTGCGTCTGTCATCACAAT				
CREB1	1385	NM_001320793.1	Fw	CGAGAACCAGCAGAGTGGA	137	2.08 (104%)	This thesis/ Ruso-Julve et al., 2019	
			Rv	CGGTGGGAGCAGATGATGTT				
DRD1	1812	NM_000794.4	Fw	GACCTTGTCTGTACTCATCTCCT	118	2.39 (119.81%)	This thesis/ Ruso-Julve et al., 2019	
			Rv	GTCACAGTTGTCTATGGTCTCAG				
DRD2	1813	NM_000795.3	Fw	CCCCGCCAAACCAGAGAAG	89	2.19 (109.32%)	This thesis/ Ruso-Julve et al., 2019	
			Rv	TTTTGCCATTGGGCATGGTCT				
ENTPD2	954	NM_001246.3	Fw	GCCAGCACTACCGAGTCTAC	137	1.86 (92.81%)	This thesis/ Ruso-Julve et al., 2019	
			Rv	CAGCACTTGGGTGGAAAAGC				
FOS	2353	NM_005252.3	Fw	GCAGAGCATTGGCAGGAG	74	2.1 (105%)	This thesis/ Ruso-Julve et al., 2019	
			Rv	CCCTTCGGATTCTCCTTTTCT				
GAPDH	2597	NM_001289746.1	Fw	GAGTCAACGGATTTGGTCGT	238	N/D	Donated by A. Pazos group	
			Rv	TTGATTTTGGAGGGATCTCG				
HTR1A	3350	NM_000524.3	Fw	TGTATCAGGTGCTCAACAAGTG	214	2.31 (115.92%)	This thesis/ Ruso-Julve et al., 2019	
			Rv	AGGAAGCCAATAAGCCAAGTG				
HTR2A	3356	NM_000621.4	Fw	CTTTGTGCAGTCTGGATTTACCT	173	2.00 (100%)	This thesis/ Ruso-Julve et al., 2019	
			Rv	ACTGATATGGTCCAAACAGCAAT				
RFX2	5990	NM_000635.3	Fw	TTCGGCTCCTTCCACCTCAT	156	2.35 (117.57%)	This thesis/ Ruso-Julve et al., 2019	
			Rv	ATCTTTGTCGAGCAGCGTCA				
UNC45B	146862	NM_001033576.1	Fw	GGACTCTGTAAGCTCGGCTC	99	2.49 (124.46%)	This thesis/ Ruso-Julve et al., 2019	
			Rv	CCACTTGCGACACTGTTTGG				
Actb	11461	NM_007393.5	Fw	GGCTGTATTCCCCTCCATCG	154	N/D	This thesis/ Lopez-Gimenez et al., 2017	Mouse (*M. musculus*)
			Rv	CCAGTTGGTAACAATGCCATGT				
Adamts2	216725	NM_175643.3	Fw	ACGCCTTTTCTACAACCTCAC	216	N/D	This thesis/ Lopez-Gimenez et al., 2017	
			Rv	GCCAGCCCATCACAGTTACT				
Cd177	68891	NM_026862.3	Fw	GGGTGTTATGAAACAGTGATGCT	102	N/D	This thesis/ Lopez-Gimenez et al., 2017	
			Rv	GGTGAGCCGAGCCTTTTTGT				
Cntnap3	238680	NM_001081129.1	Fw	CTTTGCCTCAGTTATCCTTCAGC	161	N/D	This thesis/ Lopez-Gimenez et al., 2017	
			Rv	GACCTCCATTCGTTCTCCAAG				
Entpd2	12496	NM_009849.2	Fw	ATGGCTGGAAAGTTGGTGTCA	92	N/D	This thesis/ Lopez-Gimenez et al., 2017	
			Rv	TCTTGGGTAGGGACGCACA				
Rfx2	19725	NM_027787.1	Fw	TCCAGTTTCAGTACATTGAGAAGC	150	N/D	This thesis/ Lopez-Gimenez et al., 2017	
			Rv	CACACTTACACAGAGAGATGAGC				
Unc45b	217012	NM_178680.4	Fw	AGCTACACTCTACCGGAATCG	160	N/D	This thesis/ Lopez-Gimenez et al., 2017	
			Rv	CTGGTCCAACTTGCCAAGATG				

Quantitative PCRs were analysed using the SDS 2.2.2 software (Applied Biosystems). Threshold cycles (Ct) were determined by default at the beginning of DNA amplification in the exponential phase. The mRNA expression of genes of interest was normalized to mRNA expression of housekeeping genes (GAPDH and/or β-actin) using the comparative $2^{-\Delta\Delta Ct}$ method (described in (Livak and Schmittgen 2001)):

$$\Delta\Delta Ct = \left(Ct_{targetgene} - Ct_{HouseKeeping}\right)_{condition} - \left(Ct_{targetgene} - Ct_{HouseKeeping}\right)_{control}$$

$$Relativeexpressionlevel(FoldChange) = 2^{-\Delta\Delta Ct}$$

3.4.4. In silico study of the *ADAMTS2* gene promoter

To reveal the predicted localization of specific transcription factors binding sites (TFBS) and analysis of the human *ADAMTS2* gene promoter, we take advantage of the MatInspector software (Genomatrix, Germany; https://www.genomatix.de/matinspector.html). This tool utilizes a large library of matrix descriptions for TFBS to locate matches in DNA sequences, assigning a quality rating to matches. All algorithms and implementation are described in (Quandt et al. 1995) and (Cartharius et al. 2005).

3.4.5. Chromatin immunoprecipitation (ChIP) of peripheral blood mononuclear cells (PBMCs).

Chromatin immunoprecipitation technique was used to determine whether a protein is bound to a concrete region in DNA, in this case, at the regulatory region of the *ADAMTS2* gene promoter.

Generally, 2-4·10^6 PBMCs were used for each chromatin immunoprecipitation, using the Pierce Magnetic ChIP kit (Thermo Scientific), following the manufacturer's instructions. First, cells previously isolated from blood in PBS were fixed to cross-linked proteins and DNA adding a final concentration of 1 % formaldehyde (v/v) (stock concentration= 16 %; Thermo Scientific) for 10 min with gentle agitation (wheel). Next, a final concentration

of 125 mM glycine was added to stop cross-linking reactions. Cells were washed twice with ice-cold PBS with Halt protease/phosphatase inhibitor cocktail and centrifuged 3 min at 1500 rpm. Then, cell pellet was resuspended in 200 µl of membrane extraction buffer containing protease/phosphatase inhibitors, vortexed 15 s and incubated on ice for 10 min. After that, lysate was centrifuged at 9000 g for 3 min and supernatant was removed. Nuclei was resuspended in 200 µl of MNase digestion buffer containing a final concentration of 1 mM DTT and 2 µl MNase (ChIP grade) (1 U/µl), vortexing and incubate in a 37 ºC water bath for 15 min, mixing by inversion every 5 min. Then, 20 µl MNase stop solution to stop the reaction was added, vortexing, incubate on ice for 5 min and centrifuged 9000 g for 5 min to recover the nuclei. After remove supernatant, nuclei was resuspended in 100 µl of 1X IP dilution buffer containing protease/phosphatase inhibitors and, then, sonicated to shear chromatin in sizes of 200 to 600 bp using the Bioruptor Plus sonication device (Diagenode, NJ, USA). The sonicator device was set at high power setting for 10-15 cycles of 30 s ON and 30 s OFF at 4 ºC. After sonication, nuclei lysate was centrifuged at 9000 g for 5 min and supernatants containing the digested chromatin were recollected, taken 10 µl of this supernatant and stored at -20 ºC to use as input sample from one ChIP.

For immunoprecipitation, remaining supernatant was mixed with 410 µl of 1X IP dilution buffer and incubated with the specific primary antibody (see **table 3.6.**), anti-RNA Pol II antibody as positive control or normal rabbit IgG as negative control overnight at 4 ºC with mixing. Then, 20 µl of magnetic beads were added to each IP and incubated for 2 hours at 4 ºC with mixing. After incubation, beads were collected with a magnetic stand and supernatant was removed carefully. Next, beads were washed three times with IP wash buffer 1 and twice with IP wash buffer 2. Then, beads were incubated with 150 µl of 1X IP elution buffer at 65 ºC for 30 min with shaking. Next, for IP elution, 150 µl of 1X IP Elution buffer was added to the washed beads and were incubated at 65 ºC for 30 min, resuspending the beads by vortexing the tube every 10 minutes. After incubation,

Table 3.6. Primary and secondary antibodies used in this thesis.

Antibody	Immunogen	Host/Type	Reference	Origin	Use and dilution
ADAMTS2 (F-4)	C-terminal (human)	Mouse (monoclonal)	sc-393562	Santa Cruz Biotechnologies	IHC (1:50)
CREB (82B514)	Synthetic peptide (human)	Mouse (monoclonal)	40961	Active motif	ELISA (1:1000)
CREB (86B10)	Recombinant peptide (human)	Mouse (monoclonal)	9104	Cell signaling	WB (1:500)
CREB (LB9)	Recombinant human CREB-A protein	Mouse (monoclonal)	MA1-083	Invitrogen	ChIP (2.5 µg/1x10^6 cells)
Mouse dye 680		Goat	35519	Invitrogen	WB (1:5000)
Mouse dye 800		Goat	SA535521	Invitrogen	WB (1:5000)
p38 MAPK (D13E1)	C-terminal (human)	Rabbit (monoclonal)	8690	Cell signaling	WB (1:500)
p44/42 MAPK (Erk1/2) (137F5)	C-terminus (rat)	Rabbit (monoclonal)	4695	Cell signaling	WB (1:1000)
Phospho-CREB (Ser133)	Synthetic Ser133 peptide (human)	Rabbit (monoclonal)	43096	Active motif	ELISA (1:500)
Phospho-CREB (Ser133) (87G3)	Synthetic Ser133 peptide (human)	Rabbit (monoclonal)	9198	Cell signaling	WB (1:500)
Phospho-p38 MAPK (Thr180/Tyr182)	Synthetic Thr180/Tyr182 peptide (human)	Rabbit (monoclonal)	9211	Cell signaling	WB (1:500)
Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) (D13.14.4E)	Synthetic Thr202/Tyr204 peptide (human)	Rabbit (monoclonal)	4370	Cell signaling	WB (1:500)
Phospho-PKA Substrate (RRXS*/T*) (100G7E)	Synthetic peptides (human)	Rabbit (monoclonal)	9624	Cell signaling	WB (1:500)
Rabbit dye 680		Goat	35569	Invitrogen	WB (1:5000)
β-Tubulin Anticuerpo (37)	Recombinant peptide (human)	Mouse	sc-101527	Santa Cruz Biotechnologies	WB (1:10000)

the beads were collected with a magnetic stand and the eluted protein-chromatin complex supernatant was removed and dispensed into a new tube with 6 µl of 5M NaCl and 2 µl of 2 mg/ml Proteinase K. Also, the 10 % total input sample was thawed and diluted in 150 µl of 1X IP Elution buffer with 6 µl of 5M NaCl and 2 µl of 2 mg/ml Proteinase K. Then, all IP and total-input samples were vortexed and incubated at 65 ºC for 1.5 hours in a heat block.

For DNA recovery, each eluted IP and total input sample were diluted in 750 µl of DNA Binding buffer and centrifuged at 10000 g for 1 min in a DNA Clean-Up column into a 2

ml collection tube. Flow-through was discarded. Each column was washed with 750 µl of DNA Column Wash buffer and centrifuged 10000 g for 1 min. Flow-through was discarded. Then, DNA was eluted adding 50 µl of DNA Column Elution buffer directly into the centre of each column. Finally, columns were centrifuged at 10000 g for 1 min. The resulting solution was the purified DNA.

Finally, to quantify the binding and transcription activity of the protein targeted, we performed quantitative PCRs (as described above). Specific primers for human ADAMTS2 promoter regions (based on the TFBS predicted above) were designed using Primer-Blast (NCBI) (**table 3.7.**). The qPCR reaction was prepared as follows: 5 µl of Power SYBR™ Green PCR Master Mix were mixed with 0.3 µM of each primer (forward and reverse; stock concentration 10 µM), 1.5 µl of purified DNA and DEPC-water until 10 µl of final volume. Then, reaction mix was added to a 384-well PCR plate in duplicates. Reaction mix without DNA was used as no template control (NTC) to detect possible amplification signals from contaminant DNA or primer dimers. Samples included in this PCR were: total input samples, CREB-IP samples, Pol-II-IP samples (as positive control) and IgG samples (as negative control). The protocol used for amplification was the following:

Time	Temperature	Cycle
10 min	95 ºC	Hold
15 s	95 ºC	x40 cycles
15 s	58 ºC	

The data from each immunoprecipitate (IgG, Pol II and CREB) was normalized to the corresponding inputs of chromatin before IP, normalized to IgG/input data, and expressed as relative to the control. Four experiments (two cases and one control) were combined to determine CREB binding to gene promoter.

Table 3.7. Primer sequences used for (ChIP)-coupled quantitative PCR analysis in this thesis work.

Primer pair		Primer sequences	Amplicon size (bp)	Promoter region	Origin/Publication
A	Fw	TTCATCATGGGCACTGTAGC	115	-5000 bp	This Thesis
	Rv	ATGTCCTTCCATCCAGTCCA			
B	Fw	CTCTGTGAACCTGTTAGAAGATGC	157	CRE site (-2500/-2000 bp)	This Thesis
	Rv	ACCGAAGGCTGAAGCTGATTA			
C	Fw	CTGCGGAAGAGTTTTCCAGT	150	+5000 bp	This Thesis
	Rv	CAAAAGGCTTCTCACTGGGT			

3.4.6. Luciferase reporter assay.

Luciferase report assays were performed to analyse and quantify transcription factor CREB activity in SK-N-SH cells, using Dual-Glo Luciferase Assay System (Promega).

Cells were plated in 12-well plates and, the day after, were transfected in transient conditions with Lipofectamine LTX with PLUS reagents (Invitrogen), as described above, with 0.5 µg DNA plasmids mix (relation 3/1): 0.38 µg of pGL4.29 [luc2P/CRE/Hygro] containing firefly (*Photinus pyralis*) luciferase gene reporter vector carrying 5'-CRE transcription regulatory sequence to analyse; alongside 0.12 µg of pRL-Null containing renilla (*Renilla reniformis*) luciferase gene construct that is constitutively expressed used as control of transfection efficiency. Also, cells without DNA plasmids transfected were used as blank to reduce background luminescence signals from cells. See **table 3.4.** for more information about DNA constructs.

Passive lysis and quantification of Renilla and Firefly levels were performed as described in manufacturer's directions of Dual-Luciferase Reporter Assay System kit (Promega). Briefly, cells were lysed in plate with 100 µl of 1X PLB (Passive lysis buffer) diluted in distilled water rotating for 30 min at room temperature, harvested in a 96-well plate and stored at -80 ºC until assay. Plate was centrifuged at 14000 rpm for 10 min and then, 20 µl of supernatant cell lysate was load into a new 96-well plate. Then, 100 µl of Luciferase assay reagent (LARII) containing luciferin (firefly luciferase substrate) was added and

luminescence was measured within 1 min after addition. After that, firefly luciferase reaction was quenched with 100 µl of Stop&Glo reagent that also contains coelenterazine (renilla luciferase substrate), initiating second luciferase reaction. Luminescence from both reactions was measured with GloMax-Multi apparatus (Promega).

Firefly luminescence values were normalized against Renilla luminescence values used as control of transfection for each sample. Samples were blanked using the blank wells. Measurements were done in parallel triplicates and values were averaged. Relative light units were obtained of experimental values related to untreated values (control vehicle).

3.4.7. RNA in situ hybridization (ISH) images.

To reveal the localization of specific mRNA sequence of ADAMTS2 expression on mouse embryo brain at stage E14.5, we take advantage of a RNA in situ hybridization (ISH) images collected in the Transcriptome Atlas Database for Mouse Embryo from Eurexpress project web page (Link: http://www.eurexpress.org), described in (Diez-Roux et al. 2011).

3.5. Protein analyses.

3.5.1. Immunoblotting (Western blot).

Phosphorylation and protein level quantification were analysed by immunoblotting (Western blot).

Cell lysis was performed using RIPA buffer (Sigma) supplemented with phosphatase and protease inhibitors cocktails (Roche, Germany). Adherent cells were lysed directly in the plate: culture media was removed by aspiration, cells washed once with 1X PBS and removed by aspiration, lysis buffer was added to the plate directly placed on ice and cells were scratched and collected to a 1.5 ml tube. Homogenized samples were kept on ice

for 20 min and clarified by centrifuging at 14000 rpm for 15 min at 4 ºC. Supernatant containing the proteins was collected and transferred to a new tube and stored at -20 ºC until used.

Quantification of protein concentration was carried out using DC protein assay reagent kit (Bio-Rad) in a Spark Multimode Microplate Reader (Tecan Trading AG). Calibration curve was stablished by preparation of standards using 0-10 µg/µl of BSA (concentration stock 1mg/ml, diluted in distilled water). Measuring of standards and samples was performed mixing in a 96-wells plate 1 µl of sample, 25 µl of reagent A and 200 µl of reagent B. Samples were incubated 5 min protected from light at room temperature and then, were read in the fluorometer.

Total amount protein desired to analyse was calculated, normally between 20-40 µg per sample. Protein samples were prepared by mixing the corresponding volume from protein extracts filled with lysis buffer until the total volume desired and 4X laemmli sample buffer (Bio-Rad) complemented with 2-Mercaptoethanol (VWR) as loading buffer, added to a final 1X of working concentration. Then, samples were boiled at 95 ºC for 5 min to denaturize proteins and kept on ice until using to avoid protein renaturation.

Samples were loaded and separated according to their molecular weight in a polyacrylamide-SDS gel electrophoresis (SDS-PAGE). Percentage of acrylamide/bis-acrylamide solution (29:1) (stock solution= 40%, w/v) (Bio-Basic, ON, Canada) used for the gel preparation was dependent on the molecular weight of the protein to be analysed (ranging from 8-115 %). Electrophoresis was carried out in a Mini-PROTEAN Tetra cell cuvette (Bio-Rad) powered by a basic PowerPAC supply (Bio-Rad) at constant voltage of 100-150 V for 2-3 hours, using electrophoresis running buffer[2]. PageRuler plus prestained protein ladder (Thermo Scientific) was used to evaluate protein migration and separation

[2] *Running buffer: 25 mM Tris pH 8.3, 192 mM glycine, 0.1 % SDS (w/v); stock concentration 10X, stored at room temperature.*

during gel electrophoresis. Proteins were transferred from acrylamide gel to a 0.45 µm nitrocellulose blotting membrane (Amersham Protran, GE Healthcare life science, Germany) in a Mini-Trans blot cell (Bio-Rad) using cold transfer buffer[3] at constant amperage of 350 mA for 60-90 min depending on the molecular weight of the proteins of interest. After protein transference, nitrocellulose membrane containing proteins was stained with Red Ponceau[4] solution for 5 min at room temperature and distained with distilled water until proteins bands were seen to check protein load and integrity. Then, membrane was blocked for 45-60 min in agitation at room temperature in order to avoid unspecific antibody binding during the following steps. Blocking step was performed in 5 % non-fat dry milk (w/v) or 5 % of BSA (w/v), according to antibody recommendations. Both, milk and BSA were dissolved in TBS-T buffer[5,6] (15 ml per membrane). Membrane was washed twice with TBS-T for 10 min and incubated with the specific primary antibody diluted in TBS-T with 1 % BSA (w/v) as indicated in **table 3.6.** overnight at 4 ºC in agitation. After that, membrane was washed three times with TBS-T for 10 min and then, incubated at room temperature for 1 hour with the corresponding secondary fluorochrome-conjugated antibody diluted in TBS-T with 1 % BSA (w/v) as indicated in **table 3.6**. Finally, membrane was washed three times for 10 min with TBS-T and signals were visualized and recorded with an Odyssey Infrared Imaging scanner (Li-Cor biosciences, NE, USA). Immunoblot densitometry analysis on every band was calculated

---

[3] *Transfer buffer: 25 mM Tris pH 8.3, 192 mM glycine, 10 % methanol (v/v); stock concentration 10X, stored at room temperature.*

[4] *Red Ponceau solution: 0.1% Ponceau S (w/v) (Bio-Rad) in 5 % acetic acid (v/v); stock solution 1X, stored at room temperature.*

[5] TBS: 20 mM Tris-HCl pH 7.5, 150 mM NaCl; stock concentration 10X, stored at room temperature.

[6] TBS-T: 0.05 % Tween 20 (v/v) diluted in TBS; stock solution 1X, stored at room temperature protected from light.

using Image Studio Software (LI-COR). Phosphorylation and total protein densitometry values were normalized to β-Tubulin signal.

3.5.2. CREB transcription factor ELISA-based binding assays.

To detect and quantify transcription factor CREB binding capacity to its consensus binding sites in PBMC from SCZ patients, an ELISA-based assay was performed using a TransAM CREB transcription factor assay kit (Active Motif, CA, USA), following the manufacturer's instructions.

Cell nuclei from patient PBMC were lysate using a Nuclear extract kit (Active motif), following the manufacturer's instructions. Protein concentration was determined using DC protein assay reagent kit (Bio-Rad), as described above (150 mg of nuclear protein for $9 \cdot 10^6$ cells).

For binding of CREB from samples to their consensus sequence, 30 µl complete binding buffer[7] was added to each well. Next, 5 µg of sample or positive control (provided by kit) diluted in 20 µl of complete lysis buffer[8] were added and incubated for 3 hours at room temperature with mild agitation (rocking platform). For blank, 20 µl complete lysis buffer was added only in a well. Next, each well was washed three times with 1X wash buffer[9], flicking the plate over a sink to empty the wells. Then, each well was incubated with the specific primary antibody (CREB 1:1000) (see **table 3.6**.) diluted in 100 µl of 1X antibody binding buffer[10] for 1 hour at room temperature without agitation. After that, wells were

[7] *Complete binding buffer: 10 µl of 1 µg/µl Herring sperm DNA and 1 µl 1M DTT per 1ml of binding buffer AM1 (provided by kit). Any remaining should be discarded if not used at the same day.*

[8] *Complete lysis buffer: 0.23 µl protease inhibitor cocktail, 0.02 µl of 1M DTT in 22.25 µl lysis buffer AM1, for 1 well. Any remaining should be discarded if not used at the same day.*

[9] *1X wash buffer: 10 ml of 10X Wash buffer AM1 with 90 ml of distilled water, for every 100 ml of buffer; stored at 4 ºC for one week.*

[10] *1X antibody binding buffer: 1 ml of 10X antibody binding buffer AM1 with 9 ml distilled water, for every 10 ml of buffer; discard remaining after use.*

washed three times with 1X wash buffer as described above and then, incubated at room temperature for 1 hour with the corresponding secondary HRP-conjugated antibody (anti-mouse IgG for CREB) (see **table 3.6**.) diluted in 1X antibody binding buffer. Then, wells were washed four times, developing solution was added at each well and incubated for 10 min at room temperature protected from light to develop blue colour of each reaction. Finally, stop solution was added to each well (in presence of acid the blue colour turns yellow) and absorbance was read on a Spark Multimode Microplate Reader (Tecan Trading AG) within 5 min at 450 nm with an optimal reference wavelength of 655 nm. Plate was blanked using the blank wells.

3.5.3. Immunohistochemical staining.

ADAMTS2 protein expression and localization in mice brains during development were analysed by immunohistochemical staining.

Five mice embryos at stage E18.5 and five postnatal P7 mice were anesthetized, perfused transcardially with PBS and followed by 4 % paraformaldehyde (v/v) in PBS. Brains were removed and post-fixed overnight at 4°C in the same fixative and then, dehydrated progressively in ethanol and paraffin-embedded. Next, 10 µm-thick coronal sections were obtained and mounted in parallel series. The sections were dewaxed and rehydrated for ADAMTS2 immunostaining.

The tissue was rinsed with phosphate buffer solution and 0.075 % Triton X-100 in PBS-T[11]. This was followed by 1h incubation in PBS-T with blocking solution[12] to avoid unspecific antigen binding in the following steps. Sections were then incubated with the ADAMTS2 primary antibody (Santa Cruz biotechnology, CA, USA) (see **table 3.6.**) diluted in EnVision FLEX Antibody Diluent (DAKO, Denmark) overnight at 4ºC. The following day,

[11] *PBS-T: 0.05 % Tween 20 (v/v) diluted in PBS.*

[12] *Blocking solution: 10 % goat serum (GS) (w/v) in PBS.*

sections were rinsed three times at room temperature and then, incubated with the appropriate secondary antibody during 1h (see **table 3.6.**). Afterwards, sections were washed with PBS-T at room temperature. Sections were mounted on glass slides with 10:1 Mowiol (Calbiochem)-NPG (Sigma). For the colorimetric detection the tissue was incubated with 1% 3,39-Diaminobenzidine (DAB; Vector Laboratories SK-4100) and 0.0018 % H2O2 in PBS.

3.6. Statistical analyses.

Kolmogorov-Smirnov test and Levene test were used to test normality and equality of variances respectively.

For patients' studies, in order to ensure group comparability between healthy volunteers and patients, sociodemographic and clinical characteristics at baseline were tested by unpaired Student t-test or one-way analysis of variance (ANOVA) for continuous variables as necessary, and by Fisher's Exact test for qualitative variables (**Tables 3.1., 3.2., 4.1. and 4.2.**).

Wilcoxon Singed-Rank test for independent data was used to compare CRGAs mRNA expression levels among healthy volunteers and drug-naïve patients at baseline. Wilcoxon Matched-Pairs Signed-Rank test for paired data was performed to compare the change in the mRNA expression levels from baseline to 3 months following the antipsychotic treatment ($\alpha = 0.05 / 6 = 0.008$). STATA 15.1 was used for statistical analysis.

For in vitro studies, unless otherwise specified, all experiments were independent and numerical data were summarized as the mean ± SEM (standard error of the mean) using GraphPad Prism6 software (CA, USA). Each global mean was compared using two tailed unpaired Student's t-test ($\alpha = 0.05$), described in each figure legend. Statistical tests were two-tailed with a 95% confidence interval.

RESULTS

4. RESULTS.

In the present thesis, we have followed a bottom-up strategy using the transcriptional data obtained in SCZ patients, mice samples and taking advantage of neuronal-derived cells towards the identification of membrane receptors and the intra-cellular mechanism controlled by them that affect the CRGAs expression. Initially, we analysed the dynamic transcriptional responses of CRGAs to APDs incubation in neuronal-like cells. Then, in a preclinical model of SCZ, we observed that all six genes are expressed in PFC from mice brains, and five of them are overexpressed in affected mice. Furthermore, we performed an independent validation using a new cohort of drug-naïve non-affective psychosis patients. We confirmed that *ADAMTS2* expression was significantly up-regulated at diagnosis and that treatment with APDs reduced their expression levels back to "healthy" values which, in turn, was associated with clinical response (positive symptoms). Moreover, this inhibition of *ADAMTS2* was observed in patients after 12 months of treatment with APDs. Finally, we provide new evidences that *ADAMTS2* expression in mice brains is aligned with that of the progenitors of neurons from the mesocortical and mesolimbic dopaminergic pathways (extensively associated with schizophrenia etiopathology). Accordingly, we show that in our experimental settings, *ADAMTS2* transcription is controlled by dopaminergic signalling cascade (D_1-class receptors) through Erk and cAMP/CREB.

4.1. Dynamic transcriptional control of Clinical Response Genes to Antipsychotics (CRGAs) by APDs, in neuronal-like cells.

To gain mechanistic insight into the transcriptional control of CRGAs, we analysed the effects of APDs in human neuroblastoma SK-N-SH cells, which express cell surface receptors that are well known targets of the main APDs (D_1, D_2, 5-HT_{1A} and 5-HT_{2A} receptors). We first tested potential anti-proliferative effects of clozapine, haloperidol,

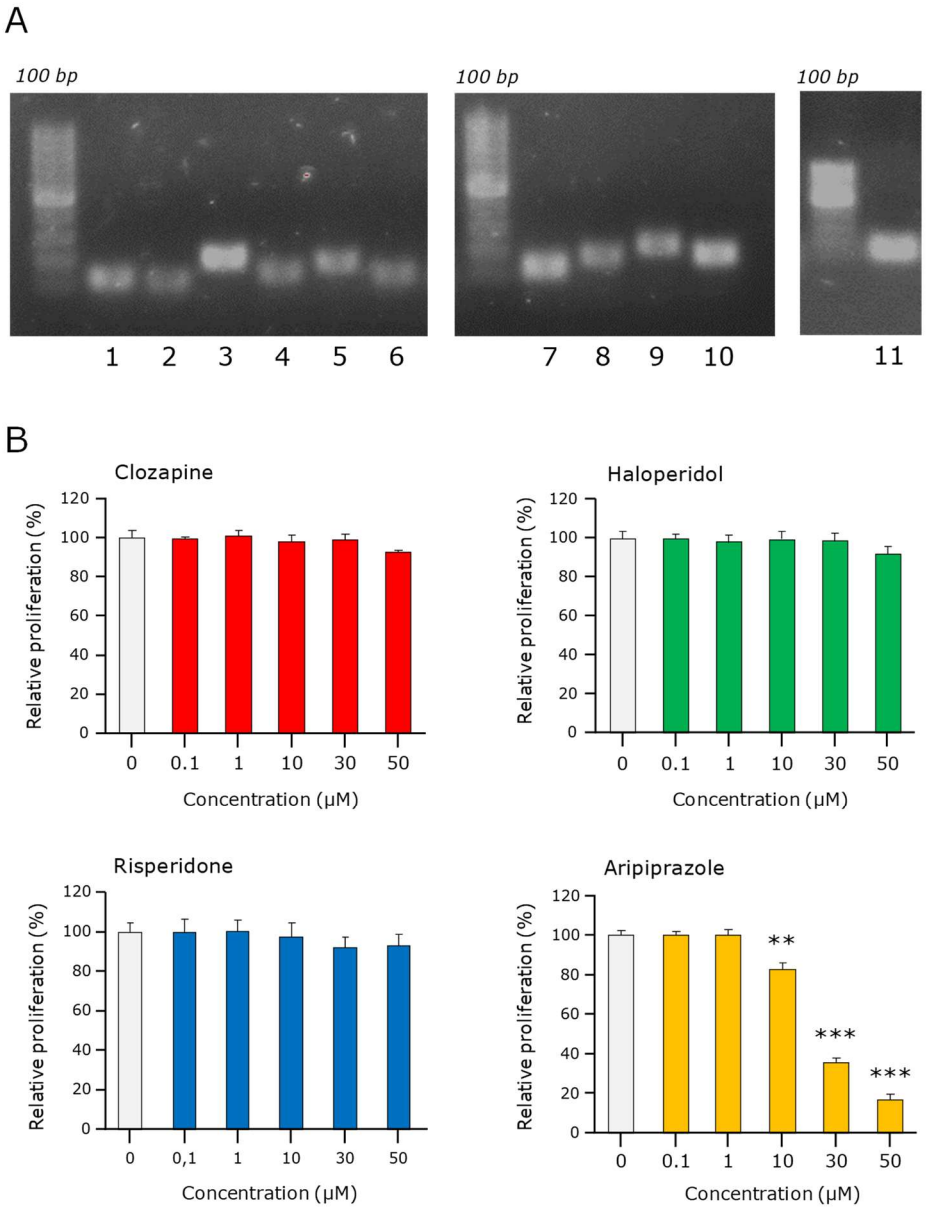


Figure 4.1. Basal gene expression of the six clinical response genes to antipsychotics (CRGAs) and the main receptors targeted by antipsychotics (RTAPs). Cell viability after APD incubation in SK-N-SH cells. (A) Figure shows PCR amplicons corresponding to a semi-quantitative (35 cycles) RT-qPCR analysis of the mRNA expression of the indicated genes in exponentially growing human SK-N-SH cells: (1) ADAMTS2, (2) CD177, (3) CNTNAP3, (4) ENTPD2, (5) RFX2, (6) UNC45B, (7) DRD1, (8) DRD2, (9) HTR1A, (10) HTR2A, and (11) ACTB. (B) Relative SK-N-SH cell proliferation after 48 hours incubation with the indicated antipsychotic drugs (Drug concentration: 0-50 μM) (N=6). Data are mean ± SEM; unpaired Student's t test: **p < 0.01 and ***p < 0.001 show significance with respect to control (Vehicle, drug 0 μM).

risperidone and aripiprazole by incubating these cells with different concentrations of each drug. We decided to work using specific APD concentrations that do not to elicit proliferative arrest (**Figure 4.1.**).

In these settings, incubation of cells with APDs induced dynamic transcriptional responses of CRGAs across time (**Figure 4.2.**). Intriguingly, different gene expression responses to antipsychotic treatment can be distinguished: (1) fast (30 min) and sustained responses (up to 24 h) (i.e. *ADAMTS2* in cells incubated with clozapine or haloperidol); (2) fast and transitory responses (i.e. *ADAMTS2* in cells incubated with haloperidol or aripiprazole); and (3) delayed modulation (24 hours) (i.e. *ENTPD2* in cells treated with clozapine). Of interest, *ADAMTS2* was the only gene reacting significantly to the four APDs used: fast and sustained in cells incubated with haloperidol and clozapine, and fast and transitory in risperidone and aripiprazole incubations. Also, only *CNTNAP3* and *UNC45B* showed expression modulation in cells when incubated with the typical APD, haloperidol. Finally, the data showed that the other five CRGAs do not show a significant response to atypical APDs, risperidone and aripiprazole, but did it noteworthy in cells incubated with clozapine.

4.2. Validation of Clinical Response genes to Antipsychotics (CRGAs) expression profiles.

4.2.1. Validation of CRGAs expression profile in a preclinical model of maternal infection during pregnancy.

In collaboration with Dr. Javier González-Maeso group and in order to explore whether our previous data obtained from blood human samples could be reproduced using a different approach, we proceeded to analyse the expression of the orthologous mice version of these genes in the CNS. For this purpose, we took advantage of a preclinical

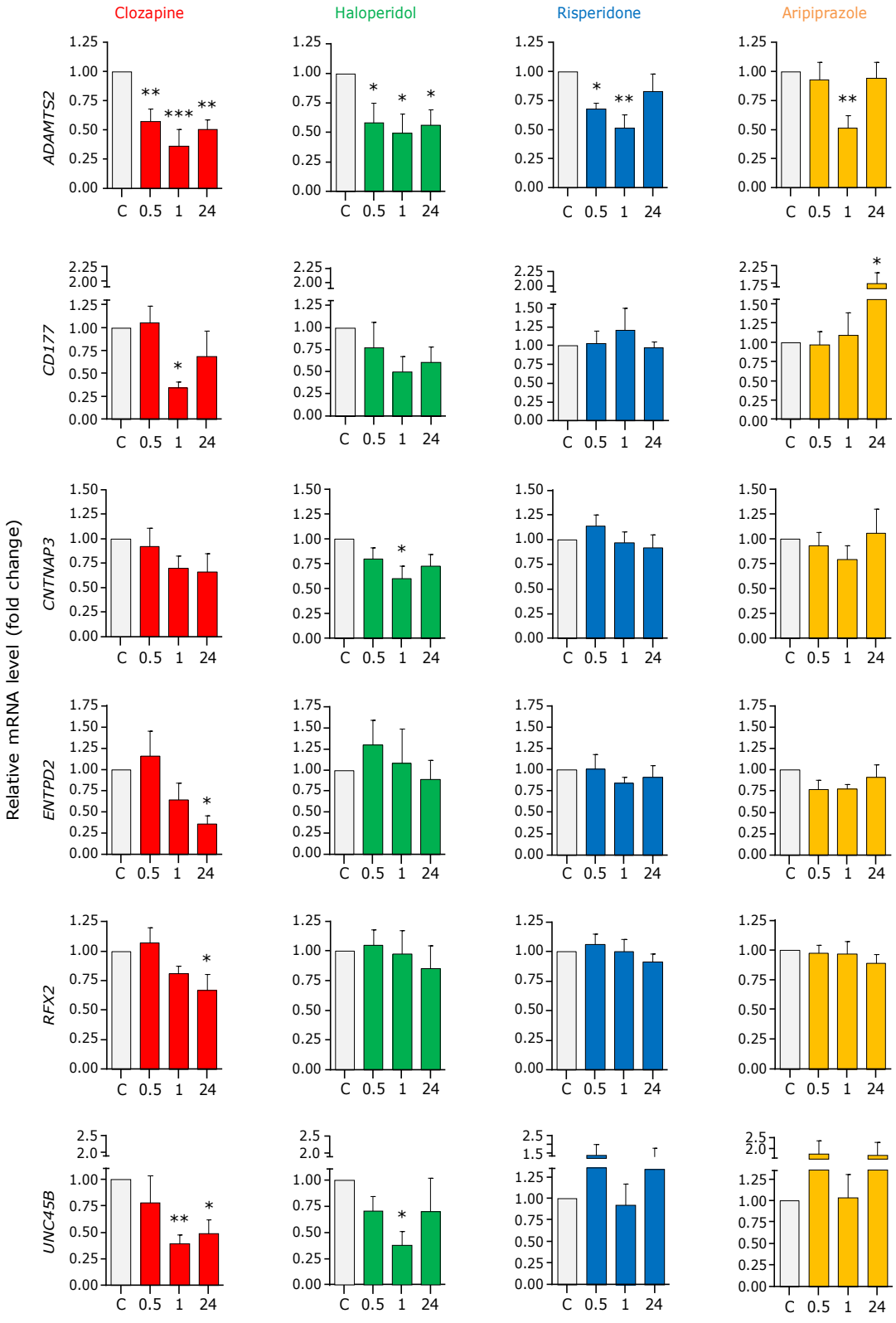

Clozapine
Haloperidol
Risperidone
Aripiprazole
ADAMTS2
CD177
CNTNAP3
ENTPD2
RFX2
UNC45B
Relative mRNA level (fold change)
C 0.5 1 24
Time (hours)

Figure 4.2. Dynamic control of CRGAs mRNA expression by APDs in SK-N-SH cells. RT-qPCR in cells incubated with antipsychotics for the indicated times as follows: risperidone (10 μM; blue bars), aripiprazole (1 μM; yellow bars), clozapine (1 μM; red bars) and haloperidol (1 μM; green bars) (N=5). Data are mean ± S.E.M; One-way ANOVA for multiple comparations: *p < 0.05, **p < 0.01, ***p < 0.001 shows significance with respect to control (C: vehicle; grey bars).

model consisting on adult mice born to mothers submitted to influenza viral infection during gestation. Pregnant CD1 mice were subjected to influenza viral infection according to the protocol previously described (Moreno et al. 2011) and adult offspring were assessed for behavioural and neurochemical traits corresponding to schizophrenia-like phenotype, essentially increased head-twitch response elicited by treatment with the hallucinogenic drug DOI and changes in the relative 5-HT_{2A} and mGlu2 receptor expression levels detected by *ex vivo* radioligand binding assays. A group of eight animals that statistically presented these alterations were pair matched with control mice. Tissue samples from mouse frontal cortex were obtained and mRNA was extracted to conduct subsequent quantitative real-time PCR (RT-qPCR) experiments as previously described with minor modifications (Gonzalez-Maeso et al. 2003).

The results first demonstrated that these six genes are expressed in cortical tissue from mice brains. Second, the statistical analysis comparing control and influenza-infected descendant mice (**Figure 4.3.**) revealed a significant overexpression of at least three of the six total genes considered in this study, in particular *CD177*, *CNTNAP3* and *ENTPD2* (significance at $p<0.01$). Other two genes such as *ADAMTS2*, and *RFX2* although not reaching statistical significance showed a tendency to be overexpressed when compared with control values.

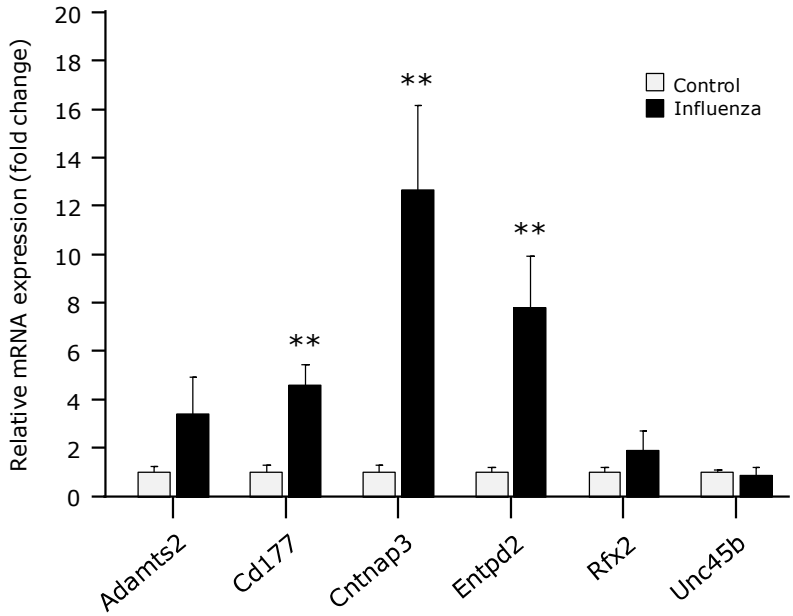


Figure 4.3. Expression of the CRGAs in frontal cortex of adult mice with schizophrenia-like behavioral. Relative mRNA expression levels of *Adamts2*, *Cd177*, *Cntnap3*, *Entpd2*, *Rfx2* and *Unc45b* determined by RT-qPCR assays in frontal cortex of adult mice born to influenza virus-infected mothers (blue bars) and controls (grey bars). N=8 animals per group. Data are mean ± SEM; unpaired Student's t test: **p < 0.01 shows significance with respect to control.

4.2.2. Independent validation of CRGAs expression in drug-naïve first episode SCZ patients before and after treatment with APDs.

4.2.2.1. Clinical characteristics of schizophrenia patients.

To gain further insights into the mechanistic effects of treatment with atypical APDs in SCZ patients, we prepared two new cohorts of drug-naïve SCZ patients with baseline quantitative data (psychopathological characteristics) and clinical follow-up data after treatment with APDs [Cohort #1: baseline and 3-month APD treatment (N=30), **Table 4.1.** and Cohort #2: baseline, 3- and 12-month treatment (N=9), **Table 4.2.**]. We proceeded with two different cohorts due to the difficulties that are usually associated to the clinical follow up of SCZ patients during APD treatment.

Table 4.1. Psychopathological characteristics at baseline and at 3-month treatment of cohort 1. Clinical changes during the follow up period comparison between aripiprazole and risperidone. BPRS: Brief Psychiatric Rating Scale; CDSS: Calgary Depression Rating Scale for Schizophrenia; CGI: Clinical Global Impression; SANS: Scale for the Assessment of Negative Symptoms; SAPS: Scale for the Assessment of Positive Symptoms; YMRS: Young Mania Rating Scale. [a] Wilcoxon Matched-Pairs Signed-Rank test; [b] Paired T-Student test; [c] Comparison between aripiprazole and risperidone at baseline; unpaired Student's t-test; [d] Comparison between aripiprazole and risperidone following the antipsychotic treatment, using the total score of the clinical scales at baseline as covariate; analysis of covariance (ANCOVA).

Cohort #1	Total (N=30)		Statistics	p	Aripiprazole (n=16)		Risperidone (n=14)		Statistics	p
	Mean	SD			Mean	SD	Mean	SD		
CGI										
Baseline	6.5	0.6			6.56	0.51	6.43	0.65	t= 0.63 [c]	0.532
3 months	1.8	1.2			1.63	1.20	2.00	1.24		
3-month change from baseline	-4.7	1.2	z= 4.83 [a]	0.000	-4.90	0.30	-4.47	0.33	F= 0.96 [d]	0.336
YMRS										
Baseline	13.8	6.2			14.25	7.24	13.29	5.01	t= 0.42 [c]	0.679
3 months	1.2	2.3			1.56	2.63	0.79	1.93		
3-month change from baseline	-12.6	6.5	z= 4.78 [a]	0.000	-12.25	0.59	-13.00	0.63	F= 0.75 [d]	0.394
CDSS										
Baseline	1.5	3.0			2.06	3.82	0.86	1.75	t= 1.08 [c]	0.288
3 months	1.2	2.3			1.19	2.34	1.14	2.25		
3-month change from baseline	-0.3	3.7	z= 0.35 [a]	0.724	-0.34	0.59	-0.32	0.63	F= 0.00 [d]	0.985
BPRS										
Baseline	70.3	15.0			69.06	16.08	71.85	13.95	t= -0.49 [c]	0.627
3 months	29.9	7.1			29.06	7.07	30.92	7.48		
3-month change from baseline	-40.4	15.2	t= 14.32 [b]	0.000	-41.13	1.81	-39.53	2.01	F= 0.35 [d]	0.561
SAPS										
Baseline	15.9	4.2			15.63	3.50	16.07	4.94	t= -0.29 [c]	0.775
3 months	0.6	1.7			0.69	1.89	0.71	1.49		
3-month change from baseline	-15.4	4.3	t= 19.28 [b]	0.000	-15.14	0.44	-15.12	0.47	F= 0.00 [d]	0.975
SANS										
Baseline	4.8	6.9			3.47	6.56	5.93	7.43	t= -0.95 [c]	0.352
3 months	3.9	6.6			4.00	6.47	4.93	7.04		
3-month change from baseline	-0.9	5.6	z= 1.14 [a]	0.255	-0.07	1.54	-0.36	1.59	F= 0.02 [d]	0.899

From a clinical perspective, we found significant within-subject changes between baseline data and after 3-month treatment with risperidone and aripiprazole in both cohorts of patient. Interestingly, no differences were found in patients between 3- and 12-month treatment groups in cohort 2, despite both were significantly different from their baseline controls. For both cohorts, the above-mentioned changes between baseline and 3-month data, significantly affected CGI (Clinical Global Impression), YMRS (Young Mania Rating Scale), BPRS (Brief Psychiatric Rating Scale) and SAPS (Scale for the Assessment of Positive Symptoms). Importantly, and as shown in **tables 4.1. and 4.2.**, no differences were found between the APDs in the total score of the clinical scales at baseline, 3-month, and 12-month of treatment (all $p > 0.05$).

4.2.2.2. Transcriptional changes elicited by antipsychotic drugs in drug-naïve SCZ patients. Identification of dysregulated expression of *ADAMTS2* in the onset and highly responsive to APDs.

PBMCs isolated from blood samples from SCZ patients were obtained at the first episode of psychosis (onset) and after 3 months of treatment with APDs (cohort #1; **Table 4.1.**). In this context, using RT-qPCR, an independent validation of transcriptional changes by focusing on the expression of the CRGAs was performed. Interestingly, *ADAMTS2* was significantly and highly overexpressed at the onset, with respect to healthy controls, and

Table 4.2. Psychopathological characteristics at baseline, at 3- and 12 month-treatment of cohort 2. Clinical changes during the follow up period comparison between aripiprazole and risperidone. BPRS: Brief Psychiatric Rating Scale; CDSS: Calgary Depression Rating Scale for Schizophrenia; CGI: Clinical Global Impression; SANS: Scale for the Assessment of Negative Symptoms; SAPS: Scale for the Assessment of Positive Symptoms; YMRS: Young Mania Rating Scale. [a] Wilcoxon Matched-Pairs Signed-Rank test; [b] Paired T-Student test; [c] Comparison between aripiprazole and risperidone at baseline; analysis of variance (ANOVA); [d] Comparison between aripiprazole and risperidone following the antipsychotic treatment at 3-month, using the total score of the clinical scales at baseline as covariate; analysis of covariance (ANCOVA); [e] Comparison between aripiprazole and risperidone following the antipsychotic treatment at 12-month, using the total score of the clinical scales at baseline as covariate; analysis of covariance (ANCOVA).

Cohort #2	Total N=12		Statistic	p	Aripiprazole N=6		Risperidone N=6		Statistics	p
	Mean	SD			Mean	SD	Mean	SD		
CGI										
Baseline	6.67	0.49			6.67	0.52	6.67	0.52	F= 0.00 [c]	1.000
3 months	1.75	0.97			1.83	0.98	1.67	1.03		
3-month change from baseline	-4.92	1.16	t= -14.63 [b]	0.000	-4.83	1.17	-5.00	1.26	F= 2.36 [d]	0.150
12 months	1.67	1.50			1.33	0.82	2.00	2.00		
12-month change from baseline	-5.0	1.16	z= -3.08 [a]	0.002	-5.33	0.82	-4.67	2.34	F= 2.26 [e]	0.160
YMRS										
Baseline	12.5	5.58			12.0	5.10	13.0	6.48	F= 0.09 [c]	0.773
3 months	0.33	0.65			0.33	0.52	0.33	0.82		
3-month change from baseline	-12.17	5.77	z= -3.07 [a]	0.002	-11.67	5.47	-12.83	6.55	F= 0.00 [d]	0.950
12 months	0.33	0.89			0.50	1.22	0.17	0.41		
12-month change from baseline	-12.17	5.77	z= -3.07 [a]	0.002	-11.50	5.47	-12.83	6.55	F= 0.32 [e]	0.588
CDSS										
Baseline	2.33	4.22			2.17	3.49	2.5	5.21	F= 0.02 [c]	0.899
3 months	1.08	2.31			0.33	0.82	1.83	3.13		
3-month change from baseline	-1.25	5.12	t= -0.85 [b]	0.416	-1.83	3.25	-0.67	6.80	F= 1.25 [d]	0.293
12 months	0.75	1.48			0.17	0.41	1.33	1.97		
12-month change from baseline	-1.58	4.70	z= 0.12 [a]	0.902	-2.00	3.63	-1.17	5.91	F= 1.96 [e]	0.195
BPRS										
Baseline	65.42	19.56			61.0	15.15	69.83	23.77	F= 0.59 [c]	0.461
3 months	27.92	4.01			26.5	2.59	29.33	4.89		
3-month change from baseline	-37.5	21.05	t= -6.17 [b]	0.000	-34.50	14.10	-40.50	27.47	F= 2.53 [d]	0.147
12 months	28.0	10.53			24.5	1.22	31.5	14.60		
12-month change from baseline	-37.42	25.34	t= -5.12 [b]	0.000	-36.50	15.82	-38.33	34.06	F= 2.65 [e]	0.138
SAPS										
Baseline	14.42	4.87			14.33	3.93	14.5	6.06	F= 0.00 [c]	0.956
3 months	0.17	0.58			0.33	0.82	0.00	0.00		
3-month change from baseline	-14.25	4.88	z= -3.08 [a]	0.002	-14.00	3.95	-14.50	6.06	F= 0.91 [d]	0.366
12 months	0.33	1.15			0.00	0.00	0.67	1.63		
12-month change from baseline	-14.08	5.32	t= -9.18 [b]	0.000	-14.33	3.93	-13.83	6.82	F= 1.03 [e]	0.337
SANS										
Baseline	5.25	4.86			4.17	5.56	6.33	4.27	F= 0.57 [c]	0.467
3 months	3.33	2.81			3.33	2.80	3.33	3.08		
3-month change from baseline	-1.92	5.25	t= -1.27 [b]	0.230	-0.83	4.67	-3.00	6.00	F= 0.91 [d]	0.366
12 months	2.08	4.70			0.17	0.71	4.00	6.29		
12-month change from baseline	-3.17	7.16	t= -1.53 [b]	0.154	-4.00	5.72	-2.33	8.85	F= 2.55 [e]	0.144

also down-regulated to "normal" levels by APDs after 3 months of treatment (**Figure 4.4.**). Unexpectedly, this approach failed to validate the expression signature of the other five genes studied in this new cohort of patients (all p-values > 0.008) (**Figure 4.4.**).

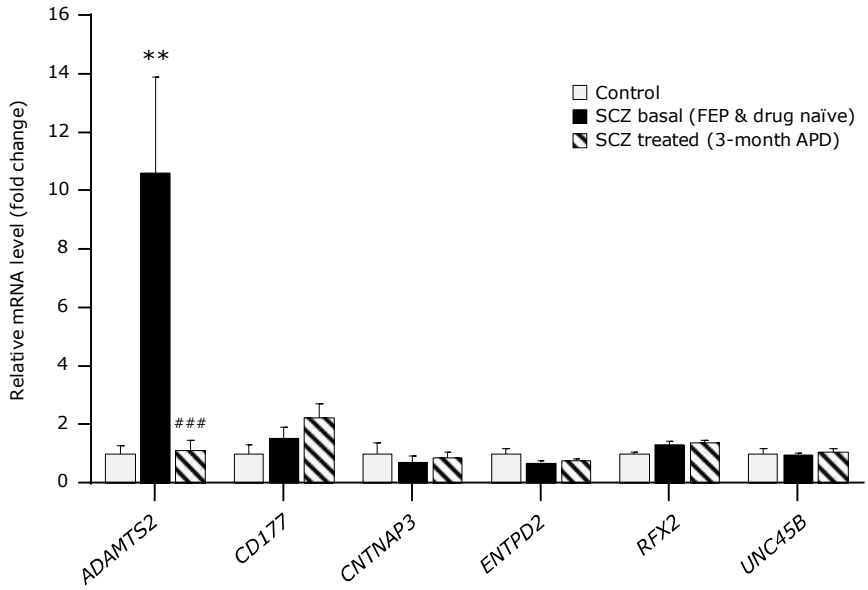


Figure 4.4. CRGAs expression in an independent cohort of drug-naïve first episode SCZ patients and regulation by APDs. Relative mRNA expression level of *ADAMTS2*, *CD177*, *CNTNAP3*, *ENTPD2*, *RFX2* and *UNC45B* genes in PBMCs from peripheral blood samples of an independent cohort of schizophrenia patients: healthy controls (basal; grey bar), untreated FEP patients (basal drug-naïve; black bars) and 3-months treated patients (striped bars). FEP: first episode of psychosis. All patients were treated with risperidone and aripiprazole drugs and shown clinic response to treatment (Cohort: patients N=30 and controls N=10). Data are mean ± S.E.M; unpaired Wilcoxon Signed-Rank test with Bonferroni correction for multiple comparisons: *p < 0.008, **p < 0.001, ***p < 0,0001 compares patients at baseline vs. healthy controls; paired Wilcoxon Signed-Rank test with Bonferroni correction for multiple comparisons: #p < 0.008, ##p < 0.001, ###p < 0,0001 compares patients at baseline vs. those after 3-month APD treatment

To evaluate if this down-regulation of *ADAMTS2* by atypical APDs was stable, we studied a second cohort of SCZ patients (cohort #2) with clinical improvement at 3-month assessment (**Table 4.2.**). Expression data by RT-qPCR validated the up-regulation of *ADAMTS2* at the onset in drug-naïve patients and its down-regulation after 3 months of

APD treatment. Interestingly, both down-regulation of *ADAMTS2* and clinical improvement were stably maintained up to 12 months (**Figure 4.5.**).

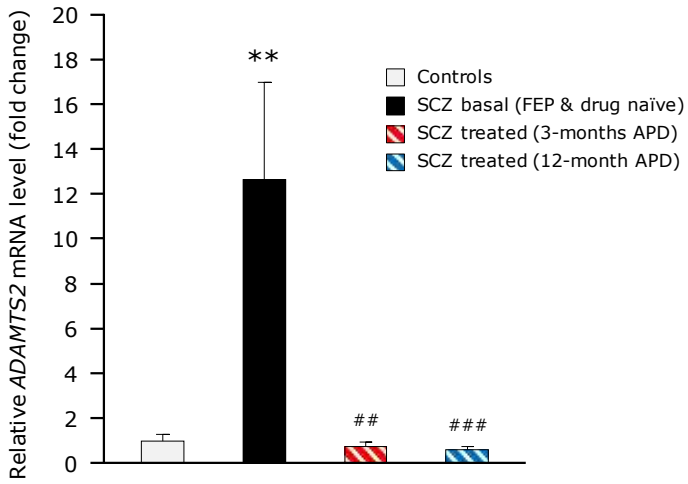


Figure 4.5. CRGAs expression in an independent cohort of drug-naïve first episode SCZ patients and regulation by APDs after 3 and 12 months. Relative expression levels in a new independent cohort: healthy controls (basal; grey bars), untreated (basal, first-episode psychosis; black bars), 3-months treated patients (red striped bars), and 12-month treated patients (blue striped bars). All patients had clinic response to APDs (Cohort: patients N=9 and controls N=9). Data are mean ± S.E.M; unpaired Student's t-test: **p < 0.01 compares patients at baseline vs. healthy controls; paired Student's t-test: ##p < 0.01, ###p < 0.001 compares patients at baseline vs. those after 3-/12-month treatment with APDs.

4.3. Neurotransmitter receptors targeted by antipsychotics and the transcriptional regulation of the CRGAs.

4.3.1. Regulation of CRGAs by selective receptor agonists.

To evaluate the possible role of the receptor targeted by APDs in the expression control of the CRGAs, we performed transcriptional analyses by RT-qPCR in SK-N-SH cells. To this end, cells were incubated for 1 hour with the selective agonist for D_1-class (SKF 83822), D_2-class (7-OH-DPAT), 5-HT_{1A} (8-OH-DPAT) and 5-$HT_{2A/2C}$ (TCB-2) receptors (**Figure 4.6.**). Results show that SKF 83822 significantly trigged *ADAMTS2* mRNA

expression as compared to 7-OH-DPAT, 8-OH-DPAT or TCB-2 (**Figure 4.6. A**). Also, we observed that SKF 83822 incubation significantly up-regulated *ENTPD2* and *UNC45B* expression. Cells incubated with 7-OH-DPAT shown significant up-regulation in *ENTPD2* expression and down-regulation in *UNC45B*. Furthermore, TCB-2 incubation trigged *CNTNAP3* expression in these cells. It is remarkable that other genes as *CD177* shown non-significant alterations after agonist incubation.

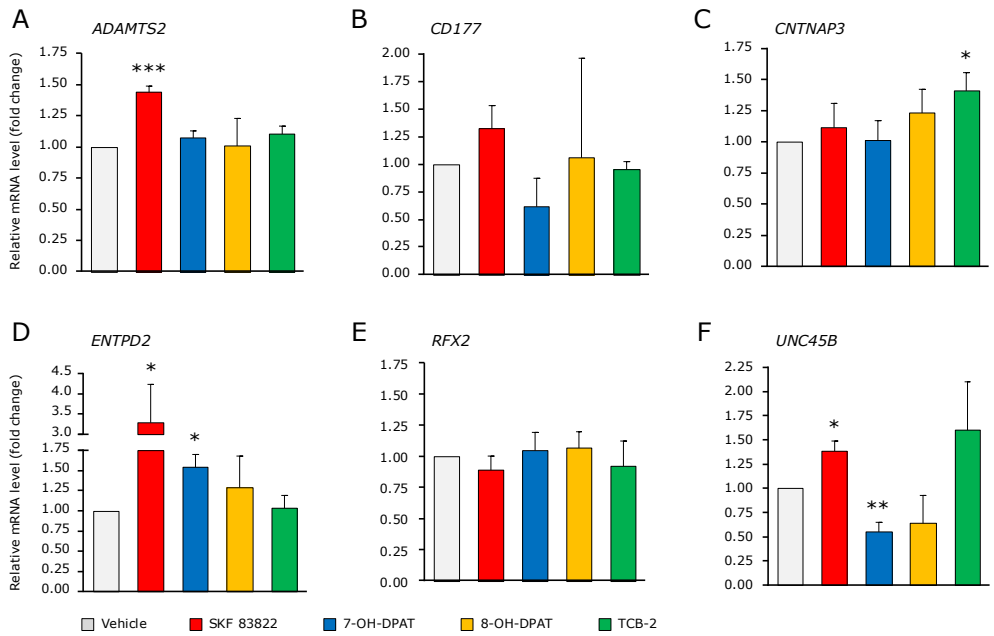


Figure 4.6. Regulation of CRGAs by selective receptor agonists. Relative *ADAMTS2*, *CD177*, *CNTNAP3*, *ENTPD2*, *RFX2* and *UNC45B* mRNA levels in SK-N-SH cells, analysed by RT-qPCR in cells incubated with the indicated selective agonist (1h): SKF 83822 (D1-class receptors, red bars), 7-OH-DPAT (D2-class receptors, blue bars), 8-OH-DPAT (5-HT1A receptor, yellow bars) and TCB-2 (5-HT2A/2C receptors, green bars) (Drug concentration 1 μM) (N=3). Data are mean ± S.E.M; Student's t test: *p < 0.05, **p < 0.01, ***p < 0.001 shows significance with respect to control (Vehicle, grey bars).

4.3.2. APDs and selective receptor antagonist can modulate the expression of *ADAMTS2* through D_1-class receptors.

Our previous data detected *ADAMTS2* as a candidate gene to participate in important SCZ biological and mechanistic activities and therefore a potential marker for diagnosis and/or therapy. Thus, we decided to depict the molecular mechanisms that can control its expression and/or activity in neuronal-like cells. In these settings, using selective antagonists for these receptors, we observed that only SCH 39166 (a D_1-class receptor antagonist) significantly down-regulated *ADAMTS2* mRNA expression, whereas L-741,626, WAY 100635 and MDL 100907 (antagonists of D_2, 5-HT_{1A} and 5-HT_{2A} receptors, respectively) were devoid of this effect (**Figure 4.7.**).

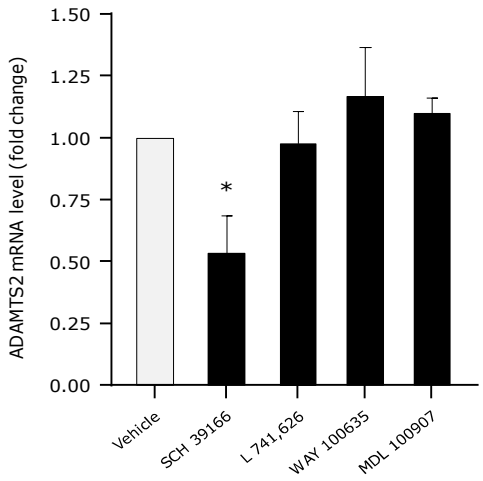


Figure 4.7. Regulation of *ADAMTS2* by selective receptor antagonist. Relative *ADAMTS2* mRNA levels in SK-N-SH cells, analysed by RT-qPCR in cells incubated with the indicated selective antagonist (1h): SCH 39166 (D1-class receptors), L 741,626 (D2-class receptors), WAY 100635 (5-HT1A receptor) and MDL 100907 (5-HT2A receptors) (Drug concentration 1 μM) (N=3-4). Data are mean ± S.E.M; Student's t test: *p < 0.05, **p < 0.01, ***p < 0.001 shows significance with respect to control (Vehicle, grey bars).

Also, in a D_1 receptor context, we first incubated SK-N-SH cells with APDs and studied *ADAMTS2* gene expression upon D_1 receptor activation using SKF 83822. Confirming our previous data, activation of the D_1 receptor triggered *ADAMTS2* mRNA expression. Supporting this observation, SCH 39166, a selective D_1 receptor antagonist, prevented D_1-mediated activation of *ADAMTS2* (**Figure 4.8.**). Interestingly, in these conditions, haloperidol and clozapine significantly blocked D_1-mediated transcriptional activation of *ADAMTS2* (**Figure 4.8.**). Finally, incubation with paliperidone (risperidone's active metabolite 9-hydroxyrisperidone) and aripiprazole did not inhibit *ADAMTS2* activation.

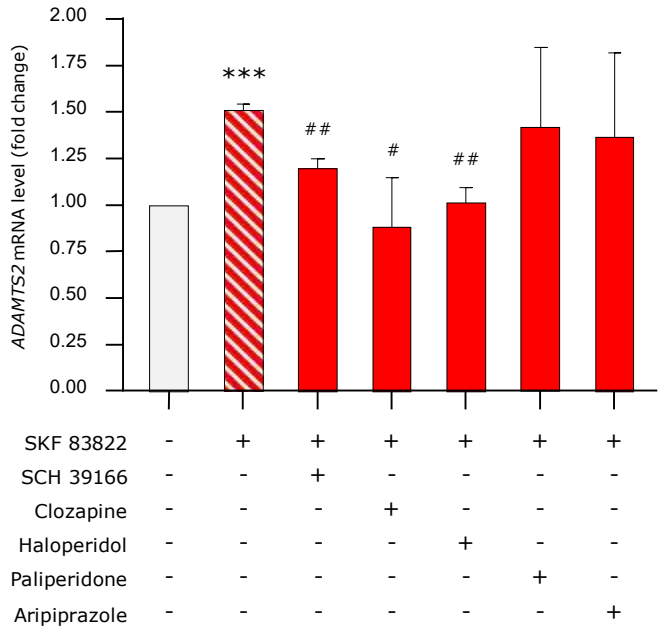


Figure 4.8. Pharmacological dopamine D1 receptor modulation involved in the control of ADAMTS2 gene expression. *ADAMTS2* mRNA levels by RT-qPCR in cells treated with SKF 83822 (1 µM, stripped bar) and pre-incubated with SCH 39166 (1 µM), clozapine (1 µM), haloperidol (1 µM), paliperidone (1 µM) and aripiprazole (1 µM) (N=4-5). Data are mean ± S.E.M; Student's t-test: *p < 0.05, **p < 0.01, ***p < 0.001 vs control condition (grey bar), and #p < 0.05, ##p < 0.01 vs SKF 83822 condition.

In addition, we decided to study whether incubation with selective receptor antagonists could modify the inhibitory effect provoked by clozapine, as it was the most potent APD in our study, over *ADAMTS2* mRNA expression in SK-N-SH cells. Results revealed that the reduction on gene expression induced by clozapine was modified by L-741,626 and MDL 100907 (a D_2 and 5-HT_{2A} receptor antagonists, respectively) but was potentiated by SCH 39166 (although not significantly) (**Figure 4.9.**).

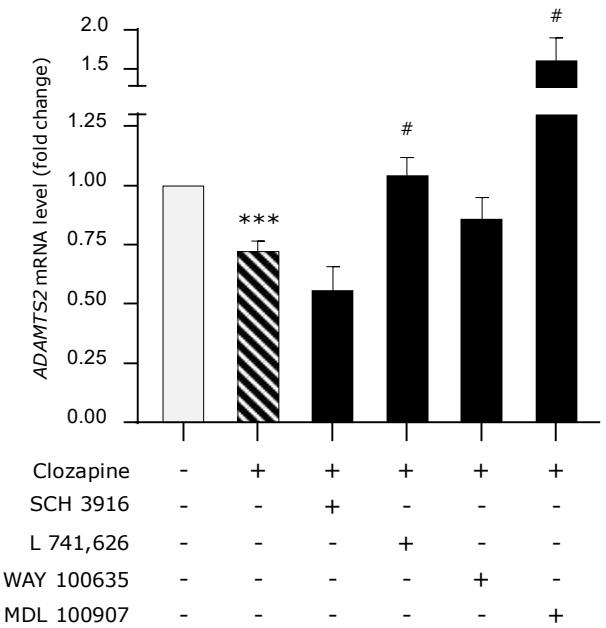


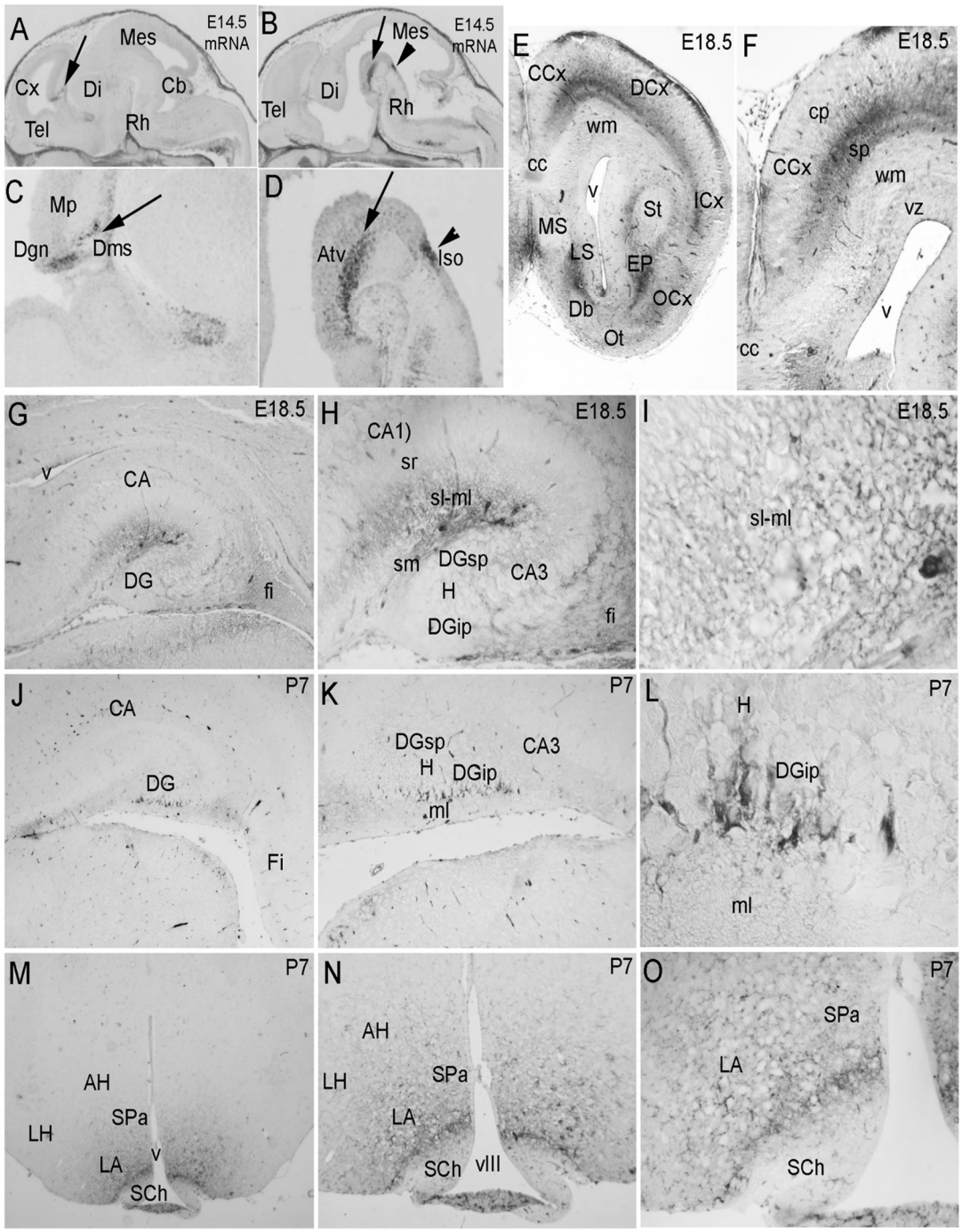
Figure 4.9. Effects of selective antagonist over clozapine-mediated repression of ADAMTS2 mRNA expression. Relative *ADAMTS2* mRNA expression level in SK-N-SH cells pre-incubated 30 min with the indicated selective antagonist: SCH 39165 (D_1-class receptors) (N=3), L 741,626 (D_2-class receptors) (N=3), WAY 100635 (5-HT_{1A} receptor) (N=5) and MDL 100907 (5-HT_{2A} receptor) (N=6), and then incubated for 1 h with clozapine (N=4) (Drug concentration 1 μM). Student's t-test: Data are mean ± S.E.M: *p < 0.05, **p < 0.01, ***p < 0.001 vs control condition (vehicle, grey bars), and #p < 0.05 vs clozapine condition (striped bar).

4.4. *ADAMTS2* gene and protein expression profile in the mouse brain during development.

4.4.1. *ADAMTS2* mRNA expression in embryo mouse brains.

Due to the scarce information that is available about the expression and function of *ADAMTS2* in the literature, we decided to study its expression with special focus in the CNS. We first took advantage of the *in situ* hybridization data publicly available at the 'eurexpress' web page (see methods above) to check for *Adamts2* mRNA expression in different sections of a mouse embryo brain at stage E14.5 (**Figure 4.10. A-D**). Interestingly, in the lateral sections, *Adamts2* expression was very specifically detected in the germinative neuroepithelium and migratory neural stream of the dentate gyrus (DGN and DMS, respectively; **Figure 4.10. A-B**) in the hippocampus, and in the progenitor cells (rhombic leap and migrator stream) of the external granular layer (EGL) and in the cerebellum (Cb). Moreover, in the midline sections (**Figure 4.10. C-D**), *Adamts2* was detected in the ventral tegmental area (VTA), in the tegmental mesencephalon (MES), and, caudally, in the progenitors of the isthmic organizer region (ISO). *Adamts2* was not expressed at this early developmental stage in the cortex (Cx) or in the diencephalon (Di). It is important to note that the regions positive for *Adamts2*

Figure 4.10. Analysis of *ADAMTS2* gene and protein expression profile in the mouse brain during development. A-D) *ADAMTS2* mRNA expression at stage E14.5. *ADAMTS2* expression in the germinative neuroepithelium (Dgn) and migratory neural stream (Dms) of the dentate gyrus (arrows in A,C); In the progenitors cells (rhombic leap and migrator stream) of the external granular layer in the cerebellum (Cb); in the Ventral Tegmental Area (Atv), in the tegmental mesencephalon (Mes), (arrow in B,D), and caudally, in the progenitors of the isthmic organizer region (Iso), (arrowhead in D). E-O). At E18.5 (E-I) and P7 ADAMTSs protein was detected in the neuropiles of the subplate of anterior cingular (CCx) and insular (ICx) cortex, lateral septum (Ls), endopiriform nucleus (EP) and striatal shell region (St)(E,F). In the hippocampus, ADAMTS2 was localized in the stratum lacunosum and moleculare of CA1 (sl-m) and proximal fimbria (fi)(G-I). J-O) At P7 ADAMTS2 protein was detected in some cells and neuropile of the infrapyramidal blade of dentate gyrus (DGip), (J-L) and its related molecular layer (ml), (J-L). Immunoreactivity was also detected in the neuropile of the lateral anterior hypothalamus (LA), with a strong expression in its limit with the suprachiasmatic nucleus (SCh), (M-O).


A
E14.5 mRNA
Mes
Cx
Di
Cb
Tel
Rh
B
E14.5 mRNA
Mes
Di
Tel
Rh
C
Mp
Dgn
Dms
D
Atv
Iso
E
E18.5
CCx
DCx
wm
cc
v
St
lCx
MS
LS
EP
Db
OCx
Ot
F
E18.5
cp
sp
CCx
wm
vz
v
cc
G
E18.5
v
CA
DG
fi
H
E18.5
CA1)
sr
sl-ml
sm
DGsp
CA3
H
DGip
fi
I
E18.5
sl-ml
J
P7
CA
DG
Fi
K
P7
DGsp
CA3
H
DGip
ml
L
P7
H
DGip
ml
M
P7
AH
SPa
LH
LA
v
SCh
N
P7
AH
LH
SPa
LA
SCh
vIII
O
P7
SPa
LA
SCh

expression align with progenitors of dopaminergic and serotonergic neurons (mesocortical and mesolimbic brain pathways) that have been extensively associated with schizophrenia etiopathology.

4.4.2. ADAMTS2 protein expression in embryo and early postnatal mouse brain.

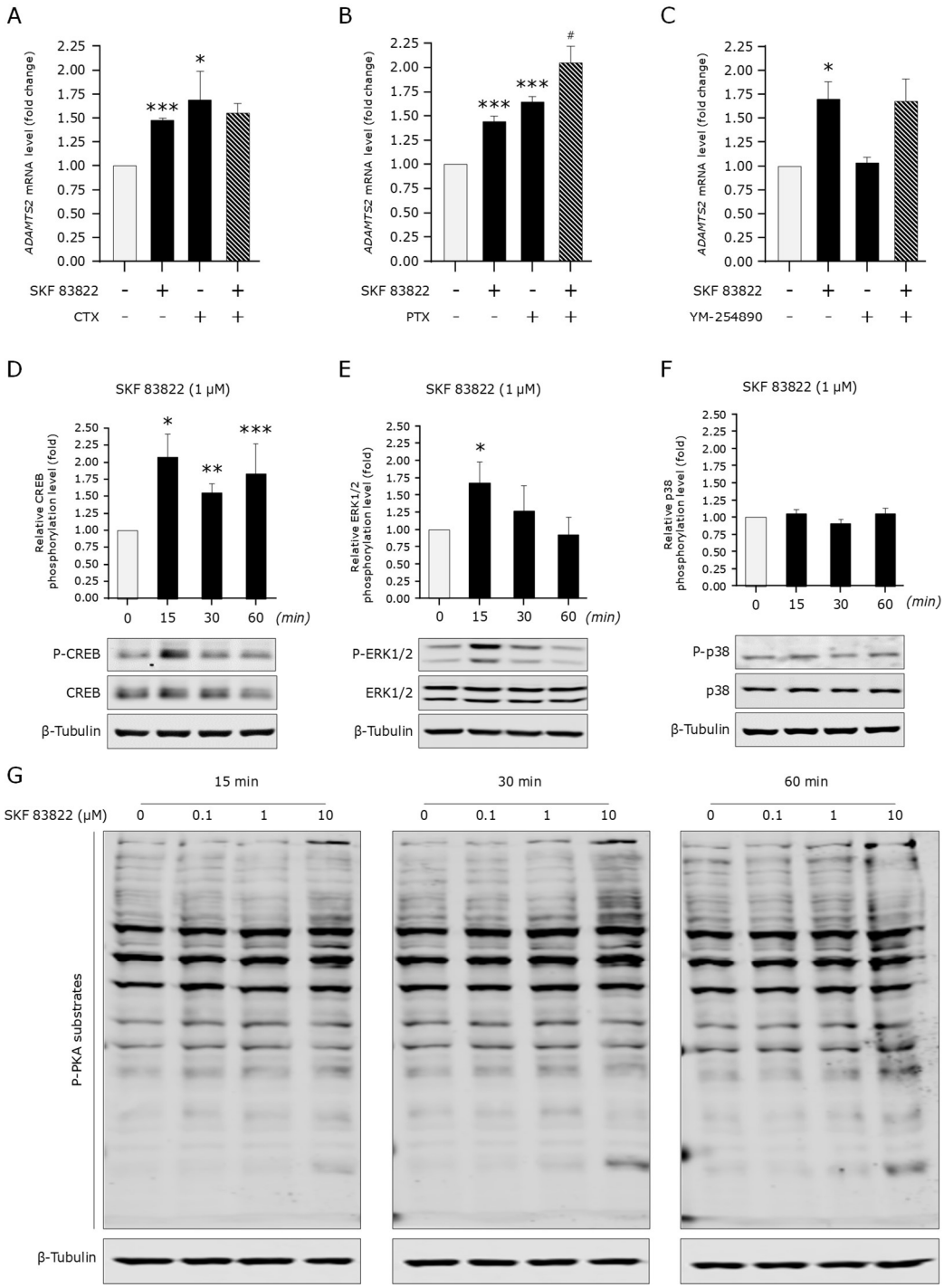
In collaboration with Prof. Salvador Martinez group, in order to detect the expression and the localization of the orthologous version of Adamts2 protein in mice brain, we perform an immunostaining in C57BL/6 wild-type mice brains. We detected ADAMTS2 protein expressed at E18.5 and P7 in the neuropiles of structures where synaptic formation and remodelling are progressing, showing a dynamic pattern (**Figure 4.10. E-L**). At E18.5 the protein was specially accumulated in the subplate of the anterior cingular (CCx) and insular (ICx) cortex, lateral septum (Ls), endopiriform nucleus (EP) and striatal shell region (St; **figure 4.10. E-F**). These regions corresponded with domains where the expression of dopaminergic D_1 and D_2 receptors was detected at this stage (as revealed by the developmental mouse map in the Allen Brain Atlas, http://developingmouse.brain-map.org/). In the hippocampus, ADAMTS2 was localized in the stratum lacunosum and moleculare of CA1 (SL-M) and proximal fimbria (fi; **figure 4.10. G-I**). At P7 the immunoreaction was strongly decreased, and only some cells and neuropiles of the infrapyramidal blade of dentate gyrus (DGip; **figure 4.10. J-L**) and its related molecular layer (ML) were immunoreactive.

4.5. D_1-receptor dependent signalling mechanisms that participate in the control of *ADAMTS2* gene expression.

4.5.1. Molecular mechanisms activated downstream of D_1-receptors in neuronal-like cells.

Since D_1 receptors are known to signal through $G_{\alpha s}$, we sought to confirm its role at controlling *ADAMTS2* expression in our system, alongside $G_{\alpha i}$ and $G_{\alpha q}$. For this purpose, we incubated SK-N-SH cells with cholera toxin ($G_{\alpha s}$ activator), pertussis toxin ($G_{\alpha i}$ inhibitor) and YM-254890 (a specific $G_{\alpha q}$ inhibitor) (Zhang et al. 2019), before activating D_1 receptors with SKF 83822. The activation of $G_{\alpha s}$ (**Figure 4.11. A**) and the inhibition of $G_{\alpha i}$ (**Figure 4.11. B**) *per se* significantly up-regulated *ADAMTS2* gene expression. Moreover, the inhibition of $G_{\alpha i}$ potentiated *ADAMTS2* expression after D_1 receptor activation (**Figure 4.11. B**). Finally, the inhibition of $G_{\alpha q}$ did not modify the basal and the SKF 83822-mediated increase of *ADAMTS2* expression (**Figure 4.11. C**). In addition, incubation of neuronal-like cells with the selective D_1 receptor agonist (SKF 83822) provoked rapid phosphorylation of CREB and ERK1/2 (15 minutes) and no changes in p38, that was used as a negative control (**Figure 4.11. D-F**). Additionally, rapid phosphorylation of PKA substrates was detected in response to SKF 83822 which, in this

Figure 4.11. Molecular mechanisms triggered downstream of dopamine D_1 activation. (A) *ADAMTS2* mRNA levels by RT-qPCR in cells incubated for 1h with SKF 83822 (D_1-class receptors selective agonist; 1 μM) (N=4) and cholera toxin (CTX; $G_{\alpha s}$ subunit activator, 100 ng/ml), or pre-incubated 30 min with CTX and then incubated with SKF 83822 for 1h (red bar) (N=3-4). (B) *ADAMTS2* mRNA levels by RT-qPCR in cells incubated with SKF 83822 and pertussis toxin (PTX; $G_{\alpha i}$ subunit inhibitor, 50 ng/ml), or pre-incubated 30 min with PTX and then incubated with SKF 83822 for 1h (blue bar) (N=5). (C) *ADAMTS2* mRNA levels by RT-qPCR in cells incubated for 1h with SKF 83822 and YM-254890 ($G_{\alpha q}$ subunit inhibitor, 1 μM), or pre-incubated 30 min with YM-254890 and then incubated with SKF 83822 for 1h (striped bar) (N=3). (D-F) Western-blots showing relative phosphorylation levels of CREB, ERK1/2 and p38 in SK-N-SH cells incubated with SKF 83822 (1 μM), as indicated for 15-, 30- and 60-min; graph bars indicate quantification of fold activation (N=3-5); one-way ANOVA for multiple comparations. And (G) Western-blots showing relative phosphorylation levels of PKA substrates in SK-N-SH cells incubated with SKF 83822 at the indicated times and agonist concentrations (N=1); pictures show a representative blot of each condition. Data are mean ± S.E.M: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ vs control condition; and #$p < 0.05$ vs activation with SKF 83822.


A
ADAMTS2 mRNA level (fold change)
SKF 83822
CTX
B
ADAMTS2 mRNA level (fold change)
SKF 83822
PTX
C
ADAMTS2 mRNA level (fold change)
SKF 83822
YM-254890
D
SKF 83822 (1 μM)
Relative CREB phosphorylation level (fold)
(min)
P-CREB
CREB
β-Tubulin
E
SKF 83822 (1 μM)
Relative ERK1/2 phosphorylation level (fold)
(min)
P-ERK1/2
ERK1/2
β-Tubulin
F
SKF 83822 (1 μM)
Relative p38 phosphorylation level (fold)
(min)
P-p38
p38
β-Tubulin
G
15 min
30 min
60 min
SKF 83822 (μM)
P-PKA substrates
β-Tubulin

setting, was also used as a positive control, to show that SKF 83822 triggers D_1 receptor downstream signalling through $G_{\alpha s}$/AC/cAMP and PKA signalling axis (**Figure 4.11. G**).

To evaluate CREB-dependent transcriptional activity in our system, we took advantage of a CRE-luciferase reporter gene (CRE-luc; see Methods). Incubation with SKF 83822 induced a concentration-dependent increase of CREB activity, which was also observed with forskolin, that was used as positive control for AC/cAMP-mediated CREB activation (**Figure 4.12. A**). Following our rationale, we analysed whether APDs could counteract the intracellular signalling activation mediated by selective D_1 receptor agonist SKF 83822. In these conditions, the results show that preincubation with clozapine, but not haloperidol, paliperidone or aripiprazole, prevented SKF 83822-induced CRE-luc activation (**Figure 4.12. B**). Also, a selective D_1 receptor antagonist like SCH 39166 prevented D_1 receptor-mediated activation of CREB (**Figure 4.12. B**).

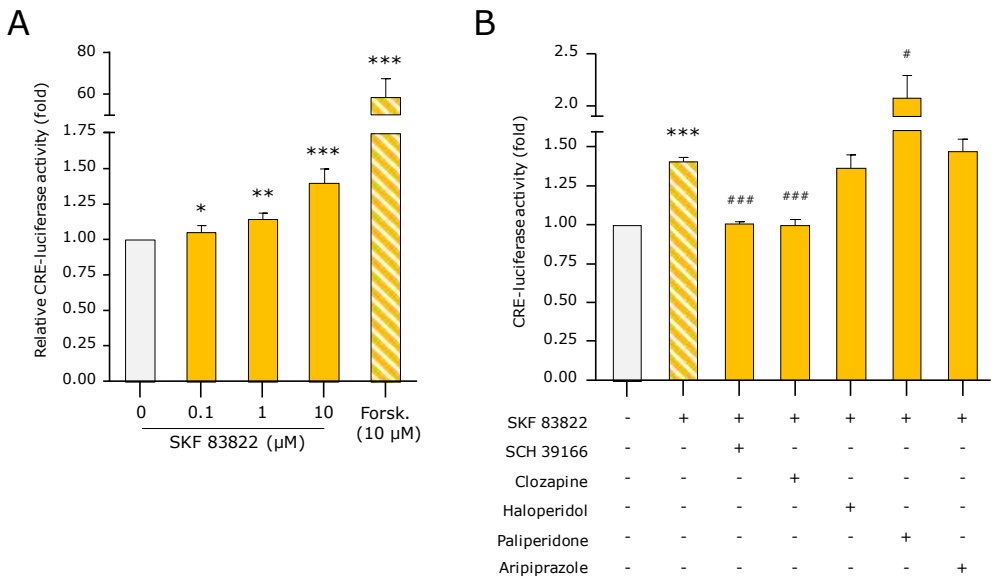


Figure 4.12. Pharmacological dopamine D_1-dependent CREB activity modulation in SK-N-SH cells. CREB activity in cells transfected with CREB-Luc alongside pRL-Null. (A) Cells were incubated for 24 h with the indicated concentrations of SKF 83822 (0.1, 1 and 10 µM) and forskolin (10 µM; stripped bar) (N=5); and (B) cells were pre-incubated for 1 h with the indicated APDs and then, incubated for 24 h with SKF 82833 (10 µM; stripped bar). Data are mean ± S.E.M; Student's t-test: *p < 0.05, **p < 0.01, ***p < 0.001 vs control condition, and #p < 0.05, ##p < 0.01 vs SKF 83822 condition.

In addition, we studied the signalling and activity of other kinases involved downstream the other receptors targeted by APDs, using selective D_2 (7-OH-DPAT), 5-HT_{1A} (8-OH-DPAT) and 5-HT_{2A} (TCB-2) receptor agonist (**Figure 4.13.**). Results show that incubating with 7-OH-DPAT agonist these cells, CREB activity decreases significantly (**Figure 4.13. A and D**). By contrast, using 5-HT_{1A} agonist no changes were detected both in signalling and CREB activity (**Figure 4.13. B and E**). Finally, using 5-HT_{2A} agonist, we observed a rapid phosphorylation of ERK but not of CREB and significant increment of NFAT activity detected by a luciferase reporter assay (**Figure 4.13. C and F**).

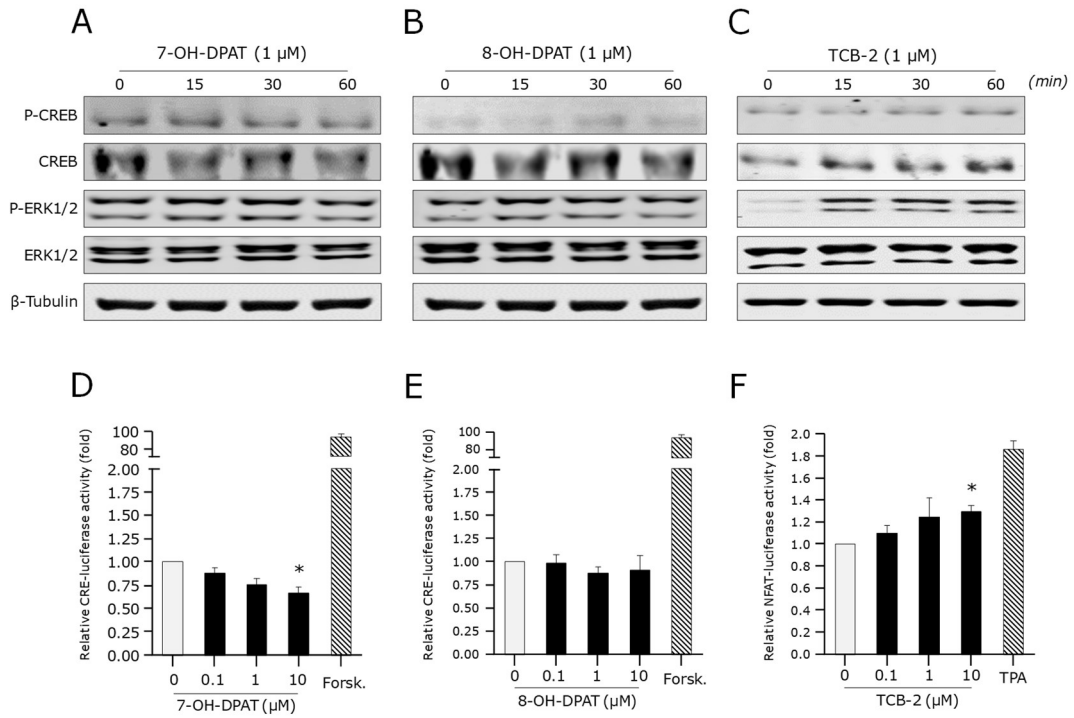


Figure 4.13. Molecular mechanisms triggered downstream of D_1, 5-HT_{1A} and 5-$HT_{2A/2C}$ receptors activation in SK-N-SH cells. SK-N-SH cells were incubated with selective agonist: 7-OH-DPAT (D_2-class receptors), 8-OH-DPAT (5-HT_{1A} receptor) and TCB-2 (5-$HT_{2A/2C}$ receptor): (A-C) show western-blots with phosphorylation levels of CREB and ERK1/2 in cells incubated with selective agonist (1 μM) for the indicated times (0, 15, 30 and 60 min) (N=3); and (D-E) show CREB activity in cells transfected with CRE-Luc and (F) shows NFAT activity in cells transfected with NFAT-Luc alongside pRL-Null, and incubated for 24 h with the indicated concentrations of selective agonist (0.1, 1 and 10 μM), and forskolin (10 μM) or TPA (10 ng/ml) used as positive control (stripped bar). N=3. Pictures show a representative blot of each condition. Data are mean ± S.E.M; Student's t-test: *p < 0.05, **p < 0.01, ***p < 0.001 vs control condition.

4.5.2. Specific inhibitors of PKA and MEK can modulate SKF 83822-dependent activation of *ADAMTS2* expression.

To evaluate the potential contribution of PKA and ERK to the activation of *ADAMTS2* expression, we incubated SK-N-SH cells with SKF 83822 D_1 receptor agonist in combination with specific MAPK/ERK (selumetinib-AZD6244) and cAMP/PKA (H89 dihydrochloride) inhibitors. Both inhibitors prevented D_1 receptor induced expression of *ADAMTS2* (**Figure 4.14. A**), although only a cAMP/PKA inhibitor abrogated CREB-dependent transcription and phosphorylation (**Figure 4.14. B-C**).

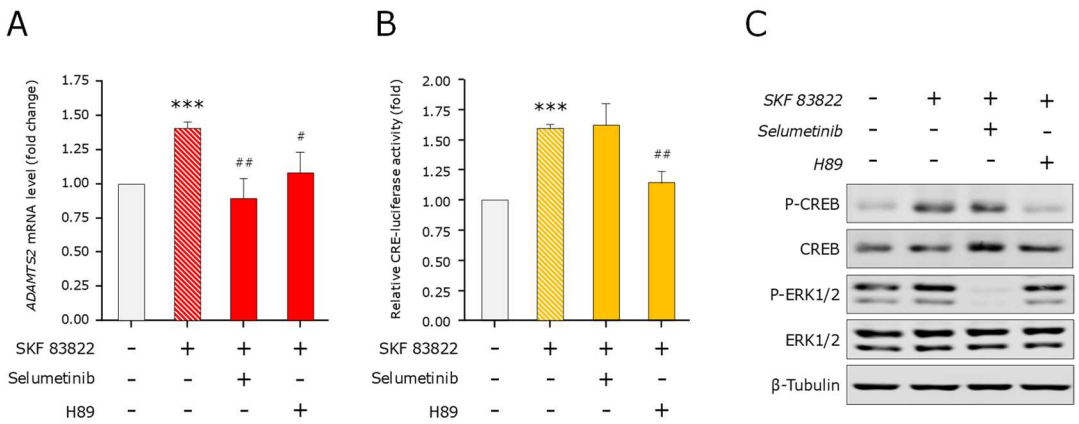


Figure 4.14. Pharmacological PKA- and MEK-dependent signalling in SK-N-SH cells. SK-N-SH cells were pre-incubated for 1 h with MAPK/ERK and cAMP-PKA inhibitors (selumetinib 1 μM and H89 10 μM, respectively) and then, incubated with SKF 82833 (RT-qPCR: 1 μM (1 h); WB: 1 μM (15 min); Luciferase: 10 μM (24 h)). (A) Shows *ADAMTS2* mRNA levels by qRT-PCR (N=6); (B) shows CREB activity in cells transfected with CREB-Luc alongside pRL-Null (N=4); and (C) western-blots showing relative phosphorylation levels of CREB and ERK1/2. Blots are representative images of each western-blot (N=3). Data are mean ± S.E.M; Student's t-test: ***p < 0.001 vs vehicle (control condition), and #p < 0.05, ##p < 0.01 vs SKF 83822 condition.

4.6. Transcriptional mechanisms controlling *ADAMTS2* gene expression downstream of dopamine D_1 receptors.

4.6.1. *ADAMTS2* expression is trigged through cAMP/PKA-dependent activation downstream D_1 receptor.

Our previous data suggest that there is a rapid activation of the signalling mechanisms controlling CREB activation after D_1 receptor activation that can be modulated pharmacologically. We decided to challenge the contribution of cAMP/CREB signalling to the transcriptional activation of *ADAMTS2*. We initially evaluated the activation of *ADAMTS2* gene expression together with *C-FOS*, an "early response gene" known to be a direct transcriptional target of CREB that was used as control. Our data showed that SKF 83822 activated mRNA expression of both *ADAMTS2* and *C-FOS*. Interestingly, incubation with forskolin alone (a specific adenylyl cyclase activator) was sufficient to trigger the transcription of both genes, whereas TPA (a PKC/MAPK activator) only activated *C-FOS* transcription (**Figure 4.15.**).

4.6.2. CREB1 is essential in the *ADAMTS2* mRNA transcription.

To explore the implication of CREB in D_1-mediated ADAMTS2 expression, we generated two stable *CREB1* knock-down SK-N-SH cells with GFP reporter by two approaches: (1) lentiviral inducible shRNA construct inducible by doxycycline (**Figure 4.16. A**); and (2) transfection of stably expression shRNA construct (**Figure 4.16. B**). Using this approach, we reduced CREB mRNA and protein expression, as shown in **figure 4.16.** by both approaches.

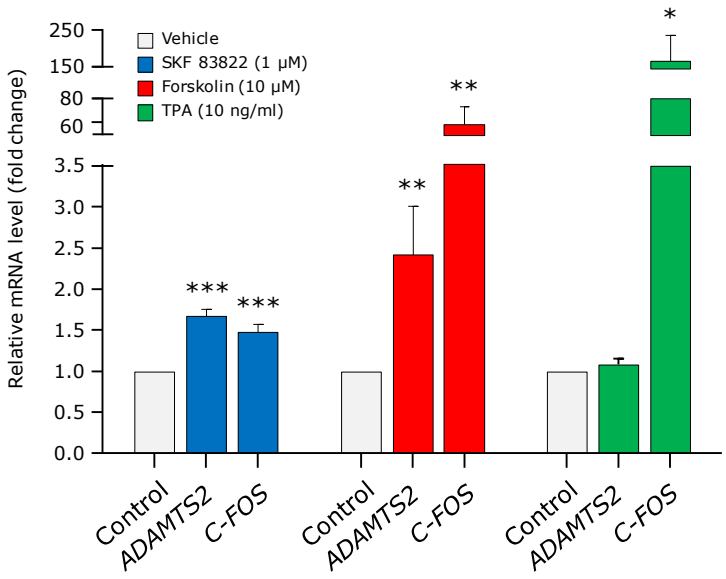


Figure 4.15. Control of ADAMTS2 mRNA expression through PKA-dependent and independent signaling activation. qRT-PCR showing relative ADAMTS2 and C-FOS mRNA expression level in cells treated for 1 hour with SKF 83822 (1 µM; N=5; blue bar), forskolin (10 µM; N=3; red bar) and TPA (10 ng/ml; N=3; green bar). Data are mean ± S.E.M; Student's t-test: *p < 0.05, **p < 0.01, ***p < 0.001 vs vehicle (control; grey bar).

In CREB knock-down cells, SKF 83822 and forskolin failed to induce the expression of *ADAMTS2* (**Figure 4.17. A and C**). Interestingly, SKF 83822 did not significantly activate *C-FOS* expression in CREB knocked down cells when compared to control cells. In contrast, TPA (a PKC/MAPK activator) indeed activated *C-FOS* expression (**Figure 4.17. B and D**).

Mechanistically, reduced CREB expression did not impair ERK phosphorylation elicited downstream of D_1 receptor activation by SKF 83822 (**Figure 4.18. A and D**). In this context, we also used forskolin and TPA as controls for specific activation of CREB (through PKA activation) and ERK, respectively (**Figure 4.18. B and C;** and **Figure 4.19.**). Thus, activation of CREB seemed to play a key role in the control of *ADAMTS2* gene expression downstream of D_1 receptors.

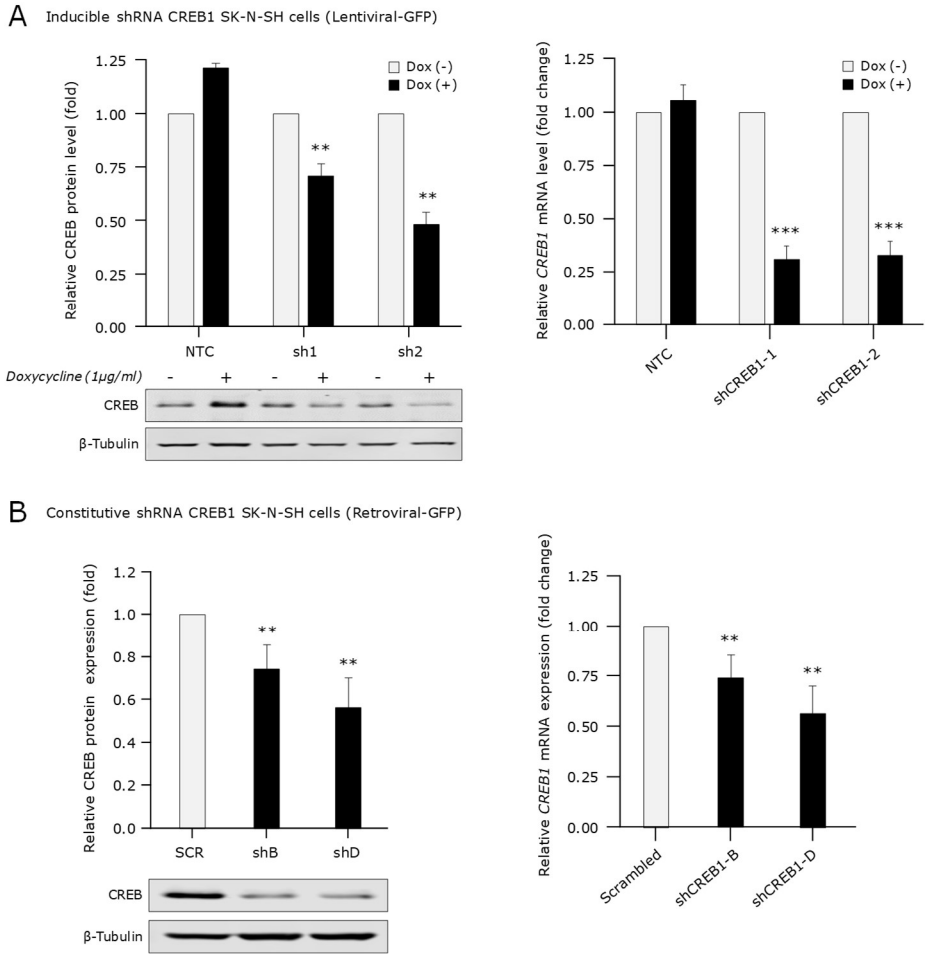


Figure 4.16. CREB1 knock-down by short hairpin (sh) RNA sequences in SK-N-SH cells. (A) CREB1 knock-down SK-N-SH cells by lentiviral inducible shRNA and GFP reporter construct (green bars), incubated with doxycycline (1 µg/ml) for 72 h, CREB protein total expression (left) and CREB1 mRNA level (right) in non-targeted control (NTC) or shCREB1 cells (N=3); and (B) CREB1 knock-down SK-N-SH cells by transfection of stably expression shRNA and GFP reporter construct (yellow bars), CREB protein total expression (left) and CREB1 mRNA level (right) in scrambled (SCR) or shCREB1 cells (N=3). Images are representative of independent experiments. All cells were selected with puromycin (1 µg/ml) at least 7 days. Data are mean ± S.E.M; Student's t-test: **p < 0.01, ***p < 0.001 vs NTC/SCR-doxycycline(-).

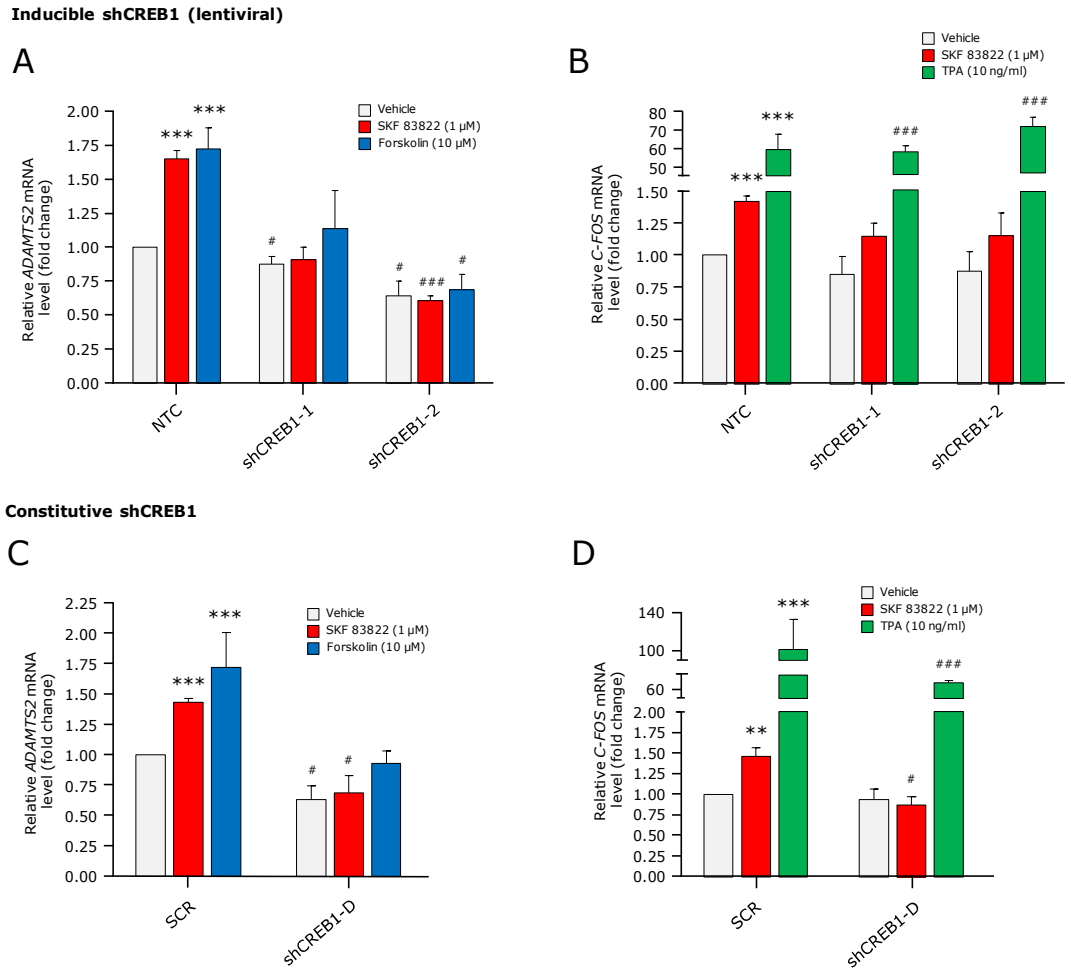


Figure 4.17. Transcriptional mechanism CREB-dependent that control *ADAMTS2* mRNA expression. CREB Knock-down SK-N-SH cells were incubated for 1 h with SKF 83822 (1 μM, red bars), forskolin (10 μM, blue bars) and TPA (10 ng/ml, green bars). RT-qPCR showing relative (A) *ADAMTS2* and (B) *C-FOS* mRNA expression in inducible shCREB1 or non-targeted control (NTC) cells incubated with doxycycline (1 μg/ml) for 72 h (N=3); and RT-qPCR showing relative (C) *ADAMTS2* and (D) *C-FOS* mRNA expression in stably expressing shCREB1 or scrambled (SCR) cells (N=3). All cells were selected with puromycin (1 μg/ml) at least 7 days. Data are mean ± S.E.M; Student's t-test: **p < 0.01, ***p < 0.001 vs NTC/SCR-vehicle; and #p < 0.05, ###p < 0.001 vs each condition in NTC/SCR cells.

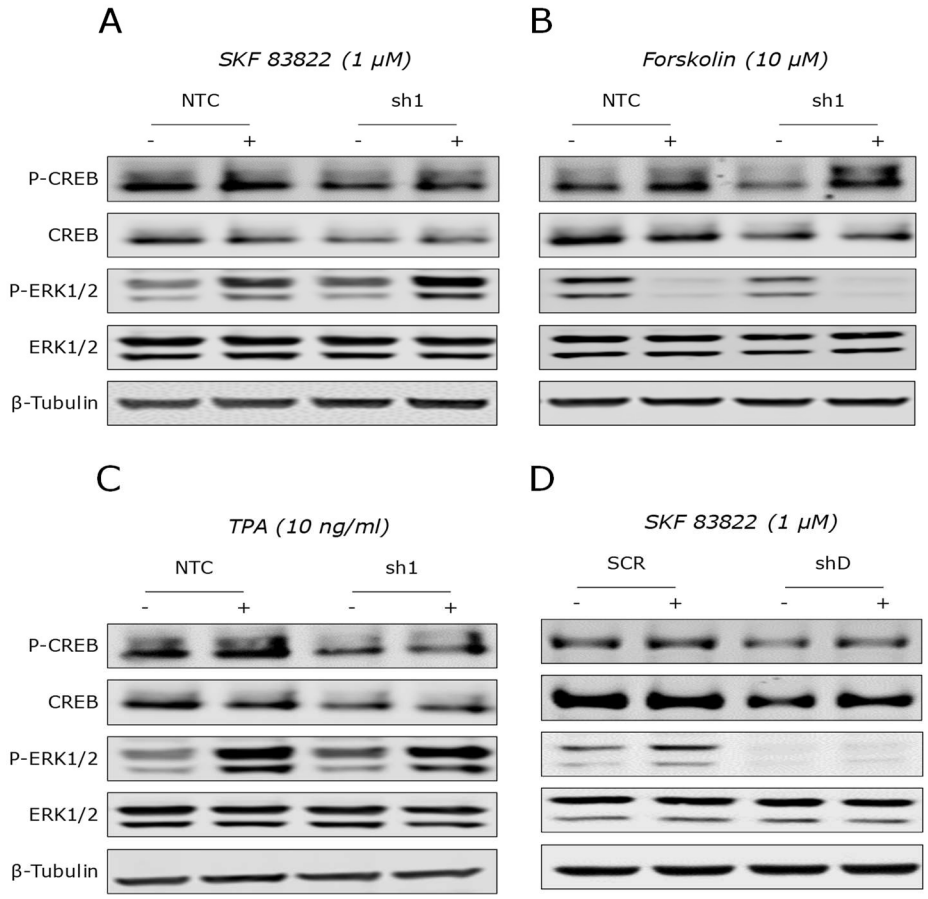


Figure 4.18. CREB-dependent and independent signalling downstream dopamine D_1 receptor in SK-N-SH cells. Western-blots using anti-phospho-CREB and anti-phospho-ERK as well as anti-CREB and anti-ERK antibodies. Inducible shCREB1 or non-targeted control (NTC) SK-N-SH cells incubated with doxycycline (1 μg/ml) for 72 h and then, incubated for 15 min with (A) SKF 83822 (1 μM), (B) forskolin (10 μM) and (C) TPA (10 ng/ml) (N=3); and (D) stably expressing shCREB1 or scrambled (SCR) SK-N-SH cells incubated for 15 min with SKF 83822 (1 μM) (N=3). All cells were selected with puromycin (1 μg/ml) at least 7 days. Blots are representative images of each western blot.

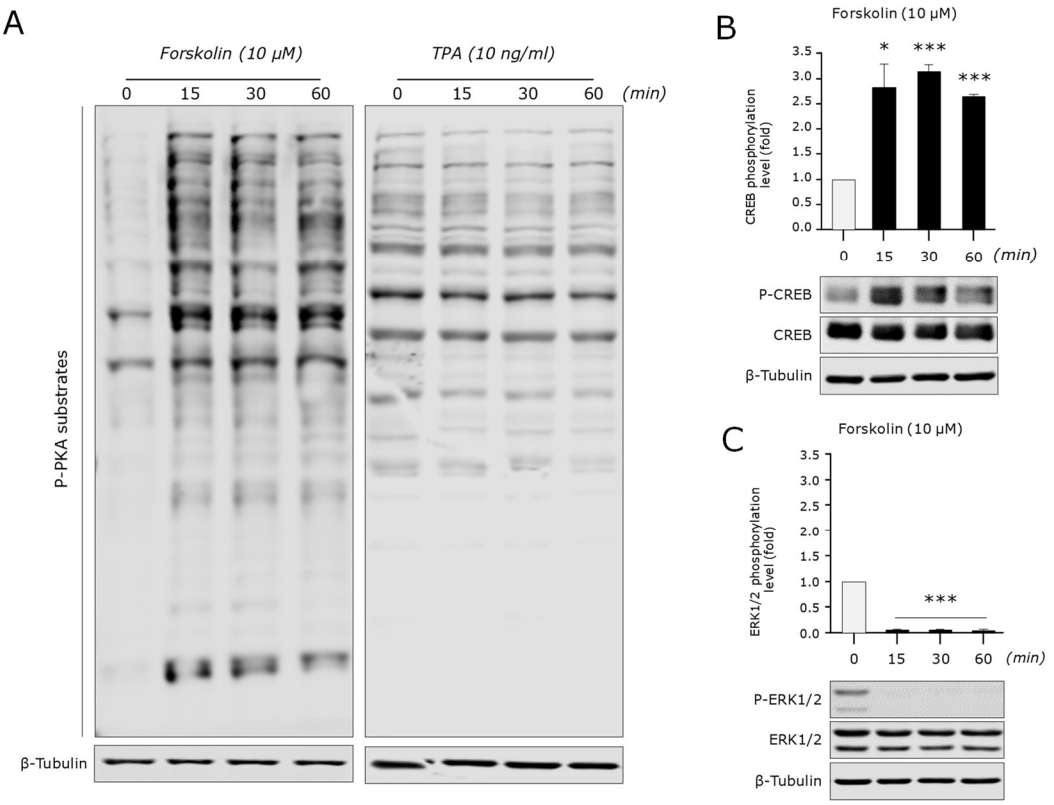


Figure 4.19. PKA-dependent and independent signaling activation. (A) Western-blots showing relative phosphorylation levels of PKA substrates in SK-N-SH cells incubated with forskolin (10 μM) and TPA (10 ng/ml), at the indicated time points (0, 15, 30 and 60 min) (N=1); and relative phosphorylation levels of CREB (C) and ERK1/2 (D) in SK-N-SH cells incubated with forskolin (10 μM), at the indicated times (N=3). Images are representative of each western blot. Data are mean ± S.E.M; Student's t-test: *p < 0.05, ***p < 0.001 vs 0 h.

4.7. Mechanism that control *ADAMTS2* in sample from clinically characterized patients.

4.7.1. Comparative CREB binding vs. relative *ADAMTS2* mRNA expression in drug-naïve and APD treated SCZ patients.

To evaluate the possible role of CREB activity in SCZ patients, we took advantage of a specific CREB DNA-binding ELISA-based assay (see methods). This approach enables to

quantify CREB binding to specific CRE elements in the DNA, using nuclear protein extracts from patient samples. For this, we used samples from the first cohort of patients. Due to the reduced availability of samples in our biobank we worked with a reduced number of cases.

Our findings, although should be taken as preliminary, showed a tendency where CREB total binding was reduced after treatment in this subset of SCZ responder patients to APDs (**Figure 4.20. A**). This observation was aligned with the downregulation of *ADAMTS2* gene expression after 3-month of APD treatment in the same patients (**Figure 4.20. B**).

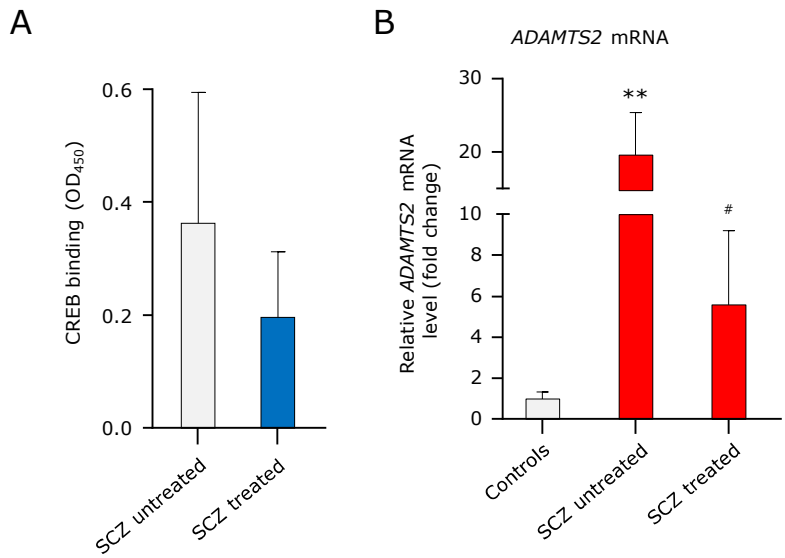


Figure 4.20. Comparative CREB binding vs. relative ADAMTS2 mRNA expression in SCZ drug-naïve and treated patients. SCZ patients and controls were selected to have a new sub-cohort for this experiment. (A) Shows CREB binding (OD_{450} units; red bars) in PBMCs from SCZ patients and controls; and (B) shows relative ADAMTS2 mRNA expression (blue bars) in PBMCs from SCZ patients and controls. Conditions: healthy controls, untreated (first-episode psychosis) and 3-months treated patients. Data are mean ± S.E.M; unpaired Student's t-test: *p < 0.05 and **p < 0.01 compares patients at baseline vs. healthy controls; paired Student's t-test: ##p < 0.01, ###p < 0.001 compares patients at baseline vs. those after 3-month treatment with APs (Cohort selected: N=7).

4.7.2. Study of the predicted binding sites in the *ADAMTS2* gene promoter.

These results strongly suggested a role for the transcription factor CREB as a regulatory element in the expression of *ADAMTS2* mRNA. We therefore hypothesized that CREB might influence the up-regulation of *ADAMTS2* mRNA in SCZ patients observed at the onset of the pathology. To confirm the binding of this TF in this promoter region, we used ChIP analysis on *ADAMTS2* regulatory sequences. As explained in the introduction, CREB proteins have a glutamine-rich Q2 domain which binds the canonical CRE site (5'-TGACGTCA-3') responsible for binding with RNA polymerase II initiation complex (Altarejos and Montminy 2011). We first analysed *ADAMTS2* with the help of 'Genomatix Matinspector' software (see methods) and found that the sequence between -2.5 kb and +2.5 kb relative to the transcription initiation site (+1), contained a number of CRE sites with the canonical nucleotide sequence TGACGTCA, as well as NFAT and AP-1 sites elements. In **figure 4.21.**, we show a schematic representation of this gene promoter, highlighting the CRE, NFAT and AP-1 sites, as well as the primers used in

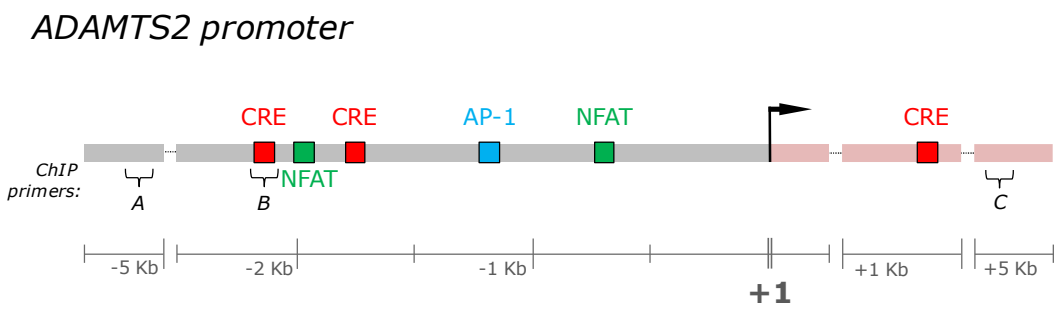


Figure 4.21. Predicted binding sites in the ADAMTS2 gene promoter region. Schematic representation of predicted CRE, NFAT and AP-1 sites in the upstream promoter region of human *ADAMTS2* gene (based on the NCBI human reference sequence), and qPCR primers used for ChIP assays. Grey colour: promoter region; pink colour: *ADAMTS2* gene.

chromatin immunoprecipitation (ChIP)-coupled quantitative PCR analysis. Also, in **table 4.3.**, we show information relative to the sequence of the predicted sites and their anchor position.

Table 4.3. List of transcription factors, location and sequence of the respective binding sites in *ADAMTS2* promoter. Binding sites predicted by 'MatInspector software' analysis. *Capital letter: Anchor position.*

Site	Location	Sequence	Transcription factor
CRE	-2135	ttgttcTGACgtgattggaag	cAMP-response element-binding protein
NFAT	-1974	atttatGGAAacatggtga	Nuclear factor of activated T-cells
CRE	-1758	tggaacccaTGACgcattcct	cAMP-response element-binding protein
AP-1	-1194	agaTGACtcaggc	Activator protein 1
NFAT	-863	ttgggtGGAAaaggtaggc	Nuclear factor of activated T-cells
CRE	1323	cgtgcgcacTGACgcccaggg	cAMP-responsive element binding protein
NFAT	2218	acgcaaGGAAacaggaaga	Nuclear factor of activated T-cells

For this purpose, we obtained PBMCs isolated from blood samples from two characterized SCZ patients obtained at the first episode of psychosis (onset) and a healthy donor (still uncharacterized but considered as control in this thesis). The reason we could only obtain limited samples in due to the complications encountered in obtaining new patient samples and controls with the appropriate informed consent, as well as the limitations in time for their collection. Therefore, we must take this data as preliminary results of a project that still in development. Chromatin from PBMCs was subjected to IP with CREB-specific antibodies and DNA fragments were amplified by qPCR. To determine where CREB binds to *ADAMTS2*, we analysed the following three different regions of *ADAMTS2*: the 5' promoter region (amplicon 1; anchor position -5000), the proximal promoter CRE site (amplicon 2; anchor position -2135) and a region used as a negative control of the CREB

recruitment (amplicon 3; anchor position +5000) (**Figure 4.21.**). We observed more binding of CREB to the regulatory region of *ADAMTS2* comprising the CRE site (position -2135) in SCZ patients than in the control (**Figure 4.22.**). These data support the idea of a positive role for CREB in the differential up-regulation expression observed in SCZ patients at the onset of the pathology, which can be explained by an enrichment of CREB direct binding to the CRE site in the regulatory region of *ADAMTS2* in PBMCs from schizophrenia patients.

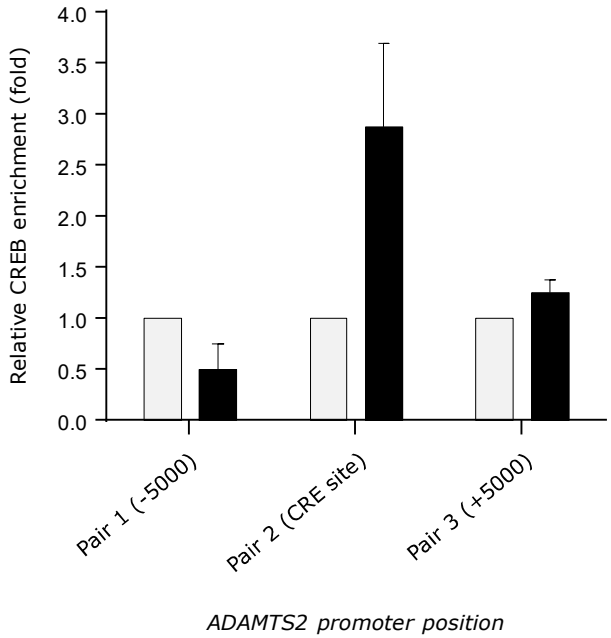


Figure 4.22. Enrichment of CREB at the *ADAMTS2* promoter in SCZ patients. ChiP-coupled quantitative PCR analysis of the enrichment of CREB at various positions along *ADAMTS2* (horizontal axis) in human PBMCs from controls (grey bar) and SCZ patients at onset (FEP, black bars), assessed with antibody to CREB (anti-CREB) and presented relative to results obtained with control IgG (N=2). Data are mean ± S.E.M.

DISCUSSION

5. DISCUSSION

Our results herein revealed that the control of *ADAMTS2* expression seems to be directly associated with dopaminergic signalling, primarily with the D_1-class receptors, and downstream of these through cAMP/CREB and MAPK signalling. *ADAMTS2* mRNA and protein were specifically expressed in mesolimbic and mesocortical dopaminergic regions in mice. Thus, D_1 receptor signalling towards CREB (cAMP response element-binding protein) activation and its effects on *ADAMTS2* gene expression might be linked to biological mechanisms involved in schizophrenia development and to clinical efficacy of antipsychotic drugs.

5.1. *ADAMTS2* expression in first episode SCZ patients and its regulation by APD treatment.

In the context of the local PAFIP program, we collected two new cohorts of drug-naïve SCZ patients with clinical data at baseline and after 3-month follow-up. These cohorts included mRNA samples from peripheral blood mononuclear cells (PBMCs) that were taken before and after treatment using APDs from individuals randomly selected out from a larger set of FEP individuals followed up in the PAFIP program. These cohorts showed significant within-subject clinical changes between baseline data and after 3 months of treatment with risperidone and aripiprazole (see clinical rate scales in **tables 4.1. and 4.2.**). Using these cases alongside healthy controls, we attempted an independent validation of our previous results using a different cohort of cases (Crespo-Facorro et al. 2015; Sainz et al. 2013). We comparatively quantified the mRNA expression of all six CRGAs (*ADAMTS2*, *CD177*, *CNTNAP3*, *ENTPD2*, *RFX2* and *UNC45B*) at baseline, 3 months after treatment (cohort #1), and also at 12-month treatment with APDs (cohort #2) in PBMCs. Remarkably, amongst the six genes analysed, *ADAMTS2* emerged as a significant

gene that was highly overexpressed at baseline in drug-naïve SCZ individuals with respect to controls and which returned to "normal" levels in clinical responders at 3-month and 12-month treatment (**Figures 4.4. and 4.5.**). Unexpectedly, in these conditions, we failed to replicate significant differences in the rest five genes studied in this new cohort of patients (all p-values > 0.008) (**Figure 4.4.**). Possibly, this can be explained by experimental design and scope differences between previous and current experiments (i.e., whole transcriptome mRNA-seq versus targeted RT-qPCR) which indeed account for these discrepancies (Fang and Cui 2011).

Due that there is not always a direct correlation between the biomarkers obtained in peripheral blood cells and CNS samples, and the problems to obtain brain biopsies from patients, we decided to analyse the expression of the orthologous version of these genes in schizophrenia-like phenotype mice. This study revealed a significant overexpression of at least three of the six total CRGAs (*Cd177*, *Cntnap3* and *Entpd2*) and other two genes (*Adamts2* and *Rfx2*) showed a tendency to be overexpressed when compared with control values (**Figure 4.3.**). This profile, despite it is not consistent with the expression data observed in the new cohort of patients, confirms two important matters: an up-regulated expression of *Adamts2* gene in a preclinical model of schizophrenia and the detectable expression of *Adamts2* in in the CNS, specifically in cortical tissue from mouse brain. This data encouraged us to explore *Adamts2* mRNA and protein expression in whole mice brains with special focus on relevant brain areas related with the etiopathology of schizophrenia (i.e. such as hippocampus and VTA).

Using neuronal-like cells (SK-N-SH), we observed that APDs can dynamically modulate CRGAs expression (**Figure 4.2.**). These dynamics include early as well as delayed transcriptional responses that may imply direct or indirect control of gene transcription through mechanisms elicited downstream of the activity of APDs over their cognate receptors but also by changes in the chromatin conformation or second and third rounds

of transcriptional regulation. In agreement with the results exposed above in patients (**Figures 4.4. and 4.5.**) but in contrast with our previous observations also in patients from a different cohort (Crespo-Facorro et al. 2015), only *ADAMTS2* mRNA expression was inhibited after treatment of neuronal-derived cells with risperidone and/or aripiprazole (two of the most currently utilized antipsychotics as first-line treatment). From a dynamic perspective, *ADAMTS2* gene expression was quickly (less than 1 hour) and robustly downregulated by all APDs (**Figure 4.2.**). Moreover, whereas the activity of risperidone and aripiprazole at downregulating *ADAMTS2* mRNA was transitory in these cells, those of clozapine and haloperidol were sustained to up to 24 h. The molecular effects exerted by clozapine and haloperidol compared to other APDs might warrant further investigations. Probably, these drugs have more complex effects beyond short-term effects by their receptor affinity, particularly, in the long term, involving intracellular mechanisms that may regulate neural functionality, neuroplasticity and neurogenesis (Aringhieri et al. 2018; Fumagalli et al. 2009; Molteni et al. 2009). Interestingly, clozapine was also highly, even more, effective at reducing CRGAs expression when compared with other APDs. The molecular effects elicited by clozapine compared to other APDs might also deserve further investigations in order to clarify its possible association with its unique established superior efficacy for treatment-resistant schizophrenia, for suicidality and its apparent ability to decrease substance use in schizophrenia patients (Khokhar et al. 2018).

Finally, blood-based biomarkers may enable prediction of the type, timing, course, and response to treatment, and may ultimately enable disease subtyping and patient stratification (Chan et al. 2011). In this regard, back to our data in patients, we found that transcription of *ADAMTS2* gene is highly associated to different SCZ clinical stages. Thus, *ADAMTS2* might play a key, and yet to be defined, mechanistic role in both the illness onset and clinical responses, regardless of the type of APD used. Furthermore, it is important to highlight that PAFIP programme standardized the methods for sample

collection, storage, and sample processing of biofluid sources of biomarkers. However, there is still a long way to go before biomarkers become part of the standard clinical care for schizophrenia patients. Before we get there, there are several things that need to be improved upon (e.g. conduct larger studies for more statistical power and candidate biomarkers need to be tested for sensitivity and specificity to resolve overlaps with related disorders) (Lakhan and Kramer 2009).

5.2. Dopaminergic signalling involved in the regulation of *ADAMTS2* expression.

5.2.1. *ADAMTS2* expression is located in 'mesocorticolimbic' system in mice brains.

There is scarce information regarding specific *ADAMTS2* expression and activity in the CNS. As it mentioned above, we demonstrate that *Adamts2* gene is expressed in mPFC in mice (**Figure 4.3.**). To better understand its potential role in SCZ, we decided to explore the orthologous *Adamts2* gene expression in the mouse CNS, specifically in C57BL/6 mice brains in collaboration with Prof. Salvador Martinez group. First, we took advantage of the *in situ* hybridization data available at the 'eurexpress' web page for mouse embryo brain. We detected *Adamts2* mRNA at stage E14.5 in the dentate gyrus (DG) in the hippocampus and the ventral tegmental area (VTA), among other brain regions (**Figure 4.10. A-D**). Interestingly, these brain regions are part of the mesolimbic and mesocortical dopamine systems (Khlghatyan et al. 2018; Phillipson 1979; Rice et al. 2016). Second, in order to detect the orthologous version of ADAMTS2 protein at a prenatal stage (E18.5) in mice brain, this protein was mapped in the neuropiles of anterior brain structures, anterior cingular cortex, superficial striatum and lateral septum, where neuronal expression of dopamine D_1 and D_2 receptors was detected by *in situ* hybridization (http://developingmouse.brain-map.org/) (**Figure 4.10. E-L**). Finally, in

human neuronal-derived cells (SK-N-SH), we observed that incubation with the selective D_1 receptor agonist (SKF 83822) significantly trigged *ADAMTS2* mRNA expression as compared to other selective receptors agonists (**Figure 4.6. A**).

The hypothesis that dopamine and dopaminergic mechanisms are essential to psychosis, and particularly to schizophrenia, has been one of the most enduring ideas about this disorder (Howes and Kapur 2009). All pharmacological drugs used in schizophrenia treatment block dopamine D_2 receptor with clinical improvement in positive symptoms for the patients (Kapur and Remington 2001) and also, an elevated presynaptic striatal dopamine levels correlates most closely with the symptom dimension of psychosis, and blockade of this heightened transmission leads to a partial resolution of symptoms (Kapur et al. 2000). Furthermore, observing our data in mice brains, *Adamts2* gene localized in the VTA region which projects to regions of the limbic system as the hippocampus (also detected), among others (see **Figure 4.10.**), until a variety of cortical regions, including the PFC. Also, as we observed in this thesis above, *Adamts2* is expressed in mice brain and up-regulated in SCZ-like phenotype mice (**Figure 4.3.**). All of this VTA innervation is part of the mesolimbic and mesocortical dopamine systems that have been extensively associated with schizophrenia etiopathology (Khlghatyan et al. 2018; Phillipson 1979; Rice et al. 2016). Thus, we can speculate that this link between *ADAMTS2* and the "mesocorticolimbic" dopamine system can have implications in the etiopathology of the schizophrenia. Finally, it is well known that D_1- and D_2-class receptors can be found at different levels throughout the target regions of the "mesocorticolimbic" system (Beaulieu and Gainetdinov 2011). *ADAMTS2* protein was located in regions where neuronal expression of dopamine D_1 and D_2 receptors was detected by *in situ* hybridization. A number of studies have indicated that the mechanism involved in this dopamine pathways regulates different aspects of behavioral and cognitive operations (Meneses 2014), specially the cognitive role of D_1-class receptors in schizophrenia (Floresco 2013). In this way, it might be of interest to perform further investigations about the potential co-

localization of *ADAMTS2* and D_1/D_2 receptors, *ADAMTS2* expression changes in mice models of schizophrenia before and after APD treatment and also alongside different stages of the development (prenatal, early postnatal and adult mice).

5.2.2. Functional association between dopaminergic system and *ADAMTS2*.

The functional association between *ADAMTS2* and the dopaminergic system has not been previously established, to our best knowledge. Also, there is scarce information regarding *ADAMTS2* expression and activity in the CNS but its expression can be detected in human and brain samples (https://www.proteinatlas.org). ADAMTS2 is a member of the ADAM Metallopeptidase with Thrombospondin family (Kelwick et al. 2015; Mead and Apte 2018). This family participates in ECM organization targeting a number of proteins such as the N-propeptides of procollagens I–III, fibronectin, decorin and Dkk3 (Bekhouche et al. 2016; Colige et al. 2005). ECM provides points of anchorage and facilitates the organization of the neural cells in the CNS regions and is key in transmission of molecular signals in several cellular processes (e.g. such as survival and growth) (Lau et al. 2013). It is known that during the life of the individual, ECM changes its composition: first, facilitating neurogenesis and plasticity changes in the brain during the early life and then, stabilizing structures such as synapses (Dityatev and Schachner 2003; Zuber et al. 2005). Alterations in the ECM as well as TGF-β and WNT signalling has been associated with SCZ (Benes et al. 2007; Berretta 2012; Hoseth et al. 2018).

Moreover, dysregulated mRNA expression of MMPs (MMP-16, -24 and -25) and ADAMTS (ADAMTS-1, -6 and -8) families of proteases have also been reported in SCZ (Pietersen et al. 2014a; 2014b). Predominantly, ADAMTS family proteins studies have been focused on the PNNs structures and neuroplasticity, mainly targeted with ADAMTS that cleavage brevican and lecticans (Demircan et al. 2013; Nakada et al. 2005). Collagen is present in two ECM structures: basal lamina (where is located the blood-brain barrier (BBB)) and

the neural interstitial matrix (a dense network of components) (see **figure 1.8.**). Therefore, in one hand, ADAMTS2 protein could be an important role in BBB permeability regulation, tightly linked with delivery of the APDs and other drugs in the CNS (Pollak et al. 2018). Other ADAMTS have been related with this process (Gurses et al. 2016; Lemarchant et al. 2013). On the other hand, ADAMTS2 could be important in the dissemination of this drugs along the neural interstitial matrix (Pollak et al. 2018). Alterations in the expression levels of MMPs, ADAM and ADAMTS proteins have been associated with SCZ (Pietersen et al. 2014a; 2014b; Sethi and Zaia 2017), including resistant schizophrenic patients (Domenici et al. 2010; Yamamori et al. 2013). Finally, ADAMTS2 could being relevant in cell-cell and ECM-cell interactions (i.e. glial cells with neurons) and activation of the plasticity, as have been observed previously (Gottschall et al. 2005; Gottschall and Howell 2015). Thus, considering *ADAMTS2* gene expression and its associated activities in the CNS, as well as the data presented in this thesis, it is conceivable to speculate that it could participate in SCZ at different stages of the disease.

5.3. CREB transcription factor have an essential role in *ADAMTS2* transcription.

5.3.1. cAMP/CREB as a primary mechanism to control *ADAMTS2* transcriptional activity.

Up to this point, results exposed *ADAMTS2* as a candidate gene to participate in important SCZ clinical and mechanistic activities. When we evaluated the possible role of the receptors targeted by APDs in the expression control of *ADAMTS2*, incubating SK-N-SH cells with selective agonists and antagonists for these receptors (D_1, D_2, 5-HT_{1A} and 5-HT_{2A}), our data strongly suggest that *ADAMTS2* gene expression is controlled by D_1-class receptors activity (**Figures 4.6. A and 4.7.**), although these regulations cannot be ruled out that other biological pathways might regulate its expression as well. D_1 receptors are

the most abundant dopaminergic receptors in CNS, and their functional crosstalk with D_2 receptor is well documented (Miller 2009a; 2009b; Paul et al. 1992). Then, we decided to study the molecular mechanisms that can control its expression and/or activity in neuronal-like cells, we observed that haloperidol and clozapine could significantly block D_1-mediated transcriptional activation of *ADAMTS2* (**Figure 4.8.**). These results could explain a repression of *ADAMTS2* expression by antagonism of both receptors (Aringhieri et al. 2018).

D_1 receptor activate adenylyl cyclase (AC), which in turn regulate intracellular cAMP levels leading to PKA activation and CREB phosphorylation (Belgacem and Borodinsky 2017; Carlezon et al. 2005; Lonze and Ginty 2002) (**Figures 1.5. and 1.6.**). Incubating neuronal-derived cells with a specific $G_{\alpha s}$ activator (CTX) and $G_{\alpha i}$ inhibitor (PTX), we observed a significant up-regulation of *ADAMTS2* expression (**Figure 4.11. A-B**). Also, using the selective D_1 receptor agonist (SKF 83822), our data showed that specific D_1 receptor activation elicited the activation of *ADAMTS2* gene expression alongside a rapid phosphorylation of CREB, ERK and PKA substrates (**Figure 4.11. D-G**). This rapid phosphorylation observed in these cells resulted in CREB-mediated transcriptional activity as detected by using specific reporter assays (**Figure 4.12. A**). Finally, incubation with forskolin alone (a specific adenylyl cyclase activator) triggered transcription of *ADAMTS2* (**Figure 4.15.**). In summary, supporting the D_1 receptor–mediated control of *ADAMTS2* transcription, our data show that it is specifically triggered by activation of D_1-class receptors (by SKF 83822), $G_{\alpha s}$ subunit (using CTX) and adenylyl cyclase (forskolin). Thus, it is possible that, as part of the dopaminergic activity, *ADAMTS2* could act as a major cAMP/CREB effector in SCZ. Interestingly, D_1 receptor-mediated CREB activation was abrogated only by clozapine (**Figure 4.12. B**), providing evidence that APDs can modulate *ADAMTS2* expression through cAMP/CREB signalling.

Although this CREB-dependent regulation, other signalling pathways might regulate its expression. In one hand, our findings reveal the contribution of D_1-class receptors over *ADAMTS2* transcription and also indicate the potential participation of D_2 and 5-HT_{2A} receptors in the effect of clozapine over *ADAMTS2* expression (**Figure 4.9.**). In this regard, it has been reported that clozapine can act as a biased agonist on 5-HT_{2A} receptor (Aringhieri et al. 2017b; Schmid et al. 2014) and/or affect the hetero-dimer D_2/5-HT_{2A} receptors (Lukasiewicz et al. 2011). On the other hand, we discarded the potential role of the $G_{\alpha q}$ subunits in the control of *ADAMTS2* gene expression. In this regard, incubation of neuronal-like cells with its specific inhibitor (YM-254890) did not modify both the basal and the SKF 83822-mediated increase of *ADAMTS2* expression (**Figure 4.11. C**). Moreover, to evaluate the potential contribution of PKA and MEK to the activation of *ADAMTS2* expression, we disrupted cAMP/CREB and ERK signalling using specific inhibitors (like H89 dihydrochloride and selumetinib-AZD6244, respectively) in SK-N-SH cells. In these conditions, we observed an inhibition of D_1 receptor-induced expression of *ADAMTS2* with both drugs, although only a cAMP-PKA inhibitor abrogated CREB-dependent transcription and phosphorylation (**Figure 4.14.**).

Thus, we observed an *ADAMTS2* expression controlling by a D_1 receptor-dependent activation through mainly a CREB and ERK signalling that can be modulated pharmacologically in SK-N-SH cells. Supporting these observations, recent data pointed at cAMP/CREB signalling as an important mechanism linking dopaminergic signalling with the pathophysiology of SCZ and mood disorders (Wang et al. 2018), and their pathological consequences are mediated through different mechanistic processes (Sakamoto et al. 2011). Intensity and duration of CREB phosphorylation with the transcriptional regulation of target genes containing CRE sites and the balance between CREB-target kinases and phosphatases determines the degree of CRE-dependent gene transcription (Hagiwara et al. 1992). In this line of evidences, neurons in different maturation stages may have distinct phenotypes regarding the phosphorylation of CREB in response to the same

antipsychotic drug (Pozzi et al. 2003; Yang et al. 2004). Hence, regulation of the phosphorylation level of CREB brain could be a suitable strategy to develop new SCZ and other mental disorders treatment.

Once observed the potential role of CREB in *ADAMTS2* expression, we decided to challenge the real contribution of cAMP/CREB signalling to the *ADAMTS2* transcription in SK-N-SH cells. Initially, our results showed that incubation with forskolin alone was sufficient to trigger the transcription of both *ADAMTS2* and *C-FOS* (an "early response gene" known to be a direct transcriptional target of CREB that was used as control) (Dey et al. 1991), that occurred alongside PKA and CREB activation (**Figures 4.15. and 4.19.**). Forskolin directly activates AC in mammals, thereby promoting a rapid phosphorylation of CREB via PKA (Seamon et al. 1981; Zhang et al. 1997). However, TPA (a PKC/MAPK activator) uniquely activated *C-FOS* transcription. Our observations suggest us that there are two proteins (among others) involved in the regulation of *ADAMTS2* but it seems that CREB activity through cAMP/PKA activation has the greatest influence over *ADAMTS2* transcription.

To elucidate the bigger role of cAMP/PKA signalling in the *ADAMTS2* gene expression, we generated stable CREB1 knock-down SK-N-SH cells with GFP reporter to reduced CREB mRNA and protein expression by two approaches: lentiviral inducible shRNA construct inducible by doxycycline and transfection of stably expression shRNA construct (**Figure 4.16.**). In CREB-knockdown cells, D_1 receptor activation (SKF 83822) and forskolin failed to increase *ADAMTS2* gene expression (**Figure 4.17.**). In contrast, SKF 83822 did not significantly activate *C-FOS* expression but TPA did it in CREB knocked down cells when compared to control cells. These data reinforce the idea that cAMP/CREB is an essential mechanism to control *ADAMTS2* transcriptional activation (**Figure 5.1.**).

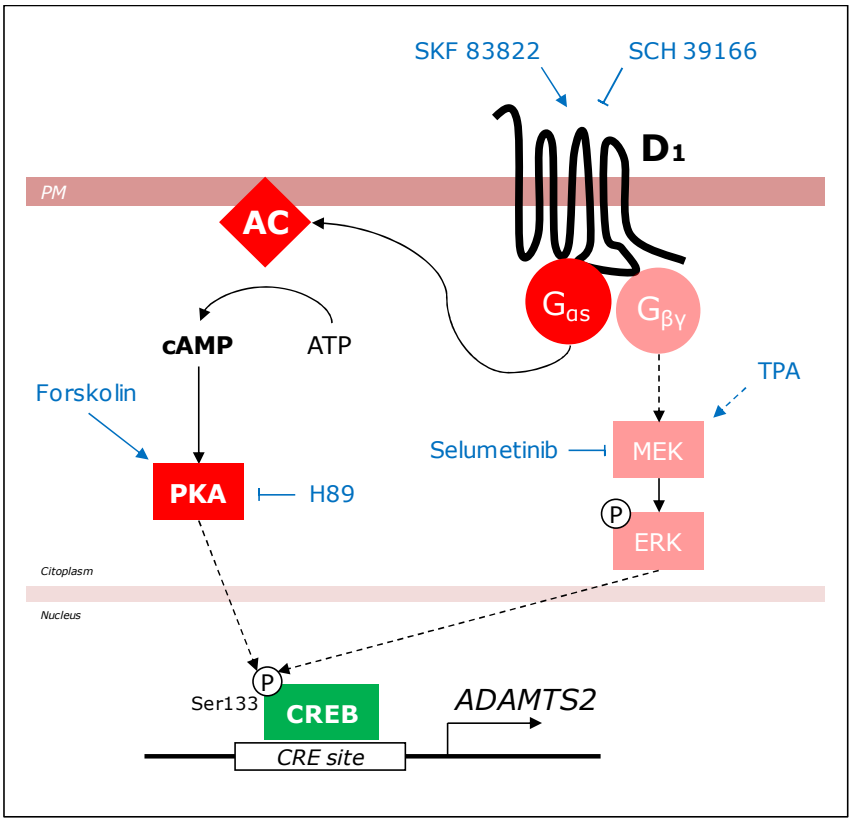


Figure 5.1. Proposed signalling model of *ADAMTS2* gene expression through D_1 activation. Two vias seem to be involved in *ADAMTS2* expression upon stimulation of G-protein coupled D_1 receptor by SKF 83822 (selective D_1 agonist): (1) $G_{\alpha s}$/AC/cAMP/PKA signalling (dark red), and (2) $G_{\beta\gamma}$/MEK/ERK1/2 signalling (light red). Both D_1 receptor-mediated activation of PKA and ERK signalling induce the phosphorylation of Ser133 CREB and *ADAMTS2* mRNA expression in SK-N-SH cells. Specific activation of PKA and inhibition of MEK by forskolin, and specific activation of MEK/ERK signalling triggered significantly CREB activation and *ADAMTS2* expression. These were retracted by selective D_1 receptor antagonist (SCH 39166) and specific inhibitors of PKA (H89) and MEK (selumetinib). Inducible shCREB1 cells were unable to induce *ADAMTS2* expression by D_1 receptors and PKA-dependent stimulation. *D_1 (dopamine D_1 receptor), $G_{\alpha s}$ ($G_{\alpha s}$ subunit protein), $G_{\beta\gamma}$ ($G_{\beta\gamma}$ subunit protein), AC (adenyl cyclase), PKA (protein kinase A), CRE (cyclic AMP-responsive element) site, PM (plasmatic membrane). Arrows: direct interaction, dashed arrows: indirect interaction, blue arrows: selective stimulation, blue lines: selective inhibition.*

5.3.2. CREB activity in samples from SCZ patients and binding to the *ADAMTS2* gene promoter.

Results exposed above support a functional role for the transcription factor CREB to regulate the expression of *ADAMTS2* gene in neuronal-like cells. To further characterize this data, we decided to challenge whether CREB activity was still directly involved in *ADAMTS2* transcriptional activation in cells from SCZ patients. Limitations of PBMCs from patients stored in the biobank might be considered, for that reason study samples were considerably reduced from the original cohort #1. For this, we quantified CREB binding to a bona-fide DNA element using DNA-binding proteins ELISA-based assays. Preliminary data show a tendency where CREB total binding is reduced after treatment in this subset of SCZ responders to APD patients that seems to be correlated with a down-regulation of *ADAMTS2* transcription in these same patients (**Figure 4.20.**). Supporting this data, a number of studies suggest that the timing of CREB regulation may be a key for the associate changes in the cellular neuronal responses (e.g. such as neuronal cell death, degeneration in brain hippocampus or excitotoxicity) (Dawson and Ginty 2002; Lopez de Armentia et al. 2007; Sakamoto et al. 2011; Zeng et al. 2016).

Data obtained in ELISA-based assay in samples from SCZ patients, encouraged us to evaluate CREB in the regulation region of *ADAMTS2* in PBMCs from SCZ patients. We hypothesized that CREB might influence the up-regulation of *ADAMTS2* mRNA in SCZ patients observed at the onset of the pathology. Again, we must take this data as preliminary results due to limitations in the collection of fresh PBMCs from blood patients (two cases and a control). Using chromatin immunoprecipitation (ChIP)-coupled quantitative PCR analysis, we observed increased binding of CREB to the regulatory region of *ADAMTS2* comprising the CRE site in SCZ patients than in the control ones (**Figure 4.22.**). These results argue in favour of CREB playing an important role at controlling the expression of *ADAMTS2* gene. In this regard, it is conceivable that CREB activity could be consider as a potential biomarker for APD response in SCZ patients. Nonetheless, as we

commented above, this data are preliminary and further efforts can be considered to explore the true role of CREB in APDs treatment in schizophrenia responder patients.

5.4. Potential limitations that might account to this work.

A number of potential limitations should be considered when interpreting our data and the impact of our findings. First, sample size in this replication study is rather small (cohort #1: N=30, and cohort #2: N=9). Noteworthy we selected a group of patients with a first episode of non-affective psychosis who had not previously been on APDs (not even a single dose) at the time of baseline blood test to avoid any interference with the mRNA levels. Only those individuals who gave written consent and had mRNA samples at baseline and at 3 months of treatment were eligible for this study. Therefore, all these stringent inclusion criteria limited the number of patients in this cohort. Further investigations to replicate our findings using larger and more heterogeneous groups are warranted. Moreover, we need to characterize a new cohort of patients with bad prognosis and non-response to treatment with APD to observe possible changes in *ADAMTS2* expression respect to responder patients. Second, although transcriptomic data obtained from blood samples might not resemble that of the brain, our study in a rodent model of schizophrenia have shown certain parallelism between the expression in human PBMCs (Crespo-Facorro et al. 2015; Sainz et al. 2013) and mouse frontal cortex (**Figure 4.3**). In this regard, it will be of interest, to address these questions, within the next future, using preclinical schizophrenia and/or *ADAMTS2* transgenic mice models. Third, we performed an independent validation using a targeted RT-qPCR gene approach that largely differs from our previous experiments using whole transcriptome analyses. Fourth, *ADAMTS2* protein and mRNA expression show modest but detectable levels in "healthy" human and mouse brain samples (https://www.proteinatlas.org). Thus, it is conceivable to detect high fold increases when analysing diseased specimens, like in this case *ADAMTS2* in SCZ. Fifth, failure of some APDs to inhibit CREB activity and/or *ADAMTS2*

mRNA expression in a D_1 receptor-context. Our data herein do not discard that APDs might regulate *ADAMTS2* by indirect mechanisms like for example those involving β-arrestin, ERK or AKT signalling (Aringhieri et al. 2017a; Del'guidice et al. 2011). Finally, our preliminary results obtained by ELISA-based assay and ChIP from PBMCs are based in a small replication subset of patients. We need to obtain a new cohort of patients with PBMCs and RNA samples collected at the same time to be compared and found the potential role of CREB and *ADAMTS2* in the etiopathology or the antipsychotic response in the schizophrenia patients.

CONCLUSIONS

6. CONCLUSIONS.

1. *ADAMTS2* is an independently validated gene with increased mRNA expression at the schizophrenia onset (FEP) and "normal" expression in responder patients after a 3- and 12-month treatment with APDs.

2. Antipsychotic drugs can dynamically regulate the expression of the CRGAs in human neuronal-like cells, been *ADAMTS2* the only gene of the study modulated by all antipsychotic drugs significantly.

3. Orthologous *Adamts2* mRNA and protein localize in embryo and postnatal mice at mesolimbic and mesocortical dopamine systems that have been extensively associated with schizophrenia etiopathology.

4. mRNA expression levels of five orthologous CRGAs (*Adamts2*, *Cd177*, *Cnapt3*, *Enpd2* and *Rfx2*) is increased in the CNS of a schizophrenia-like phenotype model when compared to control mice.

5. *ADAMTS2* gene expression is regulated by D_1 receptors in human neuronal-like cells:
    a. *ADAMTS2* gene expression elicited by D_1-class receptors is inhibited by haloperidol and clozapine but not paliperidone in neuronal-like cells.
    b. CREB activation elicited by D_1-class receptors is inhibited by clozapine but not haloperidol or paliperidone in neuronal-like cells.
    c. *ADAMTS2* mRNA expression elicited downstream of D_1-class receptors is dependent on PKA and ERK activity, whereas CREB activation is only dependent in PKA in neuronal-like cells.

d. *ADAMTS2* mRNA expression elicited downstream of D_1-class receptors requires CREB.

6. Treatment with antipsychotic drugs reduce total CREB activity in PBMCs from schizophrenia patients.

BIBLIOGRAPHY

7. BIBLIOGRAPHY

Aleman A, Kahn RS, Selten JP. 2003. Sex differences in the risk of schizophrenia: Evidence from meta-analysis. Arch Gen Psychiatry. 60(6):565-571.

Altarejos JY, Montminy M. 2011. Creb and the crtc co-activators: Sensors for hormonal and metabolic signals. Nat Rev Mol Cell Biol. 12(3):141-151.

Angrist B, Sathananthan G, Wilk S, Gershon S. 1974. Amphetamine psychosis: Behavioral and biochemical aspects. J Psychiatr Res. 11:13-23.

Anttila V, Bulik-Sullivan B, Finucane HK, Walters RK, Bras J, Duncan L, Escott-Price V, Falcone GJ, Gormley P, Malik R et al. 2018. Analysis of shared heritability in common disorders of the brain. Science. 360(6395).

Aringhieri S, Carli M, Kolachalam S, Verdesca V, Cini E, Rossi M, McCormick PJ, Corsini GU, Maggio R, Scarselli M. 2018. Molecular targets of atypical antipsychotics: From mechanism of action to clinical differences. Pharmacol Ther. 192:20-41.

Aringhieri S, Kolachalam S, Gerace C, Carli M, Verdesca V, Brunacci M, Rossi C, Ippolito C, Solini A, Corsini G et al. 2017a. Clozapine as the most efficacious antipsychotic for activating erk 1/2 kinases: Role of 5-ht2a receptor agonism. Eur Neuropsychopharmacol. 27(4):383-398.

Aringhieri S, Kolachalam S, Gerace C, Carli M, Verdesca V, Brunacci MG, Rossi C, Ippolito C, Solini A, Corsini GU et al. 2017b. Clozapine as the most efficacious antipsychotic for activating erk 1/2 kinases: Role of 5-ht2a receptor agonism. Eur Neuropsychopharmacol. 27(4):383-398.

Arning A, Hiersche M, Witten A, Kurlemann G, Kurnik K, Manner D, Stoll M, Nowak-Gottl U. 2012. A genome-wide association study identifies a gene network of adamts genes in the predisposition to pediatric stroke. Blood. 120(26):5231-5236.

Ayesa-Arriola R, Pelayo Teran JM, Setien-Suero E, Neergaard K, Ochoa S, Ramirez-Bonilla M, Perez-Iglesias R, Crespo-Facorro B. 2019. Patterns of recovery course in early intervention for first episode non-affective psychosis patients: The role of timing. Schizophr Res.

Ayesa-Arriola R, Rodriguez-Sanchez JM, Perez-Iglesias R, Gonzalez-Blanch C, Pardo-Garcia G, Tabares-Seisdedos R, Vazquez-Barquero JL, Crespo-Facorro B. 2013. The relevance of cognitive, clinical and premorbid variables in predicting functional outcome for individuals with first-episode psychosis: A 3 year longitudinal study. Psychiatry Res. 209(3):302-308.

Babulas V, Factor-Litvak P, Goetz R, Schaefer CA, Brown AS. 2006. Prenatal exposure to maternal genital and reproductive infections and adult schizophrenia. Am J Psychiatry. 163(5):927-929.

Baker K, Costain G, Fung WL, Bassett AS. 2014. Chromosomal microarray analysis-a routine clinical genetic test for patients with schizophrenia. Lancet Psychiatry. 1(5):329-331.

Baldwin P, Browne D, Scully PJ, Quinn JF, Morgan MG, Kinsella A, Owens JM, Russell V, O'Callaghan E, Waddington JL. 2005. Epidemiology of first-episode psychosis: Illustrating the challenges across diagnostic boundaries through the cavan-monaghan study at 8 years. Schizophr Bull. 31(3):624-638.

Bandtlow CE, Zimmermann DR. 2000. Proteoglycans in the developing brain: New conceptual insights for old proteins. Physiol Rev. 80(4):1267-1290.

Barrot M, Olivier JD, Perrotti LI, DiLeone RJ, Berton O, Eisch AJ, Impey S, Storm DR, Neve RL, Yin JC et al. 2002. Creb activity in the nucleus accumbens shell controls gating of behavioral responses to emotional stimuli. Proc Natl Acad Sci U S A. 99(17):11435-11440.

Beaulieu JM, Gainetdinov RR. 2011. The physiology, signaling, and pharmacology of dopamine receptors. Pharmacol Rev. 63(1):182-217.

Begni S, Popoli M, Moraschi S, Bignotti S, Tura GB, Gennarelli M. 2002. Association between the ionotropic glutamate receptor kainate 3 (grik3) ser310ala polymorphism and schizophrenia. Mol Psychiatry. 7(4):416-418.

Bekhouche M, Colige A. 2015. The procollagen n-proteinases adamts2, 3 and 14 in pathophysiology. Matrix Biol. 44-46:46-53.

Bekhouche M, Leduc C, Dupont L, Janssen L, Delolme F, Vadon-Le Goff S, Smargiasso N, Baiwir D, Mazzucchelli G, Zanella-Cleon I et al. 2016. Determination of the substrate repertoire of adamts2, 3, and 14 significantly broadens their functions and identifies extracellular matrix organization and tgf-beta signaling as primary targets. Faseb j. 30(5):1741-1756.

Belgacem YH, Borodinsky LN. 2017. Creb at the crossroads of activity-dependent regulation of nervous system development and function. Adv Exp Med Biol. 1015:19-39.

Belo VA, Souza-Costa DC, Lana CM, Caputo FL, Marcaccini AM, Gerlach RF, Bastos MG, Tanus-Santos JE. 2009. Assessment of matrix metalloproteinase (mmp)-2, mmp-8, mmp-9, and their inhibitors, the tissue inhibitors of metalloproteinase (timp)-1 and timp-2 in obese children and adolescents. Clin Biochem. 42(10-11):984-990.

Benes F, Lim B, Matzilevich D. 2007. Regulation of the gaba cell phenotype in hippocampus of schizophrenics and bipolars.

Berretta S. 2012. Extracellular matrix abnormalities in schizophrenia. Neuropharmacology. 62(3):1584-1597.

Bjorklund A, Dunnett SB. 2007. Dopamine neuron systems in the brain: An update. Trends Neurosci. 30(5):194-202.

Black D, Andreasen N. 1999. Schizophrenia, schizophreniform disorder and delusional (paranoid) disorders.; Psychiatric A, APA P, editors. Washington DC, 1999.

Boyd KN, Mailman RB. 2012. Dopamine receptor signaling and current and future antipsychotic drugs. Handb Exp Pharmacol. (212):53-86.

Bradfield JP, Taal HR, Timpson NJ, Scherag A, Lecoeur C, Warrington NM, Hypponen E, Holst C, Valcarcel B, Thiering E et al. 2012. A genome-wide association meta-analysis identifies new childhood obesity loci. Nat Genet. 44(5):526-531.

Brami-Cherrier K, Valjent E, Garcia M, Pages C, Hipskind RA, Caboche J. 2002. Dopamine induces a pi3-kinase-independent activation of akt in striatal neurons: A new route to camp response element-binding protein phosphorylation. J Neurosci. 22(20):8911-8921.

Brinkley TE, Kume N, Mitsuoka H, Phares DA, Hagberg JM. 2008. Elevated soluble lectin-like oxidized ldl receptor-1 (slox-1) levels in obese postmenopausal women. Obesity (Silver Spring). 16(6):1454-1456.

Brown AS, Begg MD, Gravenstein S, Schaefer CA, Wyatt RJ, Bresnahan M, Babulas VP, Susser ES. 2004. Serologic evidence of prenatal influenza in the etiology of schizophrenia. Arch Gen Psychiatry. 61(8):774-780.

Brown AS, Cohen P, Harkavy-Friedman J, Babulas V, Malaspina D, Gorman JM, Susser ES. 2001. A.E. Bennett research award. Prenatal rubella, premorbid abnormalities, and adult schizophrenia. Biol Psychiatry. 49(6):473-486.

Buckley PF, Miller BJ, Lehrer DS, Castle DJ. 2009. Psychiatric comorbidities and schizophrenia. Schizophr Bull. 35(2):383-402.

Cardno AG, Gottesman, II. 2000. Twin studies of schizophrenia: From bow-and-arrow concordances to star wars mx and functional genomics. Am J Med Genet. 97(1):12-17.

Carlezon WA, Jr., Duman RS, Nestler EJ. 2005. The many faces of creb. Trends Neurosci. 28(8):436-445.

Cartharius K, Frech K, Grote K, Klocke B, Haltmeier M, Klingenhoff A, Frisch M, Bayerlein M, Werner T. 2005. Matinspector and beyond: Promoter analysis based on transcription factor binding sites. Bioinformatics. 21(13):2933-2942.

Caseiro O, Perez-Iglesias R, Mata I, Martinez-Garcia O, Pelayo-Teran JM, Tabares-Seisdedos R, Ortiz-Garcia de la Foz V, Vazquez-Barquero JL, Crespo-Facorro B. 2012. Predicting relapse after a first episode of non-affective psychosis: A three-year follow-up study. J Psychiatr Res. 46(8):1099-1105.

Chakrabarti S, Davidge ST. 2012. G-protein coupled receptor 30 (gpr30): A novel regulator of endothelial inflammation. PLoS One. 7(12):e52357.

Chan MK, Guest PC, Levin Y, Umrania Y, Schwarz E, Bahn S, Rahmoune H. 2011. Converging evidence of blood-based biomarkers for schizophrenia: An update. Int Rev Neurobiol. 101:95-144.

Chan MK, Krebs MO, Cox D, Guest PC, Yolken RH, Rahmoune H, Rothermundt M, Steiner J, Leweke FM, van Beveren NJ et al. 2015. Development of a blood-based molecular biomarker test for identification of schizophrenia before disease onset. Transl Psychiatry. 5:e601.

Chartoff EH, Papadopoulou M, Konradi C, Carlezon WA, Jr. 2003. Dopamine-dependent increases in phosphorylation of camp response element binding protein (creb) during precipitated morphine withdrawal in primary cultures of rat striatum. J Neurochem. 87(1):107-118.

Cheung BM, Ong KL, Cheung RV, Wong LY, Wat NM, Tam S, Leung GM, Cheng CH, Woo J, Janus ED et al. 2008. Association between plasma alkaline phosphatase and c-reactive protein in hong kong chinese. Clin Chem Lab Med. 46(4):523-527.

Colige A, Beschin A, Samyn B, Goebels Y, Van Beeumen J, Nusgens BV, Lapiere CM. 1995. Characterization and partial amino acid sequencing of a 107-kda procollagen i n-proteinase purified by affinity chromatography on immobilized type xiv collagen. J Biol Chem. 270(28):16724-16730.

Colige A, Nuytinck L, Hausser I, van Essen AJ, Thiry M, Herens C, Ades LC, Malfait F, Paepe AD, Franck P et al. 2004. Novel types of mutation responsible for the dermatosparactic type of ehlers-danlos syndrome (type viic) and common polymorphisms in the adamts2 gene. J Invest Dermatol. 123(4):656-663.

Colige A, Ruggiero F, Vandenberghe I, Dubail J, Kesteloot F, Van Beeumen J, Beschin A, Brys L, Lapiere CM, Nusgens B. 2005. Domains and maturation processes that regulate the activity of adamts-2, a metalloproteinase cleaving the aminopropeptide of fibrillar procollagens types i-iii and v. J Biol Chem. 280(41):34397-34408.

Comuzzie AG, Cole SA, Laston SL, Voruganti VS, Haack K, Gibbs RA, Butte NF. 2012. Novel genetic loci identified for the pathophysiology of childhood obesity in the hispanic population. PLoS One. 7(12):e51954.

Crespo-Facorro B, de la Foz VO, Ayesa-Arriola R, Perez-Iglesias R, Mata I, Suarez-Pinilla P, Tabares-Seisdedos R, Vazquez-Barquero JL. 2013. Prediction of acute clinical response following a first episode of non affective psychosis: Results of a cohort of 375 patients from the spanish pafip study. Prog Neuropsychopharmacol Biol Psychiatry. 44:162-167.

Crespo-Facorro B, Prieto C, Sainz J. 2015. Schizophrenia gene expression profile reverted to normal levels by antipsychotics. Int J Neuropsychopharmacol. 18(4).

Culm KE, Lugo-Escobar N, Hope BT, Hammer RP, Jr. 2004. Repeated quinpirole treatment increases camp-dependent protein kinase activity and creb phosphorylation in nucleus accumbens and reverses quinpirole-induced sensorimotor gating deficits in rats. Neuropsychopharmacology. 29(10):1823-1830.

Dassen H, Punyadeera C, Delvoux B, Schulkens I, Marchetti C, Kamps R, Klomp J, Dijcks F, de Goeij A, D'Hooghe T et al. 2010. Olfactomedin-4 regulation by estrogen in the human endometrium requires epidermal growth factor signaling. Am J Pathol. 177(5):2495-2508.

Dawson TM, Ginty DD. 2002. Creb family transcription factors inhibit neuronal suicide. Nat med. United States. p. 450-451.

Del'guidice T, Lemasson M, Beaulieu JM. 2011. Role of beta-arrestin 2 downstream of dopamine receptors in the basal ganglia. Front Neuroanat. 5:58.

Demircan K, Yonezawa T, Takigawa T, Topcu V, Erdogan S, Ucar F, Armutcu F, Yigitoglu MR, Ninomiya Y, Hirohata S. 2013. Adamts1, adamts5, adamts9 and aggrecanase-generated proteoglycan fragments are induced following spinal cord injury in mouse. Neurosci Lett. 544:25-30.

Dey A, Nebert DW, Ozato K. 1991. The ap-1 site and the camp- and serum response elements of the c-fos gene are constitutively occupied in vivo. DNA Cell Biol. 10(7):537-544.

Diez-Roux G, Banfi S, Sultan M, Geffers L, Anand S, Rozado D, Magen A, Canidio E, Pagani M, Peluso I et al. 2011. A high-resolution anatomical atlas of the transcriptome in the mouse embryo. PLoS Biol. 9(1):e1000582.

Dityatev A, Schachner M. 2003. Extracellular matrix molecules and synaptic plasticity. Nat Rev Neurosci. 4(6):456-468.

Domenici E, Wille DR, Tozzi F, Prokopenko I, Miller S, McKeown A, Brittain C, Rujescu D, Giegling I, Turck CW et al. 2010. Plasma protein biomarkers for depression and schizophrenia by multi analyte profiling of case-control collections. PLoS One. 5(2):e9166.

Dong E, Agis-Balboa RC, Simonini MV, Grayson DR, Costa E, Guidotti A. 2005. Reelin and glutamic acid decarboxylase67 promoter remodeling in an epigenetic methionine-induced mouse model of schizophrenia. Proc Natl Acad Sci U S A. 102(35):12578-12583.

Dubail J, Apte SS. 2015. Insights on adamts proteases and adamts-like proteins from mammalian genetics. Matrix Biol. 44-46:24-37.

Dubail J, Kesteloot F, Deroanne C, Motte P, Lambert V, Rakic JM, Lapiere C, Nusgens B, Colige A. 2010. Adamts-2 functions as anti-angiogenic and anti-tumoral molecule independently of its catalytic activity. Cell Mol Life Sci. 67(24):4213-4232.

Ertugrul A, Ulug B. 2002. The influence of neurocognitive deficits and symptoms on disability in schizophrenia. Acta Psychiatr Scand. 105(3):196-201.

Fang Z, Cui X. 2011. Design and validation issues in rna-seq experiments. Brief Bioinform. 12(3):280-287.

Felinski EA, Quinn PG. 2001. The coactivator dtaf(ii)110/htaf(ii)135 is sufficient to recruit a polymerase complex and activate basal transcription mediated by creb. Proc Natl Acad Sci U S A. 98(23):13078-13083.

Finkbeiner S, Tavazoie SF, Maloratsky A, Jacobs KM, Harris KM, Greenberg ME. 1997. Creb: A major mediator of neuronal neurotrophin responses. Neuron. 19(5):1031-1047.

Fioravanti M, Carlone O, Vitale B, Cinti ME, Clare L. 2005. A meta-analysis of cognitive deficits in adults with a diagnosis of schizophrenia. Neuropsychol Rev. 15(2):73-95.

Floresco SB. 2013. Prefrontal dopamine and behavioral flexibility: Shifting from an "Inverted-u" Toward a family of functions. Front Neurosci. 7:62.

Fox CS, Liu Y, White CC, Feitosa M, Smith AV, Heard-Costa N, Lohman K, Johnson AD, Foster MC, Greenawalt DM et al. 2012. Genome-wide association for abdominal subcutaneous and visceral adipose reveals a novel locus for visceral fat in women. PLoS Genet. 8(5):e1002695.

Fumagalli F, Frasca A, Racagni G, Riva MA. 2009. Antipsychotic drugs modulate arc expression in the rat brain. Eur Neuropsychopharmacol. 19(2):109-115.

Gillespie AL, Samanaite R, Mill J, Egerton A, MacCabe JH. 2017. Is treatment-resistant schizophrenia categorically distinct from treatment-responsive schizophrenia? A systematic review. BMC Psychiatry. 17(1):12.

Gonzalez-Maeso J, Yuen T, Ebersole BJ, Wurmbach E, Lira A, Zhou M, Weisstaub N, Hen R, Gingrich JA, Sealfon SC. 2003. Transcriptome fingerprints distinguish hallucinogenic and nonhallucinogenic 5-hydroxytryptamine 2a receptor agonist effects in mouse somatosensory cortex. J Neurosci. 23(26):8836-8843.

Gottschall P, Sandy J, Zimmermann D. 2005. Substrates for metalloendopeptidades in the central nervous system. In: Conant K GP, editors., editor. Matrix metalloproteinases in the central nervous system. Imperial College Press. p. 87-118.

Gottschall PE, Howell MD. 2015. Adamts expression and function in central nervous system injury and disorders. Matrix Biol. 44-46:70-76.

Grace AA. 1991. Phasic versus tonic dopamine release and the modulation of dopamine system responsivity: A hypothesis for the etiology of schizophrenia. Neuroscience. 41(1):1-24.

Grace AA. 2012. Dopamine system dysregulation by the hippocampus: Implications for the pathophysiology and treatment of schizophrenia. Neuropharmacology. 62(3):1342-1348.

Grunder G, Hippius H, Carlsson A. 2009. The 'atypicality' of antipsychotics: A concept re-examined and re-defined. Nat Rev Drug Discov. 8(3):197-202.

Guloksuz S, van Os J. 2018. The slow death of the concept of schizophrenia and the painful birth of the psychosis spectrum. Psychol Med. 48(2):229-244.

Gulsuner S, Walsh T, Watts AC, Lee MK, Thornton AM, Casadei S, Rippey C, Shahin H, Nimgaonkar VL, Go RC et al. 2013. Spatial and temporal mapping of de novo mutations in schizophrenia to a fetal prefrontal cortical network. Cell. 154(3):518-529.

Guo H, Cheng Y, Wang C, Wu J, Zou Z, Niu B, Yu H, Wang H, Xu J. 2017. Ffpm, a pde4 inhibitor, reverses learning and memory deficits in app/ps1 transgenic mice via camp/pka/creb signaling and anti-inflammatory effects. Neuropharmacology. 116:260-269.

Gurses MS, Ural MN, Gulec MA, Akyol O, Akyol S. 2016. Pathophysiological function of adamts enzymes on molecular mechanism of alzheimer's disease. Aging dis. p. 479-490.

Haas E, Bhattacharya I, Brailoiu E, Damjanovic M, Brailoiu GC, Gao X, Mueller-Guerre L, Marjon NA, Gut A, Minotti R et al. 2009. Regulatory role of g protein-coupled estrogen receptor for vascular function and obesity. Circ Res. 104(3):288-291.

Hagiwara M, Alberts A, Brindle P, Meinkoth J, Feramisco J, Deng T, Karin M, Shenolikar S, Montminy M. 1992. Transcriptional attenuation following camp induction requires pp-1-mediated dephosphorylation of creb. Cell. 70(1):105-113.

Hall J, Trent S, Thomas KL, O'Donovan MC, Owen MJ. 2015. Genetic risk for schizophrenia: Convergence on synaptic pathways involved in plasticity. Biol Psychiatry. 77(1):52-58.

Hart AB, Engelhardt BE, Wardle MC, Sokoloff G, Stephens M, de Wit H, Palmer AA. 2012. Genome-wide association study of d-amphetamine response in healthy volunteers identifies putative associations, including cadherin 13 (cdh13). PLoS One. 7(8):e42646.

Hegarty JD, Baldessarini RJ, Tohen M, Waternaux C, Oepen G. 1994. One hundred years of schizophrenia: A meta-analysis of the outcome literature. Am J Psychiatry. 151(10):1409-1416.

Hofer TP, Frankenberger M, Mages J, Lang R, Meyer P, Hoffmann R, Colige A, Ziegler-Heitbrock L. 2008. Tissue-specific induction of adamts2 in monocytes and macrophages by glucocorticoids. J Mol Med (Berl). 86(3):323-332.

Hoseth E, Krull F, Dieset I. 2018. Exploring the wnt signaling pathway in schizophrenia and bipolar disorder. Translational Psychiatry. 8(1):55.

Howes OD, Kapur S. 2009. The dopamine hypothesis of schizophrenia: Version iii--the final common pathway. Schizophr Bull. 35(3):549-562.

Howes OD, Murray RM. 2014. Schizophrenia: An integrated sociodevelopmental-cognitive model. Lancet. 383(9929):1677-1687.

Hyman SE. 2007. Can neuroscience be integrated into the dsm-v? Nat Rev Neurosci. 8(9):725-732.

Ichikawa J, Li Z, Dai J, Meltzer HY. 2002. Atypical antipsychotic drugs, quetiapine, iloperidone, and melperone, preferentially increase dopamine and acetylcholine release in rat medial prefrontal cortex: Role of 5-ht1a receptor agonism. Brain Res. 956(2):349-357.

Ichiki T. 2006. Role of camp response element binding protein in cardiovascular remodeling: Good, bad, or both? Arterioscler Thromb Vasc Biol. 26(3):449-455.

Impagnatiello F, Guidotti AR, Pesold C, Dwivedi Y, Caruncho H, Pisu MG, Uzunov DP, Smalheiser NR, Davis JM, Pandey GN et al. 1998. A decrease of reelin expression as a putative vulnerability factor in schizophrenia. Proc Natl Acad Sci U S A. 95(26):15718-15723.

Insel TR. 2010. Rethinking schizophrenia. Nature. 468(7321):187-193.

Janowsky DS, el-Yousel MK, Davis JM, Sekerke HJ. 1973. Provocation of schizophrenic symptoms by intravenous administration of methylphenidate. Arch Gen Psychiatry. 28(2):185-191.

Jiang C, Zhou Y, Huang Y, Wang Y, Wang W, Kuai X. 2019. Overexpression of adamts-2 in tumor cells and stroma is predictive of poor clinical prognosis in gastric cancer. Hum Pathol. 84:44-51.

Jones GC, Riley GP. 2005. Adamts proteinases: A multi-domain, multi-functional family with roles in extracellular matrix turnover and arthritis. Arthritis Res Ther. 7(4):160-169.

Kahn RS, Keefe RS. 2013. Schizophrenia is a cognitive illness: Time for a change in focus. JAMA Psychiatry. 70(10):1107-1112.

Kahn RS, Sommer IE, Murray RM, Meyer-Lindenberg A, Weinberger DR, Cannon TD, O'Donovan M, Correll CU, Kane JM, van Os J et al. 2015. Schizophrenia. Nat Rev Dis Primers. 1:15067.

Kanazawa I, Yamaguchi T, Yamamoto M, Yamauchi M, Yano S, Sugimoto T. 2009. Serum osteocalcin/bone-specific alkaline phosphatase ratio is a predictor for the presence of vertebral fractures in men with type 2 diabetes. Calcif Tissue Int. 85(3):228-234.

Kapur S, Remington G. 2001. Dopamine d(2) receptors and their role in atypical antipsychotic action: Still necessary and may even be sufficient. Biol Psychiatry. 50(11):873-883.

Kapur S, Zipursky R, Jones C, Remington G, Houle S. 2000. Relationship between dopamine d(2) occupancy, clinical response, and side effects: A double-blind pet study of first-episode schizophrenia. Am J Psychiatry. 157(4):514-520.

Karin M, Smeal T. 1992. Control of transcription factors by signal transduction pathways: The beginning of the end. Trends Biochem Sci. 17(10):418-422.

Kashihara K, Ishihara T, Akiyama K, Abe K. 1999. D1/d2 receptor synergism on creb dna-binding activities in the caudate-putamen of rat. Neurol Res. 21(8):781-784.

Keefe RS, Seidman LJ, Christensen BK, Hamer RM, Sharma T, Sitskoorn MM, Lewine RR, Yurgelun-Todd DA, Gur RC, Tohen M et al. 2004. Comparative effect of atypical and conventional antipsychotic drugs on neurocognition in first-episode psychosis: A randomized, double-blind trial of olanzapine versus low doses of haloperidol. Am J Psychiatry. 161(6):985-995.

Kelwick R, Desanlis I, Wheeler GN, Edwards DR. 2015. The adamts (a disintegrin and metalloproteinase with thrombospondin motifs) family. Genome Biol. 16:113.

Khlghatyan J, Quintana C, Parent M, Beaulieu JM. 2018. High sensitivity mapping of cortical dopamine d2 receptor expressing neurons. Cereb Cortex.

Khokhar JY, Henricks AM, Sullivan EDK, Green AI. 2018. Unique effects of clozapine: A pharmacological perspective. Adv Pharmacol. 82:137-162.

Kneeland RE, Fatemi SH. 2013. Viral infection, inflammation and schizophrenia. Prog Neuropsychopharmacol Biol Psychiatry. 42:35-48.

Knight HM, Pickard BS, Maclean A, Malloy MP, Soares DC, McRae AF, Condie A, White A, Hawkins W, McGhee K et al. 2009. A cytogenetic abnormality and rare coding variants identify abca13 as a candidate gene in schizophrenia, bipolar disorder, and depression. Am J Hum Genet. 85(6):833-846.

Lai CY, Scarr E, Udawela M, Everall I, Chen WJ, Dean B. 2016. Biomarkers in schizophrenia: A focus on blood based diagnostics and theranostics. World J Psychiatry. 6(1):102-117.

Lakhan SE, Kramer A. 2009. Schizophrenia genomics and proteomics: Are we any closer to biomarker discovery? Behav Brain Funct. 5:2.

LaLumiere RT. 2014. Dopamine and memory. Identification of neural markers accompanying memory. ELSEVIER. p. 79-94.

Lapiere CM, Lenaers A, Kohn LD. 1971. Procollagen peptidase: An enzyme excising the coordination peptides of procollagen. Proc Natl Acad Sci U S A. 68(12):3054-3058.

Lasky-Su J, Anney RJ, Neale BM, Franke B, Zhou K, Maller JB, Vasquez AA, Chen W, Asherson P, Buitelaar J et al. 2008. Genome-wide association scan of the time to onset of attention deficit hyperactivity disorder. Am J Med Genet B Neuropsychiatr Genet. 147b(8):1355-1358.

Lau LW, Cua R, Keough MB, Haylock-Jacobs S, Yong VW. 2013. Pathophysiology of the brain extracellular matrix: A new target for remyelination. Nat Rev Neurosci. 14(10):722-729.

Lee CW, Hwang I, Park CS, Lee H, Park DW, Kang SJ, Lee SW, Kim YH, Park SW, Park SJ. 2012. Expression of adamts-2, -3, -13, and -14 in culprit coronary lesions in patients with acute myocardial infarction or stable angina. J Thromb Thrombolysis. 33(4):362-370.

Lemarchant S, Pruvost M, Montaner J, Emery E, Vivien D, Kanninen K, Koistinaho J. 2013. Adamts proteoglycanases in the physiological and pathological central nervous system. J Neuroinflammation. 10:133.

Leppilahti JM, Ahonen MM, Hernandez M, Munjal S, Netuschil L, Uitto VJ, Sorsa T, Mantyla P. 2011. Oral rinse mmp-8 point-of-care immuno test identifies patients with strong periodontal inflammatory burden. Oral Dis. 17(1):115-122.

Leucht S, Cipriani A, Spineli L, Mavridis D, Orey D, Richter F, Samara M, Barbui C, Engel RR, Geddes JR et al. 2013. Comparative efficacy and tolerability of 15 antipsychotic drugs in schizophrenia: A multiple-treatments meta-analysis. Lancet. 382(9896):951-962.

Leucht S, Corves C, Arbter D, Engel RR, Li C, Davis JM. 2009. Second-generation versus first-generation antipsychotic drugs for schizophrenia: A meta-analysis. Lancet. 373(9657):31-41.

Leucht S, Leucht C, Huhn M, Chaimani A, Mavridis D, Helfer B, Samara M, Rabaioli M, Bacher S, Cipriani A et al. 2017. Sixty years of placebo-controlled antipsychotic drug trials in acute schizophrenia: Systematic

review, bayesian meta-analysis, and meta-regression of efficacy predictors. Am J Psychiatry. 174(10):927-942.

Liu J, Shi Y, Tang J, Guo T, Li X, Yang Y, Chen Q, Zhao X, He G, Feng G et al. 2005. Snps and haplotypes in the s100b gene reveal association with schizophrenia. Biochem Biophys Res Commun. 328(1):335-341.

Livak KJ, Schmittgen TD. 2001. Analysis of relative gene expression data using real-time quantitative pcr and the 2(-delta delta c(t)) method. Methods. 25(4):402-408.

Lonze BE, Ginty DD. 2002. Function and regulation of creb family transcription factors in the nervous system. Neuron. 35(4):605-623.

Lopez de Armentia M, Jancic D, Olivares R, Alarcon JM, Kandel ER, Barco A. 2007. Camp response element-binding protein-mediated gene expression increases the intrinsic excitability of ca1 pyramidal neurons. J Neurosci. 27(50):13909-13918.

Lukasiewicz S, Faron-Gorecka A, Kedracka-Krok S, Dziedzicka-Wasylewska M. 2011. Effect of clozapine on the dimerization of serotonin 5-ht(2a) receptor and its genetic variant 5-ht(2a)h425y with dopamine d(2) receptor. Eur J Pharmacol. 659(2-3):114-123.

Lyketsos CG, Steinberg M, Tschanz JT, Norton MC, Steffens DC, Breitner JC. 2000. Mental and behavioral disturbances in dementia: Findings from the cache county study on memory in aging. Am J Psychiatry. 157(5):708-714.

Malhotra D, Sebat J. 2012. Cnvs: Harbingers of a rare variant revolution in psychiatric genetics. Cell. 148(6):1223-1241.

Mantamadiotis T, Lemberger T, Bleckmann SC, Kern H, Kretz O, Martin Villalba A, Tronche F, Kellendonk C, Gau D, Kapfhammer J et al. 2002. Disruption of creb function in brain leads to neurodegeneration. Nat Genet. 31(1):47-54.

Martins-de-Souza D, Schmitt A, Roder R, Lebar M, Schneider-Axmann T, Falkai P, Turck CW. 2010. Sex-specific proteome differences in the anterior cingulate cortex of schizophrenia. J Psychiatr Res. 44(14):989-991.

Mayoral-van Son J, Juncal-Ruiz M, Ortiz-Garcia de la Foz V, Cantarero-Prieto D, Blazquez-Fernandez C, Paz-Zulueta M, Paras-Bravo P, Ayuso-Mateos JL, Crespo-Facorro B. 2018. Understanding the direct and indirect costs of a first episode of psychosis program: Insights from pafip of cantabria, spain, during the first year of intervention. Early Interv Psychiatry.

McClay JL, Adkins DE, Aberg K, Bukszar J, Khachane AN, Keefe RS, Perkins DO, McEvoy JP, Stroup TS, Vann RE et al. 2011. Genome-wide pharmacogenomic study of neurocognition as an indicator of antipsychotic treatment response in schizophrenia. Neuropsychopharmacology. 36(3):616-626.

McEwen BS, Alves SE. 1999. Estrogen actions in the central nervous system. Endocr Rev. 20(3):279-307.

McGrath J, Saha S, Welham J, El Saadi O, MacCauley C, Chant D. 2004. A systematic review of the incidence of schizophrenia: The distribution of rates and the influence of sex, urbanicity, migrant status and methodology. BMC Med. 2:13.

Mead TJ, Apte SS. 2018. Adamts proteins in human disorders. Matrix Biol. 71-72:225-239.

Meltzer HY. 2013. Update on typical and atypical antipsychotic drugs. Annu Rev Med. 64:393-406.

Meltzer HY, Massey BW. 2011. The role of serotonin receptors in the action of atypical antipsychotic drugs. Curr Opin Pharmacol. 11(1):59-67.

Meneses A. 2014. Neurotransmitters and memory: Cholinergic, glutamatergic, gabaergic, dopaminergic, serotonergic, signaling, and memory. Identification of neural markers accompanying memory. ELSEVIER. p. 5-45.

Menezes NM, Arenovich T, Zipursky RB. 2006. A systematic review of longitudinal outcome studies of first-episode psychosis. Psychol Med. 36(10):1349-1362.

Miller R. 2009a. Mechanisms of action of antipsychotic drugs of different classes, refractoriness to therapeutic effects of classical neuroleptics, and individual variation in sensitivity to their actions: Part i. Curr Neuropharmacol. 7(4):302-314.

Miller R. 2009b. Mechanisms of action of antipsychotic drugs of different classes, refractoriness to therapeutic effects of classical neuroleptics, and individual variation in sensitivity to their actions: Part ii. Curr Neuropharmacol. 7(4):315-330.

Mittal VA, Willhite R, Daley M, Bearden CE, Niendam T, Ellman LM, Cannon TD. 2009. Obstetric complications and risk for conversion to psychosis among individuals at high clinical risk. Early Interv Psychiatry. 3(3):226-230.

Miyamoto S, Duncan GE, Marx CE, Lieberman JA. 2005. Treatments for schizophrenia: A critical review of pharmacology and mechanisms of action of antipsychotic drugs. Mol Psychiatry. 10(1):79-104.

Miyamoto S, Miyake N, Jarskog LF, Fleischhacker WW, Lieberman JA. 2012. Pharmacological treatment of schizophrenia: A critical review of the pharmacology and clinical effects of current and future therapeutic agents. Mol Psychiatry. 17(12):1206-1227.

Molteni R, Calabrese F, Racagni G, Fumagalli F, Riva MA. 2009. Antipsychotic drug actions on gene modulation and signaling mechanisms. Pharmacol Ther. 124(1):74-85.

Montminy M. 1997. Transcriptional regulation by cyclic amp. Annu Rev Biochem. 66:807-822.

Moreno JL, Kurita M, Holloway T, Lopez J, Cadagan R, Martinez-Sobrido L, Garcia-Sastre A, Gonzalez-Maeso J. 2011. Maternal influenza viral infection causes schizophrenia-like alterations of 5-ht(2)a and mglu(2) receptors in the adult offspring. J Neurosci. 31(5):1863-1872.

Moreno-Navarrete JM, Serrano M, Sabater M, Ortega F, Serino M, Pueyo N, Luche E, Waget A, Rodriguez-Hermosa JI, Ricart W et al. 2013. Study of lactoferrin gene expression in human and mouse adipose tissue, human preadipocytes and mouse 3t3-l1 fibroblasts. Association with adipogenic and inflammatory markers. J Nutr Biochem. 24(7):1266-1275.

Murray CJ, Lopez AD. 1996. The incremental effect of age-weighting on ylls, ylds, and dalys: A response. Bull World Health Organ. 74(4):445-446.

Nakada M, Miyamori H, Kita D, Takahashi T, Yamashita J, Sato H, Miura R, Yamaguchi Y, Okada Y. 2005. Human glioblastomas overexpress adamts-5 that degrades brevican. Acta Neuropathol. 110(3):239-246.

Negrin MA, Vazquez-Polo FJ. 2006. Bayesian cost-effectiveness analysis with two measures of effectiveness: The cost-effectiveness acceptability plane. Health Econ. 15(4):363-372.

Newton SS, Thome J, Wallace TL, Shirayama Y, Schlesinger L, Sakai N, Chen J, Neve R, Nestler EJ, Duman RS. 2002. Inhibition of camp response element-binding protein or dynorphin in the nucleus accumbens produces an antidepressant-like effect. J Neurosci. 22(24):10883-10890.

Nieto R, Kukuljan M, Silva H. 2013. Bdnf and schizophrenia: From neurodevelopment to neuronal plasticity, learning, and memory. Front Psychiatry. 4:45.

Owen MJ, O'Donovan MC, Thapar A, Craddock N. 2011. Neurodevelopmental hypothesis of schizophrenia. Br J Psychiatry. 198(3):173-175.

Owen MJ, Sawa A, Mortensen PB. 2016. Schizophrenia. Lancet. 388(10039):86-97.

Palomino A, Vallejo-Illarramendi A, Gonzalez-Pinto A, Aldama A, Gonzalez-Gomez C, Mosquera F, Gonzalez-Garcia G, Matute C. 2006. Decreased levels of plasma bdnf in first-episode schizophrenia and bipolar disorder patients. Schizophr res. Netherlands. p. 321-322.

Pantazopoulos H, Woo TU, Lim MP, Lange N, Berretta S. 2010. Extracellular matrix-glial abnormalities in the amygdala and entorhinal cortex of subjects diagnosed with schizophrenia. Arch Gen Psychiatry. 67(2):155-166.

Paul ML, Graybiel AM, David JC, Robertson HA. 1992. D1-like and d2-like dopamine receptors synergistically activate rotation and c-fos expression in the dopamine-depleted striatum in a rat model of parkinson's disease. J Neurosci. 12(10):3729-3742.

Paus T, Keshavan M, Giedd JN. 2008. Why do many psychiatric disorders emerge during adolescence? Nat Rev Neurosci. 9(12):947-957.

Pelayo-Teran JM, Perez-Iglesias R, Ramirez-Bonilla M, Gonzalez-Blanch C, Martinez-Garcia O, Pardo-Garcia G, Rodriguez-Sanchez JM, Roiz-Santianez R, Tordesillas-Gutierrez D, Mata I et al. 2008. Epidemiological factors associated with treated incidence of first-episode non-affective psychosis in cantabria: Insights from the clinical programme on early phases of psychosis. Early Interv Psychiatry. 2(3):178-187.

Pfaffl M. 2004. Quantification strategies in real-time pcr. In: Bustin SA, editor. A-z of quantitative pcr. La Jolla, CA, USA: International University Line (IUL). p. 87 - 112.

Phillipson OT. 1979. Afferent projections to the ventral tegmental area of tsai and interfascicular nucleus: A horseradish peroxidase study in the rat. J Comp Neurol. 187(1):117-143.

Pietersen CY, Mauney SA, Kim SS. 2014a. Molecular profiles of parvalbumin-immunoreactive neurons in the superior temporal cortex in schizophrenia. J Neurogenet. 28(1-2):70-85.

Pietersen CY, Mauney SA, Kim SS. 2014b. Molecular profiles of pyramidal neurons in the superior temporal cortex in schizophrenia. J Neurogenet. 28(1-2):53-69.

Pizzorusso T, Ratto GM, Putignano E, Maffei L. 2000. Brain-derived neurotrophic factor causes camp response element-binding protein phosphorylation in absence of calcium increases in slices and cultured neurons from rat visual cortex. J Neurosci. 20(8):2809-2816.

Plager DA, Kahl JC, Asmann YW, Nilson AE, Pallanch JF, Friedman O, Kita H. 2010. Gene transcription changes in asthmatic chronic rhinosinusitis with nasal polyps and comparison to those in atopic dermatitis. PLoS One. 5(7):e11450.

Pliakas AM, Carlson RR, Neve RL, Konradi C, Nestler EJ, Carlezon WA, Jr. 2001. Altered responsiveness to cocaine and increased immobility in the forced swim test associated with elevated camp response element-binding protein expression in nucleus accumbens. J Neurosci. 21(18):7397-7403.

Polderman TJ, Benyamin B, de Leeuw CA, Sullivan PF, van Bochoven A, Visscher PM, Posthuma D. 2015. Meta-analysis of the heritability of human traits based on fifty years of twin studies. Nat Genet. 47(7):702-709.

Pollak TA, Drndarski S, Stone JM, David AS, McGuire P, Abbott NJ. 2018. The blood-brain barrier in psychosis. Lancet Psychiatry. 5(1):79-92.

Pozzi L, Hakansson K, Usiello A, Borgkvist A, Lindskog M, Greengard P, Fisone G. 2003. Opposite regulation by typical and atypical anti-psychotics of erk1/2, creb and elk-1 phosphorylation in mouse dorsal striatum. J Neurochem. 86(2):451-459.

Quandt K, Frech K, Karas H, Wingender E, Werner T. 1995. Matind and matinspector: New fast and versatile tools for detection of consensus matches in nucleotide sequence data. Nucleic Acids Res. 23(23):4878-4884.

Rees E, Walters JT, Georgieva L, Isles AR, Chambert KD, Richards AL, Mahoney-Davies G, Legge SE, Moran JL, McCarroll SA et al. 2014. Analysis of copy number variations at 15 schizophrenia-associated loci. Br J Psychiatry. 204(2):108-114.

Revankar CM, Cimino DF, Sklar LA, Arterburn JB, Prossnitz ER. 2005. A transmembrane intracellular estrogen receptor mediates rapid cell signaling. Science. 307(5715):1625-1630.

Rice MW, Roberts RC, Melendez-Ferro M, Perez-Costas E. 2016. Mapping dopaminergic deficiencies in the substantia nigra/ventral tegmental area in schizophrenia. Brain Struct Funct. 221(1):185-201.

Richards JP, Bachinger HP, Goodman RH, Brennan RG. 1996. Analysis of the structural properties of camp-responsive element-binding protein (creb) and phosphorylated creb. J Biol Chem. 271(23):13716-13723.

Ripke S, Neale B, Corvin A, Walters JT FK, Holmans PA, Lee P, Bulik-Sullivan B, Collier DA, Huang H, Pers TH, Agartz I, Agerbo E, Albus M, Alexander M, Amin F, Bacanu SA, Begemann M, Belliveau RA Jr, Bene J, Bergen SE, Bevilacqua E, Bigdeli TB, Black DW, Bruggeman R, Buccola NG, Buckner RL, Byerley W, Cahn W, Cai G, Campion D, Cantor RM, Carr VJ, Carrera N, Catts SV, Chambert KD, Chan RC, Chen RY, Chen EY, Cheng W, Cheung EF, Chong SA, Cloninger CR, Cohen D, Cohen N, Cormican P, Craddock N, Crowley JJ, Curtis D, Davidson M, Davis KL, Degenhardt F, Del Favero J, Demontis D, Dikeos D, Dinan T, Djurovic S, Donohoe G, Drapeau E, Duan J, Dudbridge F, Durmishi N, Eichhammer P, Eriksson J, Escott-Price V, Essioux L, Fanous AH, Farrell MS, Frank J, Franke L, Freedman R, Freimer NB, Friedl M, Friedman JI, Fromer M, Genovese G, Georgieva L, Giegling I, Giusti-Rodríguez P, Godard S, Goldstein JI, Golimbet V, Gopal S, Gratten J, de Haan L, Hammer C, Hamshere ML, Hansen M, Hansen T, Haroutunian V, Hartmann AM, Henskens FA, Herms S, Hirschhorn JN, Hoffmann P, Hofman A, Hollegaard MV, Hougaard DM, Ikeda M, Joa I, Julià A, Kahn RS, Kalaydjieva L, Karachanak-Yankova S, Karjalainen J, Kavanagh D, Keller MC, Kennedy JL, Khrunin A, Kim Y, Klovins J, Knowles JA, Konte B, Kucinskas V, Ausrele Kucinskiene Z, Kuzelova-Ptackova H, Kähler AK, Laurent C, Keong JL, Lee SH, Legge SE, Lerer B, Li M, Li T, Liang KY, Lieberman J, Limborska S, Loughland CM, Lubinski J, Lönnqvist J, Macek M Jr, Magnusson PK, Maher BS, Maier W, Mallet J, Marsal S, Mattheisen M, Mattingsdal M, McCarley RW, McDonald C, McIntosh AM, Meier S, Meijer CJ, Melegh B, Melle I, Mesholam-Gately RI, Metspalu A, Michie PT, Milani L, Milanova V, Mokrab Y, Morris DW, Mors O, Murphy KC, Murray RM, Myin-Germeys I, Müller-Myhsok B, Nelis M, Nenadic I, Nertney DA, Nestadt G, Nicodemus KK, Nikitina-Zake L, Nisenbaum L, Nordin A, O'Callaghan E,

O'Dushlaine C, O'Neill FA, Oh SY, Olincy A, Olsen L, Van Os J, Pantelis C, Papadimitriou GN, Papiol S, Parkhomenko E, Pato MT, Paunio T, Pejovic-Milovancevic M, Perkins DO, Pietiläinen O, Pimm J, Pocklington AJ, Powell J, Price A, Pulver AE, Purcell SM, Quested D, Rasmussen HB, Reichenberg A, Reimers MA, Richards AL, Roffman JL, Roussos P, Ruderfer DM, Salomaa V, Sanders AR, Schall U, Schubert CR, Schulze TG, Schwab SG, Scolnick EM, Scott RJ, Seidman LJ, Shi J, Sigurdsson E, Silagadze T, Silverman JM, Sim K, Slominsky P, Smoller JW, So HC, Spencer CA, Stahl EA, Stefansson H, Steinberg S, Stogmann E, Straub RE, Strengman E, Strohmaier J, Stroup TS, Subramaniam M, Suvisaari J, Svrakic DM, Szatkiewicz JP, Söderman E, Thirumalai S, Toncheva D, Tosato S, Veijola J, Waddington J, Walsh D, Wang D, Wang Q, Webb BT, Weiser M, Wildenauer DB, Williams NM, Williams S, Witt SH, Wolen AR, Wong EH, Wormley BK, Xi HS, Zai CC, Zheng X, Zimprich F, Wray NR, Stefansson K, Visscher PM, Adolfsson R, Andreassen OA, Blackwood DH, Bramon E, Buxbaum JD, Børglum AD, Cichon S, Darvasi A, Domenici E, Ehrenreich H, Esko T, Gejman PV, Gill M, Gurling H, Hultman CM, Iwata N, Jablensky AV, Jönsson EG, Kendler KS, Kirov G, Knight J, Lencz T, Levinson DF, Li QS, Liu J, Malhotra AK, McCarroll SA, McQuillin A, Moran JL, Mortensen PB, Mowry BJ, Nöthen MM, Ophoff RA, Owen MJ, Palotie A, Pato CN, Petryshen TL, Posthuma D, Rietschel M, Riley BP, Rujescu D, Sham PC, Sklar P, St Clair D, Weinberger DR, Wendland JR, Werge T, Daly MJ, Sullivan PF, O'Donovan MC. 2014. Biological insights from 108 schizophrenia-associated genetic loci. Nature. 511(7510):421-427.

Ripke S, O'Dushlaine C, Chambert K, Moran JL, Kahler AK, Akterin S, Bergen SE, Collins AL, Crowley JJ, Fromer M et al. 2013. Genome-wide association analysis identifies 13 new risk loci for schizophrenia. Nat Genet. 45(10):1150-1159.

Ripke S, Sanders A, Kendler K, Levinson D. 2011. Genome-wide association study identifies five new schizophrenia loci. Nat Genet. 43(10):969-976.

Rossler W, Salize HJ, van Os J, Riecher-Rossler A. 2005. Size of burden of schizophrenia and psychotic disorders. Eur Neuropsychopharmacol. 15(4):399-409.

Saddock. Ka. 2008. Concise textbook of clinical psychiatry.

Saha S, Chant D, Welham J, McGrath J. 2005. A systematic review of the prevalence of schizophrenia. PLoS Med. 2(5):e141.

Sainz J, Mata I, Barrera J, Perez-Iglesias R, Varela I, Arranz MJ, Rodriguez MC, Crespo-Facorro B. 2013. Inflammatory and immune response genes have significantly altered expression in schizophrenia. Mol psychiatry. England. p. 1056-1057.

Sakamoto K, Karelina K, Obrietan K. 2011. Creb: A multifaceted regulator of neuronal plasticity and protection. J Neurochem. 116(1):1-9.

Sakamoto KM, Frank DA. 2009. Creb in the pathophysiology of cancer: Implications for targeting transcription factors for cancer therapy. Clin Cancer Res. 15(8):2583-2587.

Schmid CL, Streicher JM, Meltzer HY, Bohn LM. 2014. Clozapine acts as an agonist at serotonin 2a receptors to counter mk-801-induced behaviors through a betaarrestin2-independent activation of akt. Neuropsychopharmacology. 39(8):1902-1913.

Schmidt MJ, Mirnics K. 2015. Neurodevelopment, gaba system dysfunction, and schizophrenia. Neuropsychopharmacology. 40(1):190-206.

Schulze B, Rossler W. 2005. Caregiver burden in mental illness: Review of measurement, findings and interventions in 2004-2005. Curr Opin Psychiatry. 18(6):684-691.

Schumacher MA, Goodman RH, Brennan RG. 2000. The structure of a creb bzip.Somatostatin cre complex reveals the basis for selective dimerization and divalent cation-enhanced dna binding. J Biol Chem. 275(45):35242-35247.

Seamon KB, Padgett W, Daly JW. 1981. Forskolin: Unique diterpene activator of adenylate cyclase in membranes and in intact cells. Proc Natl Acad Sci U S A. 78(6):3363-3367.

Selten JP, Cantor-Graae E. 2005. Social defeat: Risk factor for schizophrenia? Br j psychiatry. England. p. 101-102.

Seshadri S, DeStefano AL, Au R, Massaro JM, Beiser AS, Kelly-Hayes M, Kase CS, D'Agostino RB, Sr., Decarli C, Atwood LD et al. 2007. Genetic correlates of brain aging on mri and cognitive test measures: A genome-wide association and linkage analysis in the framingham study. BMC Med Genet. 8 Suppl 1:S15.

Sethi MK, Zaia J. 2017. Extracellular matrix proteomics in schizophrenia and alzheimer's disease. Anal Bioanal Chem. 409(2):379-394.

Shi Y. 2009. Serine/threonine phosphatases: Mechanism through structure. Cell. 139(3):468-484.

Shirazi-Southall S, Rodriguez DE, Nomikos GG. 2002. Effects of typical and atypical antipsychotics and receptor selective compounds on acetylcholine efflux in the hippocampus of the rat. Neuropsychopharmacology. 26(5):583-594.

Siskind D, McCartney L, Goldschlager R, Kisely S. 2016. Clozapine v. First- and second-generation antipsychotics in treatment-refractory schizophrenia: Systematic review and meta-analysis. Br J Psychiatry. 209(5):385-392.

Smyth AM, Lawrie SM. 2013. The neuroimmunology of schizophrenia. Clin Psychopharmacol Neurosci. 11(3):107-117.

Sorensen HJ, Mortensen EL, Reinisch JM, Mednick SA. 2009. Association between prenatal exposure to bacterial infection and risk of schizophrenia. Schizophr Bull. 35(3):631-637.

Sorensen HJ, Mortensen EL, Schiffman J, Reinisch JM, Maeda J, Mednick SA. 2010. Early developmental milestones and risk of schizophrenia: A 45-year follow-up of the copenhagen perinatal cohort. Schizophr Res. 118(1-3):41-47.

Steiner J, Bielau H, Bernstein HG, Bogerts B, Wunderlich MT. 2006. Increased cerebrospinal fluid and serum levels of s100b in first-onset schizophrenia are not related to a degenerative release of glial fibrillar acidic protein, myelin basic protein and neurone-specific enolase from glia or neurones. J Neurol Neurosurg Psychiatry. 77(11):1284-1287.

Suhre K, Shin SY, Petersen AK, Mohney RP, Meredith D, Wagele B, Altmaier E, Deloukas P, Erdmann J, Grundberg E et al. 2011. Human metabolic individuality in biomedical and pharmaceutical research. Nature. 477(7362):54-60.

Sun L, Zhao R, Zhang L, Zhang W, He G, Yang S, Song J, Du G. 2016. Prevention of vascular smooth muscle cell proliferation and injury-induced neointimal hyperplasia by creb-mediated p21 induction: An insight from a plant polyphenol. Biochem Pharmacol. 103:40-52.

Sykova E, Nicholson C. 2008. Diffusion in brain extracellular space. Physiol Rev. 88(4):1277-1340.

Takahashi H, Yamada M, Suhara T. 2012. Functional significance of central d1 receptors in cognition: Beyond working memory. J Cereb Blood Flow Metab. 32(7):1248-1258.

Takanabe-Mori R, Ono K, Wada H, Takaya T, Ura S, Yamakage H, Satoh-Asahara N, Shimatsu A, Takahashi Y, Fujita M et al. 2013. Lectin-like oxidized low-density lipoprotein receptor-1 plays an important role in vascular inflammation in current smokers. J Atheroscler Thromb. 20(6):585-590.

Tan KC, Shiu SW, Wong Y, Leng L, Bucala R. 2008. Soluble lectin-like oxidized low density lipoprotein receptor-1 in type 2 diabetes mellitus. J Lipid Res. 49(7):1438-1444.

Tandon R, Gaebel W, Barch DM, Bustillo J, Gur RE, Heckers S, Malaspina D, Owen MJ, Schultz S, Tsuang M et al. 2013. Definition and description of schizophrenia in the dsm-5. Schizophr Res. 150(1):3-10.

Tandon R, Keshavan MS, Nasrallah HA. 2008a. Schizophrenia, "Just the facts" What we know in 2008. 2. Epidemiology and etiology. Schizophr Res. 102(1-3):1-18.

Tandon R, Keshavan MS, Nasrallah HA. 2008b. Schizophrenia, "Just the facts": What we know in 2008 part 1: Overview. Schizophr Res. 100(1-3):4-19.

Tang BL, Hong W. 1999. Adamts: A novel family of proteases with an adam protease domain and thrombospondin 1 repeats. FEBS Lett. 445(2-3):223-225.

Teng CT, Gladwell W, Beard C, Walmer D, Teng CS, Brenner R. 2002. Lactoferrin gene expression is estrogen responsive in human and rhesus monkey endometrium. Mol Hum Reprod. 8(1):58-67.

Trinh AT, Kim SH, Chang HY, Mastrocola AS, Tibbetts RS. 2013. Cyclin-dependent kinase 1-dependent phosphorylation of camp response element-binding protein decreases chromatin occupancy. J Biol Chem. 288(33):23765-23775.

Troyer JL, Nelson GW, Lautenberger JA, Chinn L, McIntosh C, Johnson RC, Sezgin E, Kessing B, Malasky M, Hendrickson SL et al. 2011. Genome-wide association study implicates pard3b-based aids restriction. J Infect Dis. 203(10):1491-1502.

Tumpey TM, Basler CF, Aguilar PV, Zeng H, Solorzano A, Swayne DE, Cox NJ, Katz JM, Taubenberger JK, Palese P et al. 2005. Characterization of the reconstructed 1918 spanish influenza pandemic virus. Science. 310(5745):77-80.

van der Zijl NJ, Hanemaaijer R, Tushuizen ME, Schindhelm RK, Boerop J, Rustemeijer C, Bilo HJ, Verheijen JH, Diamant M. 2010. Urinary matrix metalloproteinase-8 and -9 activities in type 2 diabetic subjects: A marker of incipient diabetic nephropathy? Clin Biochem. 43(7-8):635-639.

van Os J, Kapur S. 2009. Schizophrenia. Lancet. 374(9690):635-645.

van Os J, Kenis G, Rutten BP. 2010. The environment and schizophrenia. Nature. 468(7321):203-212.

Vazquez-Polo FJ, Negrin M, Cabases JM, Sanchez E, Haro JM, Salvador-Carulla L. 2005. An analysis of the costs of treating schizophrenia in spain: A hierarchical bayesian approach. J Ment Health Policy Econ. 8(3):153-165.

Vengen IT, Dale AC, Wiseth R, Midthjell K, Videm V. 2010. Lactoferrin is a novel predictor of fatal ischemic heart disease in diabetes mellitus type 2: Long-term follow-up of the hunt 1 study. Atherosclerosis. 212(2):614-620.

Wadzinski BE, Wheat WH, Jaspers S, Peruski LF, Jr., Lickteig RL, Johnson GL, Klemm DJ. 1993. Nuclear protein phosphatase 2a dephosphorylates protein kinase a-phosphorylated creb and regulates creb transcriptional stimulation. Mol Cell Biol. 13(5):2822-2834.

Walsh MT, Ryan M, Hillmann A, Condren R, Kenny D, Dinan T, Thakore JH. 2002. Elevated expression of integrin alpha(iib) beta(iiia) in drug-naive, first-episode schizophrenic patients. Biol Psychiatry. 52(9):874-879.

Wang H, Xu J, Lazarovici P, Quirion R, Zheng W. 2018. Camp response element-binding protein (creb): A possible signaling molecule link in the pathophysiology of schizophrenia. Front Mol Neurosci. 11:255.

Wang KS, Liu XF, Aragam N. 2010. A genome-wide meta-analysis identifies novel loci associated with schizophrenia and bipolar disorder. Schizophr Res. 124(1-3):192-199.

Wang WM, Lee S, Steiglitz BM, Scott IC, Lebares CC, Allen ML, Brenner MC, Takahara K, Greenspan DS. 2003. Transforming growth factor-beta induces secretion of activated adamts-2. A procollagen iii n-proteinase. J Biol Chem. 278(21):19549-19557.

Wang X, Chen W, Zhang J, Khan A, Li L, Huang F, Qiu Z, Wang L, Chen X. 2017. Critical role of adamts2 (a disintegrin and metalloproteinase with thrombospondin motifs 2) in cardiac hypertrophy induced by pressure overload. Hypertension. 69(6):1060-1069.

Weinberg ED. 2001. Human lactoferrin: A novel therapeutic with broad spectrum potential. J Pharm Pharmacol. 53(10):1303-1310.

Weinberger D, Levitt P. 2011. Schizophrenia. Blackwell Publishing Ltd.

Weinberger DR. 1987. Implications of normal brain development for the pathogenesis of schizophrenia. Arch Gen Psychiatry. 44(7):660-669.

Wen AY, Sakamoto KM, Miller LS. 2010. The role of the transcription factor creb in immune function. J Immunol. 185(11):6413-6419.

Wenthur CJ, Lindsley CW. 2013. Classics in chemical neuroscience: Clozapine. ACS Chem Neurosci. 4(7):1018-1025.

Wood JN, Grafman J. 2003. Human prefrontal cortex: Processing and representational perspectives. Nat Rev Neurosci. 4(2):139-147.

Woodward ND, Purdon SE, Meltzer HY, Zald DH. 2005. A meta-analysis of neuropsychological change to clozapine, olanzapine, quetiapine, and risperidone in schizophrenia. Int J Neuropsychopharmacol. 8(3):457-472.

Wu X, Spiro C, Owen WG, McMurray CT. 1998. Camp response element-binding protein monomers cooperatively assemble to form dimers on dna. J Biol Chem. 273(33):20820-20827.

Xie C, Wang ZC, Liu XF, Wang L, Yang MS. 2011. Association between schizophrenia and single nucleotide polymorphisms in lipoprotein lipase gene in a han chinese population. Psychiatr Genet. 21(6):307-314.

Yamamori H, Hashimoto R, Ishima T, Kishi F, Yasuda Y, Ohi K, Fujimoto M, Umeda-Yano S, Ito A, Hashimoto K et al. 2013. Plasma levels of mature brain-derived neurotrophic factor (bdnf) and matrix metalloproteinase-9 (mmp-9) in treatment-resistant schizophrenia treated with clozapine. Neurosci Lett. 556:37-41.

Yan Z, Feng J, Fienberg AA, Greengard P. 1999. D(2) dopamine receptors induce mitogen-activated protein kinase and camp response element-binding protein phosphorylation in neurons. Proc Natl Acad Sci U S A. 96(20):11607-11612.

Yang BH, Son H, Kim SH, Nam JH, Choi JH, Lee JS. 2004. Phosphorylation of erk and creb in cultured hippocampal neurons after haloperidol and risperidone administration. Psychiatry Clin Neurosci. 58(3):262-267.

Yang H, Hou H, Pahng A, Gu H, Nairn AC, Tang YP, Colombo PJ, Xia H. 2015. Protein phosphatase-1 inhibitor-2 is a novel memory suppressor. J Neurosci. 35(45):15082-15087.

Zeng B, Li Y, Niu B, Wang X, Cheng Y, Zhou Z, You T, Liu Y, Wang H, Xu J. 2016. Involvement of pi3k/akt/foxo3a and pka/creb signaling pathways in the protective effect of fluoxetine against corticosterone-induced cytotoxicity in pc12 cells. J Mol Neurosci. 59(4):567-578.

Zhang G, Liu Y, Ruoho AE, Hurley JH. 1997. Structure of the adenylyl cyclase catalytic core. Nature. 386(6622):247-253.

Zhang H, Nielsen AL, Stromgaard K. 2019. Recent achievements in developing selective gq inhibitors. Med Res Rev.

Zuber B, Nikonenko I, Klauser P, Muller D, Dubochet J. 2005. The mammalian central nervous synaptic cleft contains a high density of periodically organized complexes. Proc Natl Acad Sci U S A. 102(52):19192-19197.

RESUMEN EN CASTELLANO

8. RESUMEN EN CASTELLANO.

8.1. Introducción.

La esquizofrenia (SCZ) es un trastorno neuropsiquiátrico con un profundo efecto sobre los individuos que la padecen y la sociedad (afectando entorno al 1% de la población, con una incidencia en Cantabria de 13.8 por cada 100.000 habitantes). La mayoría de los pacientes presentan, de forma prolongada pero intermitente, alteraciones de tipo psiquiátrica cuyos síntomas pueden acabar siendo crónicos y desarrollar incapacidad en el individuo. La psicopatología de cada individuo que padece SCZ es muy variable (relacionada con factores socioeconómicos, género, edad, ambiente urbano o consumo de drogas), presentándose alteraciones en la cognición, las emociones, la percepción de la realidad y otros aspectos del comportamiento. Por ello, la SCZ se presenta como una combinación heterogénea de síntomas, tradicionalmente clasificados como "positivos", "negativos" y "cognitivos". Los síntomas positivos reflejan comportamientos y pensamientos que normalmente no están presentes en los individuos y dan lugar a la "pérdida de contacto con la realidad", consistentes en delirios, alucinaciones y habla/comportamiento desorganizados. Los síntomas negativos incluyen retraimiento social, aplanamiento afectivo, anhedonia, afasia y abulia. Finalmente, los síntomas cognitivos se presentar como un amplio conjunto de disfunciones cognitivas. Debido a su complejidad, la SCZ es un trastorno difícil de diagnosticar (basado principalmente en la observación clínica) y sin test biológicos válidos disponibles.

En la actualidad, se piensa que la esquizofrenia es el resultado de una interacción compleja entre factores de riesgo genético y ambientales que influyen en el desarrollo cerebral temprano y la adaptación biológica de las experiencias durante el trascurso de la vida del individuo. En las últimas décadas, numerosos estudios han demostrado que los factores genéticos contribuyen sustancialmente en este trastorno mental, aunque no exclusivamente. En este sentido, estudios en gemelos han sugerido una heredabilidad

entorno al 80% de este trastorno. Además, numerosas mutaciones raras (como son los CNVs o los SNPs, por sus siglas en inglés) han sido relacionadas con un alto riesgo de padecer la enfermedad y con diferentes procesos biológicos (como por ejemplo, rutas dopaminérgicas/glutaminérgicas, sinapsis o canales de calcio), aunque actualmente se desconoce el efecto real de estas alteraciones genéticas sobre el desarrollo de la patología. Por otro lado, numerosas evidencias sugieren que individuos que manifiestan esquizofrenia como adultos, presentaron complicaciones durante el neurodesarrollo temprano dando lugar a dificultades cognitivas y sociales en dichos individuos que acabarán dando lugar a complicaciones psicóticas en la edad adulta temprana (hipótesis del neurodesarrollo).

La esquizofrenia se presenta con un curso crónico con frecuentes recaídas y tendencia a desarrollar resistencia al tratamiento (baja adherencia). Dicho tratamiento se basa en el uso de agentes farmacológicos, conocidos como fármacos antipsicóticos (APs), que tienen como diana receptores de neurotransmisores como la dopamina y la serotonina, entre muchos otros. Históricamente se han clasificados en APs típicos y atípicos, basándose en que, estos últimos, además de mejorar los síntomas positivos (como en el caso de los típicos), también reducían los efectos extrapiramidales (como parkinsonismo o discinesia tardía) y presentaban mejoría de los síntomas negativos y cognitivos. A pesar de la efectividad de los APs en la clínica, hay una marcada disparidad en la respuesta de los pacientes en cuanto a síntomas y efectos secundarios: mientras que entre el 50 y el 60% de los pacientes logran un grado óptimo de mejoría clínica de los síntomas positivos, es común poca o ninguna mejoría de los síntomas negativos o de los déficits cognitivos. Se estima que entre el 20-50% de los pacientes desarrollan resistencia al tratamiento y, de estos, alrededor del 30-60% sí que responden a clozapina. Estas evidencias sugieren que aunque el receptor D_2 es la diana clásica utilizada por los APs, su bloqueo puede no abordar la anomalía biológica principal en un porcentaje significativo de pacientes.

Desde una perspectiva molecular, los efectos de los APs también pueden incluir la modulación de mecanismos independientes del receptor D_2 a través de efectos indirectos, como por ejemplo aquellos relacionados con otros receptores metabotrópicos implicados en el control de la señalización dependiente de cAMP y/o aquellos relacionados con los efectos del agonismo sesgado. Los receptores dopaminérgicos son receptores acoplados a proteínas G que promueven la fosforilación de CREB a través de diferentes vías (activación de la señalización de cAMP/PKA o de MEK/ERK), siendo este factor de transcripción esencial en la transducción de señales de la dopamina. La proteína CREB está presente en diferentes áreas del cerebro y alteraciones de su actividad en neuronas dopaminérgicas han sido relacionadas con cambios en el comportamiento en animales. Además, alteraciones en la expresión de CREB han comprometido la supervivencia neuronal y la neurogénesis. El papel de CREB en el tratamiento de la SCZ también ha sido estudiado en animales, observándose que los diferentes APs pueden alterar la fosforilación y activación de CREB de diferentes maneras, cuyo efecto es diferente en función del estadio neuronal. Aunque se requieren mayor número de investigaciones, algunos estudios han sugerido que los niveles de fosforilación de CREB influye en trastornos del estado de ánimo y su regulación puede ser objeto de nuevas estrategias aplicadas al tratamiento de esta clase de trastornos.

El estudio de patrones transcripcionales asociados con la respuesta clínica al tratamiento con APs en pacientes con SCZ ha ampliado las posibilidades de estudio de nuevos mecanismos moleculares desconocidos asociados con la acción antipsicótica que pueden ser cruciales para la mejoría de la respuesta clínica. En esta línea, anteriores estudios de nuestro grupo de investigación, basados en secuenciación masiva del transcriptoma de pacientes, caracterizados en el ámbito del programa regional 'PAFIP' en el hospital U. 'Marqués de Valdecilla', nos han permitido conocer una firma de seis genes (*ADAMTS2*, *CD177*, *CNTNAP3*, *ENTPD2*, *RFX2*, and *UNC45B*) con expresión diferencial en los primeros episodios de psicosis (FEP, por sus siglas en inglés) en pacientes *drug-naïve* y cuya sobre-

expressión, en estos mismos pacientes respondedores clínicos tras tratamiento con APs atípicos, se reducía a niveles de controles sanos. Entre los resultados obtenidos en estos trabajos, hay que destacar el gen *ADAMTS2* como aquel que más significativamente estaba sobre-expresado en los FEP y el que mayor modulación experimento tras el tratamiento con APs en estos pacientes. ADAMTS2 pertenece a la familia de las proteasas ADAMTS (‘*a disintegrin and metalloproteinase with thrombospondin motifs*’) cuya actividad se basa en escindir la molécula conocida como pro-colágeno, esencial en el mantenimiento de la estructura de la matriz extracelular (ECM, por sus siglas en inglés), incluyendo aquella del sistema nervioso central (SNC). La relación establecida entre las proteasas de la familia ADAMTS y su capacidad para alterar el medio extracelular, junto con la observación de que la expresión de *ADAMTS2* puede ser modulada por los APs y su relación con la respuesta clínica en pacientes de SCZ, lo convierte en un gen candidato atractivo para investigar su papel en la patología y/o eficacia en la respuesta clínica al tratamiento antipsicótico.

8.2. Hipótesis y objetivos.

8.2.1. Hipótesis.

La esquizofrenia es un trastorno neuropsiquiátrico cuyo origen puede ser explicado por alteraciones genómica y mecanísticas que regulan importantes redes neuronales y rutas de señalización en el sistema nervioso central y células periféricas. Estas alteraciones pueden ser asociadas con diferentes estadios de la patología y la heterogeneidad en la respuesta clínica al tratamiento antipsicótico.

La hipótesis principal de la presente tesis se basa en la observación de que una serie de genes alterados en los FEP en pacientes de SCZ y regulados tras el tratamiento podrían estar relacionados con la respuesta clínica a fármacos antipsicóticos. Además, estas alteraciones en la expresión génica podrían estar mediadas por mecanismos moleculares y celulares en el SNC, cuyo estudio podría revelar nuevas dianas farmacológicas para futuros tratamientos.

8.2.2. Objetivos.

El objetivo principal de la presente tesis es diseccionar los mecanismos esenciales que participan en la biología de la esquizofrenia con un potencial papel para el diagnóstico y/o tratamiento. Además, tratará de comprender aspectos fisiológicos noveles de la enfermedad.

Esta tesis se centrará en tres objetivos específicos:

1. Estudio de los perfiles de expresión de los genes con respuesta clínica a los APs en diferentes condiciones y modelos experimentales:

    1.1. Validar la expresión diferencial de estos genes asociada con diferentes estadios de la esquizofrenia usando cohortes independientes de pacientes.

1.2. Estudio de la regulación de la transcripción de estos genes en respuesta a agonistas/antagonistas selectivos de receptores y fármacos antipsicóticos en células SK-N-SH.

1.3. Analizar la expresión de estos genes en diferentes estadios del desarrollo en cerebro de ratones sanos y en un modelo preclínico de comportamiento de SCZ.

2. Elucidar la señalización y mecanismos intracelulares relacionados con la expresión de *ADAMTS2* y los receptores diana de fármacos APs.

3. Investigar el papel de CREB y la señalización de los receptores de la familia D_1 en la regulación de la expresión de *ADAMTS2*.

8.3. Resultados y discusión.

8.3.1. La expresión de *ADAMTS2* en los primeros episodios de psicosis (FEP) de pacientes con esquizofrenia y su regulación por tratamiento antipsicótico.

Resultados previos en nuestro laboratorio basados en secuenciación del transcriptoma por mRNA-seq a partir de células mononucleares de sangre periférica (PBMCs, por sus siglas en inglés) de pacientes de SCZ caracterizados como respondedores clínicos, revelaron una firma de seis genes (*ADAMTS2*, *CD177*, *CNTNAP3*, *ENTPD2*, *RFX2*, and *UNC45B*) cuya expresión estaba significativamente alterada (elevada) en los primeros episodios de psicosis (FEP) en pacientes *drug-naïve* y que podía ser revertida a niveles 'sanos' tras el tratamiento con APs atípicos. A estos genes decidimos nombrarlos como 'genes con respuesta clínica a APs' (CRGAs, por sus siglas en inglés). Por tanto, en un primer momento, decidimos replicar el estudio de expresión en PBMCs mediante RT-qPCR en dos nuevas cohortes de pacientes respondedores y controles sanos. Los resultados mostraron a *ADAMTS2* como el gen significativamente más sobre-expresado en el basal y el que cuya expresión más se modulaba tras tres meses y un año de tratamiento con APs. Inesperadamente, en estas condiciones, la validación falló en la replicación del resto de genes que estudiábamos, posiblemente por las diferencias técnicas existentes entre ambas aproximaciones (RT-qPCR y mRNA-seq).

Debido a que no siempre existe una correlación entre los datos obtenidos en SNC y células de sangre periférica, decidimos analizar la expresión de la versión ortóloga de estos CRGAs en un ratón de comportamiento esquizofrénico, basado en un modelo preclínico de SCZ inducido por la infección por virus de la influenza. Los resultados en mPFC de cerebro de ratones 'enfermos', mostraron un patrón de sobre-expresión de cinco de los seis genes (*Adamts2, Cd177*, *Cntnap3*, *Entpd2 y Rfx2*). Estos datos, aunque no coinciden plenamente con los datos obtenidos en las cohortes anteriores, nos muestra que,

efectivamente, *Adamts2* está sobre-expresado en un modelo preclínico de SCZ y que su expresión puede ser detectada en SNC.

Finalmente, trabajamos con la línea celular humana de neuroblastoma llamaba SK-N-SH. Tras la incubación con APs (clozapina, haloperidol, risperidona y aripiprazol), observamos que estos fármacos pueden modular dinámicamente la expresión de los CRGAs. De acuerdo con nuestros datos previos en pacientes, sólo la expresión de *ADAMTS2* fue inhibido de forma rápida y robusta en células SK-N-SH después de la incubación con todos los APs: siendo transitoria con risperidona y aripiprazol; y mantenida con clozapina y haloperidol. Destaca, además, clozapina como el AP que mayor efecto tubo sobre la expresión de todos los CRGAs. Es posible que este efecto sea producido por cambios que involucren mecanismos intracelulares más complejos a largo plazo, que puedan estar regulando la funcionalidad neural, la neuroplasticidad o la neurogénesis, y, que a su vez, le otorguen mayor eficacia frente a la aparición de resistencia al tratamiento en los pacientes.

8.3.2. Señalización dopaminérgica involucrada en la regulación de la expresión de *ADAMTS2*.

Se dispone de escasa información sobre la expresión de *ADAMTS2* y su actividad en el SNC. Por ello, decidimos estudiar su expresión en cerebros de ratón C57BL/6 sano. Primero, mediante hibridación *in situ* de RNA de resultados obtenidos a partir de la base de datos de 'Eurexpress', observamos que el mRNA de *Adamts2* se detectó en el giro dentado (DG) del hipocampo y el área tegmental ventral (VTA) de cerebro de embrión de estadio E14.5 de ratones. Por otro lado, mediante tinción inmunohistoquímica contra la proteína de Adamts2 en ratón postnatal temprano, los datos revelaron su expresión en regiones cerebrales cuyas neuronas expresaban los receptores D_1 y D_2. Finalmente, en las células SK-N-SH, se observó que tras la incubación con el agonista selectivo del

receptor D_1 (SKF 83822), se incrementó significativamente la expresión de ADAMTS2, en comparación con otros agonistas selectivos.

Uno de los pilares clave en el estudio del trastorno de la SCZ ha sido la hipótesis que los mecanismos dopaminérgicos juegan un papel esencial en la psicosis y la esquizofrenia. La mayoría de los fármacos antipsicóticos utilizados en la actualidad se basan en el bloqueo del receptor D_2 y la mejoría clínica en los síntomas positivos. Aunque la relación entre sistema dopaminérgico y la expresión de *ADAMTS2* no había sido previamente establecido, para nuestro conocimiento, si observamos nuestros datos en cerebros de ratones, *Adamts2* se localizó en regiones que forman parte de los sistemas mesolímbicos y mesocorticales dopaminérgicos del cerebro (VTA, mPFC, etc.) ampliamente relacionados con el trastorno de la esquizofrenia. Además, estas regiones expresan ambas familias de receptores dopaminérgicos, que regulan diferentes aspectos del comportamiento y la cognición, especialmente la familia de receptores D_1. Por otro lado, el papel de *ADAMTS2* en el entorno de la ECM neural no ha sido estudiado en profundidad. Esta proteína forma parte de la familia de proteasas ADAMTS, las cuales participan en la organización del ECM. Las ADAMTS en el SNC, son clave en los cambios de composición del ECM del entorno neural: facilitando la neurogénesis y la neuroplasticidad en el desarrollo temprano, y estabilizando conexiones sinápticas en la edad adulta. Además, cambios en el ECM están relacionados con la difusión de fármacos a través de la barrera hematoencefálica. Esto nos permitiría especular que ADAMTS2 pueda tener un papel fundamental en la aparición de las bases del trastorno en la edad temprana del individuo y, también, en la respuesta clínica al tratamiento con APs.

8.3.3. CREB como factor de transcripción clave en la expresión de *ADAMTS2*.

Nuestros datos en células de SK-N-SH sugieren que la regulación de la expresión de *ADAMTS2* se realiza a través de los receptores D_1, uno de los receptores más abundantes

en el SNC. Esta familia de receptores señaliza a través de la adenilil ciclasa (AC) que regula los niveles intracelulares de cAMP, activando la PKA y fosforilando CREB. Por ello, decidimos estudiar los mecanismos moleculares que puedan estar interviniendo en el control de la expresión de *ADAMTS2* en estas células. Nuestros datos mediante RT-qPCR, además de sugerir una estimulación de la expresión dependiente del receptor D_1 (por SKF 83822), mostraron un incremento del mRNA de *ADAMTS2* al activar la subunidad $G_{\alpha s}$ (por cholera toxin) y AC (por forskolina), poniendo en escena la importancia del eje $G_{\alpha s}$/cAMP/CREB en la regulación de dicho gen. Además, mediante ensayos de gen reportero por luciferasa (CRE-luc), sólo clozapina era capaz de revertir la activación de CREB por SKF 83822. Posteriormente, para evaluar la contribución de PKA y MEK a la activación de *ADAMTS2*, mediante el uso de inhibidores específicos de estas dos proteínas, observamos que ambos inhibidores son capaces de bloquear la activación de *ADAMTS2* dependiente del receptor D_1. Finalmente, mediante el silenciamiento de CREB por infección lentiviral de vectores con shRNA para CREB1, observamos que la activación de la expresión de *ADAMTS2* dependiente del receptor D_1 (SKF 83822) y PKA (por forskolina), es incapaz de producirse. Estos datos refuerzan la idea que el eje cAMP/CREB es un mecanismo esencial en la transcripción de *ADAMTS2*.

Una gran cantidad de estudios recientes han puesto en evidencia la señalización basada en cAMP/CREB como un importante mecanismo en la relación de la señalización dopaminérgica y la fisiopatología de la SCZ. Por ello, nos propusimos evaluar si CREB pudiera estar influyendo en la sobreexpresión de *ADAMTS2* en los FEP de pacientes de SCZ *drug-naïve*. Nuestros resultados en PBMCs (todavía preliminares), nos mostraron: (1) una disminución de la cantidad total de CREB unido al DNA de los pacientes tras el tratamiento con APs (usando ensayos ELISA); y (2) un mayor enriqueciendo de CREB unido a la región CRE (sitio especifico de unión como factor de transcripción) en la región promotora del gen *ADAMTS2* en pacientes en el comienzo de la enfermedad (*drug-naïve*) (mediante ensayos ChIP contra CREB).

En conclusión, los resultados expuestos en la presente tesis argumentan a favor que el factor de transcripción CREB podría estar jugando un papel esencial en el control de la expresión del gen *ADAMTS2*. Además, muestran a *ADAMTS2* como un potencial gen candidato, junto con la actividad de CREB, para ser considerado como biomarcador para el diagnóstico en diferentes estadios de la esquizofrenia y/o para la respuesta clínica al tratamiento con APs.

ANNEXES

ANNEXES

Annexed Table 1

geneID	Gene Symbol	baseMean	baseMeanA	baseMeanB	foldChange	log2FoldChange	pval	padj
9509	ADAMTS2	24.026600	45.834033	4.399911	0.095997	-3.380873	6.32907E-74	1.3236E-69
10232	MSLN	19.902828	2.957421	35.153693	11.886603	3.571265	4.04424E-49	4.22886E-45
3429	IFI27	75.198695	33.505473	112.722595	3.364304	1.750308	5.94483E-34	4.14414E-30
680	BRS3	2.507595	4.974239	0.287615	0.057821	-4.112263	1.78269E-25	9.32034E-22
84873	GPR128	6.945074	8.904480	5.181608	0.581910	-0.781131	3.17266E-23	1.327E-19
105	ADARB2	171.335909	119.550223	217.943026	1.823025	0.866334	3.49048E-21	1.21661E-17
23500	DAAM2	107.383519	162.735641	57.566609	0.353743	-1.499226	4.72498E-21	1.41162E-17
3048	HBG2	815.737293	470.213913	1126.708335	2.396161	1.260725	2.38445E-18	6.23324E-15
55301	OLAH	8.718639	13.984260	3.979580	0.284576	-1.813116	5.68551E-18	1.32112E-14
722	C4BPA	87.844973	52.460749	119.690776	2.281530	1.190002	4.96129E-16	1.03756E-12
6422	SFRP1	5.137860	1.842809	8.103405	4.397311	2.136622	1.25359E-15	2.38331E-12
283726	FAM154B	17.664389	25.802382	10.340195	0.400746	-1.319241	2.18555E-15	3.80886E-12
10349	ABCA10	39.828973	52.485733	28.437888	0.541821	-0.884111	4.77E-15	7.67347E-12
91543	RSAD2	1695.646906	1053.050547	2273.983629	2.159425	1.110647	1.00982E-14	1.50846E-11
3047	HBG1	355.697607	228.284645	470.369272	2.060451	1.042960	1.78018E-13	2.48193E-10
146862	UNC45B	229.458589	336.931785	132.732712	0.393945	-1.343933	8.73578E-13	1.14182E-09
30848	CTAG2	1.097797	2.297113	0.018412	0.008015	-6.963062	1.54775E-12	1.904E-09
9381	OTOF	57.931752	41.195009	72.994821	1.771934	0.825325	5.50169E-12	6.39205E-09
26153	KIF26A	10.644450	16.129551	5.707859	0.353876	-1.498685	8.05665E-12	8.86783E-09
10964	IFI44L	1504.272893	959.158358	1994.875974	2.079819	1.056458	9.02943E-12	9.44162E-09
346171	ZFP57	12.563949	8.363857	16.344033	1.954126	0.966524	9.73699E-12	9.69665E-09
1675	CFD	1334.446577	1754.339473	956.542971	0.545244	-0.875026	1.58022E-11	1.50214E-08
57126	CD177	126.918303	170.698824	87.515833	0.512691	-0.963837	2.97617E-10	2.70612E-07
389792	IER5L	74.242768	100.023292	51.040296	0.510284	-0.970627	6.26795E-10	5.4002E-07
5239	PGM5	50.343508	67.754069	34.674004	0.511763	-0.966453	6.45555E-10	5.4002E-07
152573	SHISA3	2.039628	3.637135	0.601871	0.165480	-2.595275	1.47145E-09	1.18356E-06
374407	DNAJB13	84.830385	63.024624	104.455570	1.657377	0.728902	4.39699E-09	3.28408E-06
954	ENTPD2	26.792888	35.713767	18.764096	0.525402	-0.928506	4.28886E-09	3.28408E-06
79937	CNTNAP3	229.521550	304.886418	161.693169	0.530339	-0.915013	5.53349E-09	3.99041E-06
3434	IFIT1	1683.606350	1174.892272	2141.449021	1.822677	0.866059	7.48191E-09	5.21564E-06
6035	RNASE1	16.681659	23.099121	10.905943	0.472137	-1.082723	9.47775E-09	6.39382E-06
100128071	LOC100128071	25.304834	34.134849	17.357820	0.508507	-0.975660	1.36062E-08	8.89204E-06
401115	C4orf48	59.906978	73.665168	47.524607	0.645144	-0.632308	1.50516E-08	9.53864E-06
#N/D	SH3D20	76.357583	99.886571	55.181493	0.552442	-0.856106	1.62509E-08	9.99572E-06
64105	CENPK	263.769115	185.487901	334.222207	1.801854	0.849483	1.95993E-08	1.17109E-05
389813	C9orf172	44.204997	55.049531	34.444916	0.625708	-0.676439	2.12993E-08	1.23731E-05
146439	CCDC64B	56.512991	75.634308	39.303806	0.519656	-0.944372	2.67713E-08	1.51316E-05
55194	FAM176B	80.058148	103.893588	58.606251	0.564099	-0.825980	2.8702E-08	1.57959E-05
2812	GP1BB	455.557098	600.593229	325.024581	0.541173	-0.885839	4.06221E-08	2.17828E-05
56163	RNF17	3.873280	6.039121	1.924023	0.318593	-1.650213	4.17216E-08	2.18131E-05
51375	SNX7	4.482696	6.289891	2.856221	0.454097	-1.138928	5.41432E-08	2.7617E-05
171484	FAM9C	4.072980	6.203126	2.155848	0.347542	-1.524740	7.32818E-08	3.64891E-05
2850	GPR27	111.188114	150.714810	75.614088	0.501703	-0.995094	7.80255E-08	3.70852E-05
100500847	MIR3615	49.281767	61.032256	38.706327	0.634195	-0.657002	7.68651E-08	3.70852E-05
4070	TACSTD2	26.455666	33.859943	19.791817	0.584520	-0.774675	1.70679E-07	7.93203E-05
2899	GRIK3	5.978323	8.586254	3.631186	0.422907	-1.241588	2.16646E-07	9.63981E-05
3811	KIR3DL1	93.226172	117.598700	71.290897	0.606222	-0.722082	2.14966E-07	9.63981E-05
6614	SIGLEC1	741.597466	558.415261	906.461450	1.623275	0.698907	2.34659E-07	0.000102238
2848	GPR25	34.863794	41.927778	28.506208	0.679888	-0.556630	2.44785E-07	0.000102557
266629	SEC14L3	69.795312	56.022441	82.190896	1.467107	0.552974	2.45198E-07	0.000102557
399697	CTXN2	23.513187	32.331522	15.576685	0.481780	-1.053553	3.20572E-07	0.000131453
5657	PRTN3	23.700488	29.995986	18.034540	0.601232	-0.734007	3.67935E-07	0.000147974
3437	IFIT3	3314.679672	2456.412326	4087.120283	1.663858	0.734532	3.90592E-07	0.000154104
56241	SUSD2	14.317989	17.159409	11.760710	0.685380	-0.545025	3.97917E-07	0.000154104
6557	SLC12A1	1036.958111	1372.766452	734.730604	0.535219	-0.901799	4.23484E-07	0.000161024
6521	SLC4A1	2646.590174	2134.126208	3107.807744	1.456244	0.542252	4.57429E-07	0.000170825
222553	SLC35F1	13.787400	18.449576	9.591441	0.519873	-0.943768	4.84007E-07	0.00017758
22986	SORCS3	29.783529	23.890663	35.087108	1.468654	0.554494	5.32349E-07	0.000191949
765	CA6	274.614630	215.039196	328.232522	1.526385	0.610119	1.02502E-06	0.000363326
284207	METRNL	519.056148	672.387687	381.057762	0.566723	-0.819284	1.05263E-06	0.000366894
6285	S100B	102.721023	126.108870	81.671960	0.647631	-0.626757	1.10416E-06	0.000378547
3090	HIC1	49.404848	66.191915	34.296488	0.518137	-0.948594	1.13508E-06	0.000382871
64478	CSMD1	23.768552	18.266715	28.720206	1.572270	0.652849	1.15682E-06	0.000384011
8460	TPST1	153.797938	196.089630	115.735415	0.590217	-0.760683	1.24006E-06	0.000405208
11274	USP18	81.579680	59.857889	101.129292	1.689490	0.756588	1.31044E-06	0.00042162
55437	STRADB	644.864731	496.213797	778.650571	1.569184	0.650014	1.7176E-06	0.000544245

geneID	Gene Symbol	baseMean	baseMeanA	baseMeanB	foldChange	log2FoldChange	pval	padj
212	ALAS2	4770.872855	3841.677686	5607.148508	1.459557	0.545531	1.75894E-06	0.000549025
2038	EPB42	426.724352	350.141173	495.649213	1.415570	0.501383	2.01147E-06	0.000618616
10468	FST	2.452094	3.969268	1.086638	0.273763	-1.869001	2.40965E-06	0.000730333
8991	SELENBP1	572.263913	464.648873	669.117449	1.440050	0.526119	2.49097E-06	0.000733713
79776	ZFHX4	2.519362	3.652107	1.499891	0.410692	-1.283872	2.4573E-06	0.000733713
65990	FAM173A	107.831672	131.750824	86.304435	0.655058	-0.610305	2.70923E-06	0.000786129
112703	FAM71E1	46.116139	58.541067	34.933704	0.596738	-0.744829	2.7441E-06	0.000786129
5990	RFX2	499.317912	642.283741	370.648665	0.577079	-0.793158	2.89908E-06	0.000819305
730152	GSTA7P	0.747582	1.416990	0.145115	0.102411	-3.287562	3.1889E-06	0.000889192
1052	CEBPD	1668.282048	2137.440507	1246.039436	0.582959	-0.778535	3.41549E-06	0.000939843
548594	KIR3DP1	73.452433	89.933800	58.619202	0.651804	-0.617490	3.55978E-06	0.000966827
3848	KRT1	293.352563	231.622022	348.910050	1.506377	0.591083	3.90506E-06	0.001022088
4854	NOTCH3	17.301117	23.102207	12.080135	0.522900	-0.935394	3.90987E-06	0.001022088
5909	RAP1GAP	42.718989	31.810777	52.536380	1.651528	0.723801	3.85031E-06	0.001022088
2069	EREG	35.135187	45.313069	25.975093	0.573236	-0.802798	4.27929E-06	0.001104849
10501	SEMA6B	44.129218	63.864927	26.367080	0.412857	-1.276286	4.38926E-06	0.001119422
9022	CLIC3	172.779797	210.700445	138.651213	0.658049	-0.603733	5.259E-06	0.001325078
1051	CEBPB	2172.036846	2759.691951	1643.147251	0.595410	-0.748045	5.58503E-06	0.001390473
83656	C8orf12	42.845258	50.262875	36.169403	0.719605	-0.474723	5.83713E-06	0.001436141
2352	FOLR3	271.071413	359.526107	191.462189	0.532540	-0.909037	5.94215E-06	0.001437037
3802	KIR2DL1	35.884463	43.633659	28.910187	0.662566	-0.593864	5.97821E-06	0.001437037
126003	TRAPPC5	592.700663	742.447694	457.928334	0.616782	-0.697168	7.18992E-06	0.001708668
100423062	IGLL5	1241.859855	1496.678287	1012.523266	0.676514	-0.563809	7.79104E-06	0.001807324
3766	KCNJ10	7.893270	5.127188	10.382743	2.025037	1.017948	7.86432E-06	0.001807324
56666	PANX2	438.955406	587.974394	304.838317	0.518455	-0.947709	7.7597E-06	0.001807324
2161	F12	31.640038	40.471031	23.692144	0.585410	-0.772481	7.95654E-06	0.001808644
116835	HSPA12B	7.319957	8.425416	6.325044	0.750710	-0.413672	8.64009E-06	0.001942906
#N/D	C9orf140	31.163448	38.403265	24.647612	0.641810	-0.639781	8.8386E-06	0.001948437
23349	KIAA1045	21.239073	28.008236	15.146826	0.540799	-0.886836	8.94219E-06	0.001948437
4037	LRP3	119.223571	150.269008	91.282678	0.607462	-0.719134	8.9442E-06	0.001948437
#N/D	NCRNA00200	3.459063	2.155659	4.632127	2.148821	1.103546	9.07751E-06	0.001957093
5654	HTRA1	27.316698	34.760209	20.617537	0.593136	-0.753565	1.02743E-05	0.002192518
11326	VSIG4	67.625430	82.392997	54.334619	0.659457	-0.600650	1.17398E-05	0.002479934
56603	CYP26B1	20.337010	24.926296	16.206652	0.650183	-0.621082	1.23503E-05	0.002532175

Annexed Table 2

geneID	Gene Symbol	Base Mean	Base Mean Not Medicated	Base Mean Medicated	Fold Change	Log2 Fold Change	Pval	Padj
9509	ADAMTS2	23.8633998	43.5827381	4.14406151	0.09508493	-3.39463952	1.9322E-45	3.7708E-41
57126	CD177	125.214203	182.642966	67.7854394	0.37113633	-1.42997888	4.1559E-15	4.0551E-11
146862	UNC45B	228.318709	355.590532	101.046886	0.28416641	-1.81519204	9.3573E-13	6.0869E-09
10321	CRISP3	115.798684	67.5920582	164.005309	2.4263991	1.27881687	4.8714E-09	2.3766E-05
85495	RPPH1	26.0470251	39.6458922	12.4481581	0.31398355	-1.67123911	3.214E-07	0.00125443
249	ALPL	4431.74611	5880.32097	2983.17125	0.50731436	-0.97904811	1.4913E-06	0.00344926
4317	MMP8	183.666354	123.534818	243.79789	1.9735156	0.98076792	1.8018E-06	0.00344926
4973	OLR1	33.1319214	20.8596342	45.4042086	2.17665412	1.12211217	1.4058E-06	0.00344926
10562	OLFM4	137.50003	93.0789384	181.921122	1.95448213	0.96678639	1.9442E-06	0.00344926
1088	CEACAM8	187.212884	127.349844	247.075924	1.94013527	0.95615724	1.5981E-06	0.00344926
154664	ABCA13	92.1790157	61.8853678	122.472664	1.97902457	0.98478952	1.3657E-06	0.00344926
5990	RFX2	520.355823	681.16962	359.542026	0.52783039	-0.92185368	7.0039E-06	0.01051397
4057	LTF	1632.34997	1170.32361	2094.37632	1.78957026	0.83961318	6.5125E-06	0.01051397
2852	GPER1	28.3369356	36.7709215	19.9029497	0.54126872	-0.88558307	1.0099E-05	0.01407669
79937	CNTNAP3	195.227907	249.033421	141.422394	0.5678852	-0.81632878	1.6829E-05	0.02189388
954	ENTPD2	29.04178	36.5123712	21.5711888	0.59079123	-0.75927968	2.5164E-05	0.03069279
387755	INSC	31.7387972	41.6047807	21.8728136	0.52572837	-0.9276105	3.8283E-05	0.04394647
1991	ELANE	76.6833597	91.053017	62.3137025	0.68436725	-0.54715738	5.9052E-05	0.06402204
389813	C9orf172	63.3846919	77.315243	49.4541408	0.63964283	-0.64466155	9.0179E-05	0.09262339
3625	INHBB	16.1028163	21.4796452	10.7259874	0.49935589	-1.0018597	0.00014204	0.13621689
7180	CRISP2	2.58667534	1.09607432	4.07727636	3.71989042	1.89526012	0.00014658	0.13621689
3848	KRT1	210.908544	165.251102	256.565986	1.5525826	0.63467003	0.00020037	0.17000946
285696	AC091878.1	60.0669474	75.0119349	45.1219599	0.60153041	-0.73329042	0.00019487	0.17000946
25775	C22orf24	154.570123	194.726421	114.413824	0.58756189	-0.76718727	0.00021003	0.17078147
3934	LCN2	370.412837	285.536593	455.28908	1.59450344	0.67310721	0.00022267	0.17381696
389792	IER5L	101.576133	126.697917	76.4543477	0.60343808	-0.72872236	0.0003056	0.22937299
8120	AP3B2	20.2059925	25.8766688	14.5353161	0.56171512	-0.83208946	0.00041963	0.2669202
5657	PRTN3	32.3901001	38.120854	26.6593463	0.69933759	-0.51593904	0.00046237	0.2669202
199675	C19orf59	418.288918	515.723317	320.854519	0.62214468	-0.68467797	0.00046504	0.2669202
4680	CEACAM6	141.107548	106.264272	175.950824	1.65578534	0.72751565	0.00038084	0.2669202
83999	KREMEN1	698.705581	859.837907	537.573254	0.62520302	-0.67760336	0.00042501	0.2669202
2172	FABP6	78.7903286	98.8146349	58.7660223	0.5947097	-0.74974248	0.00041365	0.2669202
10842	PPP1R17	6.97056195	8.95676199	4.98436191	0.5564915	-0.84556845	0.00044593	0.2669202
212	ALAS2	4171.91918	3378.20145	4965.63692	1.46990551	0.55572342	0.00041266	0.2669202
65990	FAM173A	139.144942	170.96511	107.324774	0.62775834	-0.6717188	0.00050991	0.26894367
79623	GALNT14	70.2697348	84.6043458	55.9351237	0.66113771	-0.59697728	0.00050902	0.26894367
554226	ANKRD30BL	5.98277107	9.23809637	2.72744577	0.29523894	-1.76004508	0.00050877	0.26894367
131540	ZDHHC19	62.1731299	76.6546163	47.6916435	0.62216271	-0.68463618	0.00060146	0.30888182
5654	HTRA1	29.0901674	36.3559096	21.8244253	0.60029925	-0.73624622	0.00075618	0.37837951
11007	CCDC85B	446.035191	531.689649	360.380733	0.6778028	-0.5610625	0.00091942	0.43953719
1675	CFD	1920.56635	2290.19601	1550.93668	0.67720696	-0.56233129	0.00092344	0.43953719
25790	CCDC19	92.536496	116.299622	68.7733698	0.59134646	-0.75792447	0.00094761	0.44029874
55301	OLAH	6.5652293	8.12671964	5.00373896	0.61571448	-0.6996666	0.0010178	0.46191584
283692	RP11-752G15	17.3727069	21.9906507	12.754763	0.58000844	-0.7858542	0.00108605	0.48168932
165530	CLEC4F	34.5274924	41.402309	27.6526759	0.66790178	-0.58229213	0.00117412	0.50917517
55194	EVA1B	111.197559	134.522134	87.8729848	0.65322324	-0.61435197	0.00125516	0.51029906
2826	CCR10	23.7737706	28.938547	18.6089943	0.64305213	-0.6369924	0.00123591	0.51029906
6948	TCN2	136.228865	162.895748	109.561982	0.67258958	-0.57220168	0.00122068	0.51029906
6768	ST14	636.627845	770.341036	502.914654	0.65284677	-0.61518369	0.0013781	0.53787181
60675	PROK2	2208.63282	2584.44468	1832.82097	0.709174	-0.49578846	0.00135066	0.53787181
306	ANXA3	622.558781	735.632189	509.485372	0.69258167	-0.52994388	0.00144447	0.552723
10461	MERTK	108.002303	127.493851	88.5107545	0.69423547	-0.52650301	0.00151342	0.56796852
5493	PPL	135.17282	165.722681	104.622959	0.63131346	-0.66357159	0.00160804	0.59209236
3249	HPN	5.84741561	7.89193517	3.80289605	0.48187117	-1.0532806	0.00166642	0.60222717
6521	SLC4A1	2212.55151	1832.14597	2592.95704	1.4152568	0.50106385	0.00182132	0.61281238
6234	RPS28	90.5153964	100.158649	80.8721434	0.80744043	-0.30857226	0.00180754	0.61281238
101929768	CTD-2127H9.	1.90027367	1.28367974	2.5168676	1.9606663	0.97134401	0.00176589	0.61281238
90249	UNC5A	31.8374377	40.6771772	22.9976983	0.56537105	-0.82273008	0.0017856	0.61281238
89958	SAPCD2	34.5784916	40.1570633	28.9999199	0.72216237	-0.46960485	0.00209563	0.69315574
133	ADM	382.260851	465.210699	299.311003	0.64338805	-0.63623896	0.00213874	0.69562517
1258	CNGB1	15.820273	19.6290501	12.0114958	0.61192445	-0.70857454	0.00227366	0.72738435
4282	MIF	953.411705	1116.11504	790.708367	0.70844701	-0.49726814	0.00241432	0.75992555
6037	RNASE3	64.4890359	52.8888079	76.0892638	1.43866475	0.52473045	0.00253793	0.78026713
100652740	C16orf98	600.57391	713.944414	487.203407	0.68241084	-0.55128753	0.00255891	0.78026713
25893	TRIM58	1547.66628	1328.46688	1766.86568	1.33000356	0.41143011	0.00293195	0.85553843
2735	GLI1	326.667436	401.156165	252.178708	0.62862977	-0.6697175	0.00293728	0.85553843
126003	TRAPPC5	743.257067	902.881061	583.633073	0.64641191	-0.62947431	0.00285439	0.85553843

geneID	Gene Symbol	Base Mean	Base Mean Not Medicated	Base Mean Medicated	Fold Change	Log2 Fold Change	Pval	Padj
374454	KRT77	1.40356996	0.67078025	2.13635967	3.18488755	1.67124244	0.00302245	0.86739795
932	MS4A3	196.874214	155.851151	237.897278	1.52643902	0.61016996	0.00314315	0.86885528
26153	KIF26A	16.9051087	19.4103811	14.3998363	0.74186263	-0.43077603	0.00307355	0.86885528
51208	CLDN18	5.61762669	3.75171251	7.48354087	1.99469998	0.99617177	0.00316109	0.86885528
112464	PRKCDBP	9.49303397	11.6320097	7.35405823	0.63222594	-0.66148788	0.00339957	0.89138534
388564	TMEM238	91.2333212	108.105427	74.3612151	0.6878583	-0.53981671	0.00340357	0.89138534
1051	CEBPB	2698.91643	3253.62066	2144.21219	0.65902341	-0.60159837	0.00342577	0.89138534
728637	LOC728637	11.5487481	13.4242287	9.67326744	0.72058273	-0.47276401	0.00337335	0.89138534
5304	PIP	3.3981026	4.6816574	2.11454781	0.4516665	-1.14667018	0.00355621	0.91315153
8991	SELENBP1	472.421	406.041892	538.800108	1.32695694	0.40812156	0.00395743	0.91693922
55701	ARHGEF40	1700.91266	2025.07833	1376.747	0.67984876	-0.55671426	0.00402461	0.91693922
2038	EPB42	355.878587	307.682652	404.074521	1.31328341	0.39317828	0.00396543	0.91693922
146439	CCDC64B	54.8915246	65.9722014	43.8108479	0.66408043	-0.59057011	0.00370157	0.91693922
55437	STRADB	478.619398	385.302151	571.936645	1.48438477	0.5698651	0.00405697	0.91693922
284889	LOC284889	991.399674	1154.97127	827.828075	0.71675209	-0.48045389	0.00381341	0.91693922
2850	GPR27	148.5482	181.333534	115.762866	0.63839745	-0.6474732	0.00381004	0.91693922
604	BCL6	9343.4998	11103.9902	7583.00944	0.68290852	-0.55023577	0.0041348	0.91693922
731424	RP11-701P16	117.419044	138.092394	96.7456929	0.70058669	-0.51336452	0.0037614	0.91693922
339975	RP11-138B4.	11.0282549	9.69427613	12.3622337	1.27520957	0.35073436	0.00389893	0.91693922
101929639	RP11-326F20	125.568055	148.307303	102.828806	0.69334958	-0.52834517	0.00410575	0.91693922
11326	VSIG4	60.1352312	69.385745	50.8847174	0.73335982	-0.44740687	0.00400501	0.91693922
2848	GPR25	53.6297004	62.735161	44.5242398	0.70971747	-0.49468327	0.00440109	0.92991042
150147	C21orf128	8.36237019	10.22945	6.49529041	0.63495989	-0.65526264	0.00432756	0.92991042
9609	RAB36	121.968536	145.470238	98.4668342	0.67688646	-0.56301424	0.00438993	0.92991042
2180	ACSL1	12238.8882	14255.602	10222.1743	0.71706367	-0.47982687	0.00435337	0.92991042
10409	BASP1	3541.67911	4230.7871	2852.57111	0.67424123	-0.56866324	0.00443155	0.92991042
2999	GZMH	1135.69789	984.711688	1286.6841	1.30666073	0.3858846	0.00457603	0.93070776
8632	DNAH17	905.988781	1085.98613	725.991437	0.66850894	-0.58098123	0.00457842	0.93070776
84634	KISS1R	17.8459605	22.0552333	13.6366878	0.61829715	-0.69362775	0.0045763	0.93070776
55049	C19orf60	493.646752	589.879997	397.413507	0.67371925	-0.56978058	0.0046922	0.93471505
54958	TMEM160	141.893354	167.899404	115.887304	0.69021868	-0.53487458	0.00469393	0.93471505
100128071	FAM229A	36.2089873	44.6397456	27.7782291	0.6222757	-0.68437418	0.00480545	0.94656441
100423062	IGLL5	1021.23335	1234.18027	808.286439	0.65491765	-0.61061459	0.00494745	0.94656441

PUBLICATIONS

PUBLICATIONS

RELATED TO THIS THESIS:

- **Ruso-Julve F**, Pombero A, Pilar-Cuéllar F, García-Díaz N, Garcia-Lopez R, Juncal-Ruiz M, Castro E, Díaz A, Vazquez-Bourgón J, García-Blanco A, Garro-Martinez E, Pisonero E, Estirado A, Ayesa-Arriola R, López-Giménez J, Mayor Jr. F, Valdizán E, Meana J, Gonzalez-Maeso J, Martínez S, Vaqué JP, Crespo-Facorro B. **Dopaminergic control of *ADAMTS2* expression through cAMP/CREB and ERK. Molecular effects of antipsychotics.** *Translational Psychiatry*. 2019 *(Provisional acceptance).*

- Lopez-Gimenez JF, de la Fuente Revenga M, **Ruso-Julve F**, Saunders JM, Moreno JL, Crespo-Facorro B, González-Maeso J. **Validation of schizophrenia gene expression profile in a preclinical model of maternal infection during pregnancy.** *Schizophrenia Research*. 2017, Nov;189:217-218. doi: 10.1016/j.schres.2017.02.005.

OTHER PUBLICATIONS:

- Gilabert-Juan J, López-Campos G, Sebastiá-Ortega N, Guara-Ciurana S, **Ruso-Julve F**, Prieto C, Crespo-Facorro B, Sanjuán J, Moltó MD. **Time dependent expression of the blood biomarkers EIF2D and TOX in patients with schizophrenia.** *Brain, Behavior, and Immunity*. 2019, Aug;80:909-915. doi: 10.1016/j.bbi.2019.05.015.

- Sainz J, Prieto C, **Ruso-Julve F**, Crespo-Facorro B. **Blood Gene Expression Profile Predicts Response to Antipsychotics.** *Frontiers in Molecular Neuroscience*. 2018, Mar 6;11:73. doi: 10.3389/fnmol.2018.00073.

DOPAMINERGIC CONTROL OF ADAMTS2 EXPRESSION THROUGH cAMP/CREB AND ERK MOLECULAR EFFECTS OF ANTIPSYCHOTICS.

Fulgencio Ruso-Julve[1,2,3], Ana Pombero[3,4], Fuencisla Pilar-Cuéllar[3,5,6], Nuria García-Díaz[2,7], Raquel Garcia-Lopez[3,4], María Juncal-Ruiz[3,9], Elena Castro[3,5,6], Álvaro Díaz[3,5,6], Javier Vazquez-Bourgón[1,3], Agustín García-Blanco[2,7], Emilio Garro-Martinez[3,5,6], Helena Pisonero[2,7], Alicia Estirado[3,4], Rosa Ayesa-Arriola[1,3], Juan López-Giménez[10], Federico Mayor Jr.[11,12], Elsa Valdizán[3,5,6], Javier Meana[3,13], Javier Gonzalez-Maeso[14], Salvador Martínez[3,4], José Pedro Vaqué[2,7,*,#], Benedicto Crespo-Facorro[1,3,8,*,#].

***Senior authors that contributed equally; # Corresponding authors.**

1. Department of Psychiatry, University Hospital Marqués de Valdecilla-IDIVAL, Santander, Spain.
2. Department of Molecular Biology, School of Medicine, University of Cantabria, Santander, Spain.
3. Centro de Investigación Biomédica en Red de Salud Mental (CIBERSAM), Instituto de Salud Carlos III.
4. Instituto de Neurociencias, UMH-CSIC, Alicante, Spain.
5. Instituto de Biomedicina y Biotecnología de Cantabria, IBBTEC (Universidad de Cantabria, CSIC, SODERCAN), 39011 Santander, Spain
6. Department of Physiology and Pharmacology, School of Medicine, University of Cantabria, Santander, Spain.
7. Infection, Immunity and Digestive Pathology Group, IDIVAL, Santander, Spain.
8. Department of Psychiatry, School of Medicine, University Hospital Virgen del Rocio, IBiS, Sevilla, Spain.
9. Department of Psychiatry, Sierrallana Hospital, Torrelavega, Cantabria, Spain.
10. Institute of Parasitology and Biomedicine "López-Neyra" (IPBLN-CSIC), Armilla, Granada, Spain.
11. Department of Molecular Biology, Centro de Biología Molecular "Severo Ochoa" (UAM-CSIC), Universidad Autónoma de Madrid.
12. Centro de Investigación Biomédica en Red de Enfermedades Cardiovasculares (CIBERCV), Instituto de Salud Carlos III.
13. Department of Pharmacology, University of the Basque Country UPV/EHU, Leioa, Bizkaia, Spain.
14. Department of Physiology and Biophysics, Virginia Commonwealth University School of Medicine, Richmond, Virginia, USA.

Corresponding author information:

Dr. Benedicto Crespo-Facorro, Fundación Instituto de Investigación Marqués de Valdecilla, IDIVAL. Address: IDIVAL, Avda. Cardenal Herrera Oria s/n, 39011 - Santander (Spain). Telephone: +34-942 31 55 15; Fax: +34-942 31 55 17.

e-mail: crespob@unican.es.

Dr. José P. Vaqué, Departamento de Biología Molecular, Universidad de Cantabria. Address: Facultad de Medicina, Avda. Cardenal Herrera Oria s/n, 39011 – Santander (Spain). Telephone: + 34-942 206858.

e-mail: jose.vaque@unican.es.

Running Title: D_1-receptors control ADAMTS2 expression through CREB

ABSTRACT

A better understanding of the molecular mechanisms that participate in the development and clinical manifestations of schizophrenia can lead to improve our ability to diagnose and treat this disease. Previous data strongly associated the levels of deregulated *ADAMTS2* expression in peripheral blood mononuclear cells (PBMCs) from patients at first episode of psychosis (up) as well as in clinical responders to treatment with antipsychotic drugs (down). In this current work, we performed an independent validation of such data and studied the mechanisms implicated in the control of ADAMTS2 gene expression. Using a new cohort of drug-naïve schizophrenia patients with clinical follow up, we confirmed that expression of *ADAMTS2* was highly up-regulated in PBMCs at the onset (drug-naïve patients) and downregulated, in clinical responders, after treatment with antipsychotics. Mechanistically, *ADAMTS2* expression was activated by dopaminergic signalling (D_1-class receptors), and downstream, by cAMP/CREB and MAPK/ERK signalling. Incubation with antipsychotic drugs and selective PKA and MEK inhibitors abrogated D_1-mediated activation of *ADAMTS2* in neuronal-like cells. Thus, D_1 receptors signalling towards CREB activation might participate in the onset and clinical responses to therapy in schizophrenia patients, by controlling *ADAMTS2* expression and activity. The unbiased investigation of molecular mechanisms triggered by antipsychotic drugs may provide a new landscape of novel targets potentially associated with clinical efficacy.

INTRODUCTION

Antipsychotic drugs (APDs) remain the standard pharmacological treatment for schizophrenia (SCZ) and psychotic disorders, mainly by targeting dopamine neurotransmission (primarily D_2) [1-3]. APDs can be classified into typical (i.e. haloperidol) and atypical (i.e. aripiprazole, risperidone or clozapine), the latter prescribed as first-line drugs and/or in refractory patients [4]. Despite the effectiveness of APDs in the clinical realm, there is a marked disparity among patients with respect to symptoms, responses and side effects [5, 6]. Whereas 50-60 % of patients achieve an optimal degree of clinical improvement of positive symptoms, little to no improvement of negative symptoms or cognitive deficits is common [5]. Overall estimates suggest that one-fifth to one-half of patients have treatment resistant schizophrenia and about 30–60% of these, respond to clozapine [6-8]. These evidences suggest that although D_2 is a direct target for all drugs used to treating schizophrenia, its blockade may not tackle the primary biological anomaly in a significant percentage of patients [9]. In this respect, the combined D_1 and D_2 receptors antagonism has been proposed to have synergic effects, which could account for the atypical clinical effectiveness of clozapine [10]. From a molecular perspective, the effects of APDs may also include the modulation of D_2 receptor-independent mechanisms through indirect effects, like for example, those related to other metabotropic receptors implicated in the control cAMP-dependent signalling [11] and/or those related with receptor biased agonism [4, 12]. Downstream of cAMP, the activation of PKA (protein kinase A) promotes phosphorylation of DARPP-32 (a cytosolic protein) which has been associated with the pathophysiology of SCZ [13, 14]. Additionally, the glutamate hypothesis of schizophrenia led to explore its potential clinical application as targets for therapy [15]. The investigation of the mechanisms

downstream of the receptors targeted by APDs will certainly guide to finding out new explanations for pathophysiological mechanisms and novel approaches for therapy [4].

A new paradigm has emerged based on the investigation of transcriptional patterns associated with clinical responses in SCZ, raising the likelihood of revealing unknown molecular mechanisms associated with antipsychotic action that may be crucial in accomplishing optimal clinical responses [16, 17]. Using an unbiased transcriptome approach, we have previously described a set of genes that are differentially expressed at the onset of SCZ [16]. In addition to this, we also showed significant differential gene expression elicited after 3-month APDs treatment in responder patients [17]. Amongst the above-mentioned results, *ADAMTS2* was the highest up-regulated gene in SCZ patients at the onset of the disease, and also was the most significant down-regulated gene, back to "healthy levels", in responder patients to treatment with APDs. The observation that the expression of *ADAMTS2* can be modulated by APDs and its association with clinical response, makes it an appealing candidate to investigate its interface with illness pathology and clinical efficacy [17].

In the present work we initially performed an independent validation using a new cohort of drug-naïve non-affective psychosis patients. We confirmed that *ADAMTS2* expression was significatively overexpressed at diagnosis and that treatment with APDs reduced their expression levels back to "healthy" values which, in turn, was associated with clinical responses (positive symptoms). However, the molecular or cellular mechanisms exerted by APDs to regulate *ADAMTS2* expression are unknown. Following a bottom-up strategy starting from the transcriptional data obtained in SCZ cases and taking advantage of neuronal-like cells towards the identification of membrane receptors and the intra-cellular mechanism controlled by them, we herein

provide new evidence that *ADAMTS2* expression is regulated by dopaminergic signalling cascade (D_1-class receptors) through ERK and cAMP/CREB activities.

METHODS AND MATERIALS

Human samples and Study Setting

Human samples for this study were obtained from an ongoing epidemiological and 3-year longitudinal intervention program of first-episode psychosis (PAFIP) conducted at the University Hospital Marques de Valdecilla (Cantabria, Spain) and biological samples were provided by the IDIVAL biobank. Approved by the Cantabria Ethics Institutional Review Board (IRB), conforming to international standards for research ethics. Patients meeting inclusion criteria and their families provided written informed consent to be included in the PAFIP.

A new independent set of 30 antipsychotic drug-naïve first episode non-affective individuals and 10 healthy individuals (without a history of neuropsychiatric disorders) were used to validate gene expression profiles related to clinical response (Table 1).

Briefly, all patients underwent a head-to-head risperidone and aripiprazole randomized (simple randomization procedure), flexible-dose, open-label study (EudraCT number 2013-005399-16). More detailed information about PAFIP and treatment protocol has been published elsewhere [17, 18] and also is briefly available in supplementary information.

RNA Extraction

Peripheral blood from patients and controls was extracted using the TempusTM Blood RNA Tube (Applied Biosystems, UK). PBMCs isolation was performed using a Ficoll-Paque Premium reagent (Sigma) and total mRNA isolation using a TempusTM Spin RNA Isolation Kit (Invitrogen, CA, USA), following the manufacturer's instructions. RNA Integrity Number (RIN) was characterized with a Bioanalyzer (Agilent Technologies) and samples with a RIN>8 were selected. Total mRNA from culture cells

was extracted using Trizol reagent (Invitrogen) and its concentration was determined using a Nanodrop 2000 spectrophotometer (Thermo Scientific, IL, USA).

RT-PCR and quantitative PCR

cDNA synthesis was performed using the SuperScript IV Reverse Transcriptase (Invitrogen). cDNA was amplified using the Power SYBR™ Green PCR Master Mix (Applied Biosystems) in a 7300 Fast Real-Time PCR System (Applied Biosystems). Specific oligos for human *ADAMTS2*, *CREB1* and *C-FOS* were designed using Primer-Blast (NCBI; see sequences in Table S1). *ACTB* expression was used to normalize values. Gene expression changes were determined using $2^{(-\Delta\Delta Ct)}$ formula. A melting curve was generated for every run to confirm assay specificity.

Cell culture and treatments

Human neuroblastoma (SK-N-SH, ATCC HTB-11) and 293T (ATCC CRL-3216) cells were obtained from the American Type Cell Collection (ATCC, Rockville, MD). Cells were cultured in Modified Eagle's medium (MEM) and Dulbecco's Modified Eagle's Medium (DMEM), respectively (Corning, VA, USA). Culture medium was supplemented with 10% dialyzed fetal bovine serum (HyClone, UT, USA), glucose, L-glutamine, streptomycin sulphate and potassium penicillin (10000 U/L) (Lonza, Belgium).

Drugs and pharmacological agents

Aripiprazole, clozapine, haloperidol hydrochloride, H89 dihydrochloride, L 741,626, MDL 100907, paliperidone, risperidone, SKF 83822, SCH39166, TCB-2, WAY 100635, 7-OH-DPAT and 8-OH-DPAT were purchased from Tocris Bioscience (Spain). Forskolin and selumetinib (AZD6244) were purchased from Selleckchem (Spain). Cholera toxin (CTX), pertussis toxin (PTX) and TPA were purchased from

Sigma-Aldrich (MO, USA). YM-254890 was purchased from Adipogen life sciences (CA, USA). All were dissolved in DMSO, except CTX and WAY 100635 that were dissolved in water.

Luciferase report assays

Luciferase report assays were performed by transfection with 0.5 µg DNA of the following plasmids mix (relation 3:1): pGL4.29[luc2P/CRE/Hygro] containing firefly luciferase reporter; alongside pRL-Null containing renilla luciferase used as control (Promega, WI, USA). Cells were transfected with Lipofectamine LTX with PLUS reagents (Invitrogen) in transient conditions. Firefly and renilla levels were detected using Dual-Luciferase Reporter Assay System kit and quantified using a GloMax-Multi apparatus (Promega).

Western blot

Cells were starved overnight before treatment. Whole cell lysates were obtained using RIPA buffer (Sigma) supplemented with phosphatase and protease inhibitors (Roche, Germany). Protein expression was analysed by western-blot as described previously [19]. Briefly, antibodies used were: phospho-CREB, CREB, phospho-ERK1/2, ERK1/2, phospho-p38, p38 and phospho-PKA substrates (Cell Signaling, MA, USA), and β-Tubulin (Santa Cruz biotechnology). Fluorophore conjugate antibodies were obtained from Invitrogen. Signals were visualized and recorded with an Odyssey Infrared Imaging scanner (LI-COR biosciences, NE, USA). Immunoblot densitometry analysis on every band was calculated using Image Studio Software (LI-COR biosciences). Phosphorylation and total protein densitometry values were normalized to β-Tubulin signal.

CREB knocked down reagents and procedures

ShCREB1-inducible SK-N-SH cells were generated by lentiviral infection of SK-N-SH cells with SMARTvector carrying tGFP and human inducible CREB1 shRNA mCMV constructs or non-target control shRNA (Dharmacon, CO, USA). Lentiviral particles were produced by co-transfection of 293T cells using Trans-Lentiviral shRNA Packaging System (Dharmacon), according to the manufacture's protocol. Cells were incubated with doxycycline (1 µg/ml) (Sigma) for 72 h to induce GFP and shRNA expression. SK-N-SH cells expressing stable shCREB1 constructs were generated by direct transfection by Lipofectamine LTX with PLUS reagent (Invitrogen) using pGFP-V-RS-CREB1 shRNA expression vectors or scrambled control (Origene, MD, USA). Transfected cells were selected with puromycin (1 µg/mL) at least 7 days.

Statistical analyses

Kolmogorov-Smirnov test and Levene test were used to test normality and equality of variances respectively.

For patients' studies, in order to ensure group comparability between healthy volunteers and patients, sociodemographic and clinical characteristics at baseline were tested by unpaired Student t-test or one-way analysis of variance (ANOVA) for continuous variables as necessary, and by Fisher's Exact test for qualitative variables (Table 1 and table S2).

Wilcoxon Signed-Rank test for independent data was used to compare the ADAMTS2 mRNA expression level among healthy volunteers and drug-naïve patients at baseline. Wilcoxon Matched-Pairs Signed-Rank test for paired data was performed to compare the change in the mRNA expression level from baseline to 3-months following the antipsychotic treatment ($\alpha = 0.05$). STATA 15.1 was used for statistical analysis.

For *in vitro* studies, unless otherwise specified, all experiments were independent and numerical data were summarized as the mean ± SEM (standard error of the mean) using GraphPad Prism6 software. Each global mean was compared using unpaired Student's *t*-test (two tailed; $\alpha = 0.05$) or one-way ANOVA followed by *post-hoc* test where appropriated, as described in each figure legend.

RESULTS

ADAMTS2 expression is controlled by APDs treatment in SCZ patients.

To better evaluate the mechanistic effects of atypical APDs in SCZ patients, we prepared and studied a new cohort of 30 drug-naïve SCZ patients with baseline quantitative data (sociodemographic and clinical characteristics) and clinical follow-up data, after 3 months of treatment with APDs (Table 1). We found significant within-subject changes between baseline data and after 3 months of treatment in both risperidone and aripiprazole groups. These changes were observed in CGI (Clinical Global Impression, $p < 0.000$), YMRS (Young Mania Rating Scale, $p < 0.000$), BPRS (Brief Psychiatric Rating Scale, $p < 0.000$) and SAPS (Scale for the Assessment of Positive Symptoms, $p < 0.000$) total scores. However, as it is shown in Table 1, no differences were found in total scores of the clinical scales when the two APD groups were compared at baseline and after 3 months of treatment.

To analyse transcriptomic changes, we obtained PBMCs isolated from blood samples from SCZ patients that were obtained at the first episode of psychosis (onset) and after a 3-month period of treatment with APDs. In this context, using qRT-PCR, we performed an independent validation of transcriptional changes by focusing on *ADAMTS2*. Thus, we confirmed *ADAMTS2* as a significant gene that was highly overexpressed at the onset, with respect to healthy controls, and down-regulated by APDs after 3 months of treatment (Fig. 1).

Dynamic control of ADAMTS2 transcription by APDs in neuronal-like cells.

To gain mechanistic insight into the transcriptional control of *ADAMTS2*, we analysed the effects of APDs in SK-N-SH cells using dialyzed fetal bovine serum (dFBS). No significant differences in ADAMTS2 expression were observed when dFBS was not

present in the culture medium (data not shown). These cells express cell surface receptors that are targets of APDs (i.e., D_1, D_2, 5-HT_{1A} and 5-HT_{2A} receptors) as well as detectable basal expression of ADAMTS2 mRNA. Our data showed that incubation with APDs in these cells induced dynamic changes in ADAMTS2 mRNA expression along time. Incubation with atypical APDs like paliperidone, aripiprazole (Fig. 2) and risperidone (Fig. S1), provoked a dynamic inhibition of *ADAMTS2* basal mRNA expression in these cells with fast (less than 1 h) but transitory responses. Interestingly, clozapine (atypical APD) and haloperidol (typical APD), induced a fast and sustained (up to 24 h) inhibition of *ADAMTS2* expression (Fig. 2).

Neurotransmitter receptors and associated signalling pathways involved in the control of ADAMTS2 gene expression.

Using selective agonists for D_1, D_2, 5-HT_{1A} and 5-HT_{2A} receptors in SK-N-SH cells, we observed that SKF 83822 (a D_1-class receptor agonist) significantly triggered ADAMTS2 mRNA expression compared to the lack of effect of 7-OH-DPAT, 8-OH-DPAT and TCB-2 (agonists of D_2, 5-HT_{1A} and 5-HT_{2A} receptors, respectively) (Fig. 3A). In addition, using selective antagonists for these receptors, we observed that only SCH 39166 (a D_1-class receptor antagonist) significantly down-regulated ADAMTS2 mRNA expression, whereas L 741,626, WAY 100635 and MDL 100907 (antagonists of D_2, 5-HT_{1A} and 5-HT_{2A} receptors, respectively) were devoid of this effect (Fig. 3A). Furthermore, we analysed ADAMTS2 mRNA and protein expression profiles in the brain of developing and early postnatal mice (see supplementary methods). Interestingly and in support of our previous results in cells, ADAMTS2 was specifically localized in brain regions that are part of the mesolimbic and mesocortical dopamine systems (Fig. S2).

Next, we studied if treatment with APDs could modulate ADAMTS2 gene expression activated downstream of the D_1 receptor. Our results show that haloperidol and clozapine could significantly block SKF 83822-mediated transcriptional activation of *ADAMTS2*. As expected, SCH 39166, a selective D_1 receptor antagonist, also prevented D_1 receptor-mediated activation of *ADAMTS2* (Fig. 3B). In addition, the reduction in *ADAMTS2* expression induced by clozapine was prevented by L 741,626 and MDL 100907 (a D_2 and 5-HT_{2A} receptor antagonists, respectively) (Fig. S3).

Since D_1 receptors are known to signal through Gαs, we sought to confirm its role at controlling *ADAMTS2* expression in our system, alongside Gαi and Gαq. For this purpose, we incubated SK-N-SH cells with cholera toxin (Gαs activator), pertussis toxin (Gαi inhibitor) and YM-254890 (a specific Gαq inhibitor)[20], before activating D_1 receptors with SKF 83822. The activation of Gαs (Fig. S4A) and the inhibition of Gαi (Fig. S4B) *per se* significantly up-regulated *ADAMTS2* expression. Moreover, the inhibition of Gαi potentiated *ADAMTS2* expression after D_1 receptor activation (Fig. S4B). Finally, the inhibition of Gαq did not modify the basal and the SKF 83822-mediated increase of *ADAMTS2* expression (Fig. S4C).

Incubation of neuronal-like cells with the selective D_1 receptor agonist (SKF 83822) provoked rapid phosphorylation of CREB (cAMP response element-binding protein) and ERK1/2 (15 minutes) and no changes in p38 (Fig. S4D-F). Additionally, rapid phosphorylation of PKA substrates was detected in response to SKF 83822 (Fig. S4G). To evaluate CREB-dependent transcriptional activity in neuronal-like cells, we took advantage of a CRE-luciferase reporter. We analysed if APD could counteract the intracellular signalling activation mediated by selective SKF 83822 D_1-class receptor agonist. Our results (Fig. 3C) demonstrate that preincubation with clozapine, but not

haloperidol, paliperidone or aripiprazole, prevented SKF 83822-induced CREB activation using a specific reporter assay.

To evaluate the potential contribution of PKA and ERK to the activation of *ADAMTS2* expression, we incubated SK-N-SH cells with SKF 83822 D_1 receptor agonist in combination with selective MAPK/ERK (selumetinib-AZD6244) and cAMP/PKA (H89 dihydrochloride) inhibitors. Both inhibitors prevented D_1 receptor induced expression of *ADAMTS2* (Fig. 3D), although only a cAMP/PKA inhibitor abrogated CREB-dependent transcription and phosphorylation (Fig. 3E-F).

Transcriptional mechanisms controlling ADAMTS2 gene expression downstream of dopamine D_1 receptors.

Our previous data suggest that there is a rapid activation of the signalling mechanisms controlling CREB activation after D_1 receptor activation that can be modulated pharmacologically. We decided to challenge the contribution of cAMP/CREB signalling to the transcriptional activation of *ADAMTS2* (Fig. 4A). We initially evaluated the activation of ADAMTS2 gene expression together with that of *C-FOS*, an "early response gene" known to be a direct transcriptional target of CREB that was used as control. Our data showed that SKF 83822 activated mRNA expression of both *ADAMTS2* and *C-FOS*. Interestingly, incubation with forskolin alone (a selective adenyl cyclase activator) was sufficient to trigger the transcription of both genes, whereas TPA (a PKC/MAPK activator) uniquely activated *C-FOS* transcription.

To explore the implication of CREB in D_1 receptor-mediated *ADAMTS2* expression, we generated stable SK-N-SH cells with inducible expression of control or *CREB1* shRNAs upon addition of doxycycline to the culture media. Using this approach, we reduced

CREB1 mRNA and protein expression, as shown in figure 4B. Under these settings, SKF 83822 and forskolin failed to induce the expression of *ADAMTS2* (Fig. 4C). Interestingly, SKF 83822 did not significantly activate *C-FOS* expression in *CREB1* knocked down cells when compared to control cells; in contrast, TPA did (Fig. 4D). A schematic representation of the signalling pathways and the molecules used in this work is illustrated in figure 5 for explanatory purposes. The above-mentioned results were further confirmed using SK-N-SH cells stably expressing control or CREB1 shRNAs (Fig. S5). Mechanistically, reduced CREB expression did not impair ERK phosphorylation elicited downstream of D_1 receptor activation by SKF 83822 (Fig. 4E). In this context, we also used forskolin and TPA as controls for selective activation of CREB and ERK, respectively (Fig. 4E and fig. S6). Thus, activation of CREB seems to play a key role in the control of ADAMTS2 gene expression downstream of D_1 receptors.

DISCUSSION

Our results revealed that treatment with APDs control *ADAMTS2* expression, which is directly associated with dopaminergic signalling, primarily with the D_1-class receptors, and downstream through cAMP/CREB and MAPK signalling. Interestingly, ADAMTS2 mRNA and protein were specifically found in mesolimbic and mesocortical dopaminergic regions in mice. Our data suggest that D_1 receptor signalling towards CREB (cAMP response element-binding protein) activation and its effects on *ADAMTS2* expression may be linked to key biological mechanisms in SCZ and the clinical response to APDs [21, 22].

In the context of the local PAFIP program, we collected a new cohort of 30 drug-naïve SCZ patients with associated clinical data and follow-up. Using these cases alongside "healthy" controls, we attempted an independent validation of our previous results using a different cohort of cases [16, 17]. In this cohort, we confirmed *ADAMTS2* as a significant gene that was highly overexpressed at baseline in PBMCs from drug-naïve SCZ individuals and which returned to "normal" levels in clinical responders treated with APDs (clinical rate scales in Table 1). Although, there is not always a direct correlation between the biomarkers obtained in peripheral blood cells and CNS samples, a previous study in a rodent model of schizophrenia have shown a certain parallelism between the expression in human PBMCs [16, 17] and mouse frontal cortex samples [23]. Supporting the previously mentioned data, *ADAMTS2* mRNA expression was quickly downregulated by all APDs in neuronal-like cells. Moreover, whereas the activities of paliperidone, risperidone and aripiprazole downregulating *ADAMTS2* mRNA were transitory in such cells, those of clozapine and haloperidol were sustained to up to 24 h. The molecular effects exerted by clozapine and haloperidol as compared to other APDs might deserve further investigations. In this respect, clozapine has shown superior efficacy for

treatment-resistant and suicidality, as well as its apparent ability to decrease substance use in SCZ [24]. Thus, ADAMTS2 might play a key, and yet to be defined, mechanistic role in both the illness onset and clinical responses, regardless of the type of APD used.

There is scarce information regarding specific ADAMTS2 expression and activity in the central nervous system (CNS). Therefore, we analysed the expression profiles of ADAMTS2 mRNA and protein in mice. Interestingly, these were specifically localized in brain regions that are part of the mesolimbic and mesocortical dopamine systems (i.e., the dentate gyrus (DG) in the hippocampus and the ventral tegmental area (VTA) [21, 22, 25]. At a prenatal stage (E18.5), ADAMTS2 protein was mapped in the neuropiles of anterior brain structures, anterior cingulate cortex, superficial striatum and lateral septum, where neuronal expression of dopamine D_1 and D_2 receptors was detected by *in situ* hybridization (http://developingmouse.brain-map.org/). The hypothesis that dopamine and dopaminergic mechanisms are essential to psychosis, and particularly to schizophrenia, has been one of the most enduring ideas about this disorder [26]. Elevated presynaptic striatal dopamine correlates most closely with the symptom dimension of psychosis, and blockade of this heightened transmission leads to a resolution of symptoms for most patients [27]. Nonetheless, the functional association between ADAMTS2 and the dopaminergic system has not been previously established, to our best knowledge. ADAMTS2 is a member of the ADAM Metallopeptidase with Thrombospondin family [28, 29], with a number of targets such as the N-propeptides of procollagens I–III, fibronectin, decorin and Dkk3 participating in extracellular matrix (ECM) organization, as well as in TGF-β and WNT signalling [30, 31]. Alterations in the ECM as well as TGF-β and WNT signalling have been associated with SCZ [32-34]. Moreover, deregulated mRNA expression of MMPs (MMP-16, -24 and -25) and ADAMTS (ADAMTS-1, -6 and -8) families of proteases have also been reported in

SCZ [35, 36]. Thus, considering *ADAMTS2* expression and its associated activities in the CNS, as well as the data presented in this work, it is conceivable to speculate that it could participate in SCZ at different stages of the disease. In this regard, a selective D_1-class receptor agonist (SKF 83822) significantly activated *ADAMTS2* expression, and haloperidol and clozapine blocked this activation in neuronal-derived cells. It will be of interest, to address these appealing questions, within the next future, using SCZ and/or ADAMTS2 transgenic mice models.

D_1 receptors are the most abundant dopaminergic receptor in CNS, and their functional crosstalk with D_2 receptor is well documented [11, 37, 38]. D_1 receptor activate adenylyl cyclase (AC), which in turn regulate intracellular cAMP levels leading to PKA activation and CREB phosphorylation [39-41]. Our data have shown that selective D_1 receptor activation up-regulated *ADAMTS2* expression alongside a rapid phosphorylation of CREB and ERK proteins, which resulted in CREB-mediated transcriptional activity as detected by using specific reporter assays (Figure 3). In support of this and in our system, *ADAMTS2* transcription was specifically triggered by activation of D_1 receptors (by SKF 83822), Gαs (using CTX) and adenylyl cyclase (forskolin). Thus, it is possible that, as part of the dopaminergic activity, *ADAMTS2* could act as a major cAMP/CREB effector in SCZ. Interestingly, D_1 receptor-mediated CREB activation was abrogated by clozapine (Fig. 3C), providing evidence that cAMP/CREB signalling, and therefore *ADAMTS2* expression, can be modulated by APDs. Our findings reveal the contribution of D_1 receptors over *ADAMTS2* transcription and also indicate the participation of D_2 and 5-HT_{2A} receptors in the effect of clozapine in the control of *ADAMTS2* expression. In this regard, it has been reported that clozapine can act as a biased agonist on 5-HT_{2A} receptor [42, 43] and/or affect the hetero-dimer D_2/5-HT_{2A} [44]. Moreover, disrupting cAMP/CREB and ERK signalling

using selective PKA and MEK inhibitors also impaired *ADAMTS2* expression and CREB activity (in this case, only when it was PKA dependent) in neuronal-like cells. Supporting these observations, recent data pointed at cAMP/CREB signalling as an important mechanism linking dopaminergic signalling with the pathophysiology of SCZ [45]. Moreover, incubating SK-N-SH cells with forskolin was sufficient to trigger *ADAMTS2* expression that occurred alongside PKA and CREB activation. Forskolin directly activates AC in mammals, thereby promoting a rapid phosphorylation of CREB via PKA [46, 47]. In addition, in CREB-knockdown cells, D_1 receptor activation failed to increase ADAMTS2 gene expression, reinforcing the idea that cAMP/CREB is an essential mechanism to control *ADAMTS2* transcriptional activation.

Finally, a number of potential limitations could be considered when interpreting our data and the scope of our findings: 1) Sample size in this study is rather small (N=30). Noteworthy we selected a group of patients with a first episode of non-affective psychosis who had not previously taken APDs (not even a single dose) at the time of baseline blood test to avoid any interference with the mRNA levels. Only those individuals who gave written consent and had mRNA samples at baseline and at 3-month were eligible for this study. Therefore, all these stringent inclusion criteria limited the number of patients in this cohort. Further investigations to replicate our findings using larger and more heterogeneous groups are warranted. 2) Transcriptomic data obtained from blood samples might not resemble that of the brain. 3) ADAMTS2 protein and mRNA expression show modest but detectable levels in "healthy" human and mouse brain samples (https://www.proteinatlas.org). Thus, it is conceivable to detect high fold increases when analysing diseased specimens, like in this case ADAMTS2 in SCZ. 4) Failure of some APDs to inhibit CREB activity and/or ADAMTS2 mRNA expression in a D_1-context. Our data herein do not discard that

APDs might regulate *ADAMTS2* by indirect mechanisms like for example those involving β-arrestin, ERK or AKT signalling [42, 48].

In conclusion, we have confirmed the association between *ADAMTS2* expression and schizophrenia disease including its potential role in the clinical efficacy of APDs. Transcription of *ADAMTS2* is primarily controlled by the activity of D_1-class receptors through cAMP/CREB and MAPK signalling. The unbiased investigation of the molecular mechanisms triggered by APDs, may provide a landscape of novel targets potentially associated with improved therapeutic responses.

FUNDING AND DISCLOSURE

All authors declare no conflicts of interest and financial disclosures in connection with the article. This work was supported by: SAF2016-76046-R and SAF2013-46292-R (MINECO) to BCF, PI16/00156 (isciii and FEDER) to JPV and SAF2017-83702-R (MINECO), LUCHAMOS POR LA VIDA project to FRJ and JPV, Red TERCEL RD12/0019/0024 (ISCIII) and GVA-PROMETEO 2018/041 (Generalitat Valenciana) to SM. JPV is supported by the RyC research program (RYC-2013-14097) and FRJ by the predoctoral research program (BES-2014-070615), from MINECO.

AKNOWLEDGEMENTS

We are highly indebted to the participants and their families for their cooperation in this study. We also thank IDIVAL biobank (Inés Santiuste and Jana Arozamena) for clinical samples and data as well as the PAFIP members (Marga Corredera) for the data collection.

REFERENCES

1. Chouinard G, Samaha AN, Chouinard VA, Peretti CS, Kanahara N, Takase M, *et al.* Antipsychotic-Induced Dopamine Supersensitivity Psychosis: Pharmacology, Criteria, and Therapy. *Psychother Psychosom* 2017; **86**(4)**:** 189-219.

2. Seeman P. Targeting the dopamine D2 receptor in schizophrenia. *Expert Opin Ther Targets* 2006; **10**(4)**:** 515-531.

3. Rampino A, Marakhovskaia A, Soares-Silva T, Torretta S, Veneziani F, Beaulieu JM. Antipsychotic Drug Responsiveness and Dopamine Receptor Signaling; Old Players and New Prospects. *Front Psychiatry* 2018; **9:** 702.

4. Aringhieri S, Carli M, Kolachalam S, Verdesca V, Cini E, Rossi M, *et al.* Molecular targets of atypical antipsychotics: From mechanism of action to clinical differences. *Pharmacol Ther* 2018; **192:** 20-41.

5. Fusar-Poli P, Papanastasiou E, Stahl D, Rocchetti M, Carpenter W, Shergill S, *et al.* Treatments of Negative Symptoms in Schizophrenia: Meta-Analysis of 168 Randomized Placebo-Controlled Trials. *Schizophr Bull* 2015; **41**(4)**:** 892-899.

6. Meltzer HY. Treatment-resistant schizophrenia--the role of clozapine. *Curr Med Res Opin* 1997; **14**(1)**:** 1-20.

7. Elkis H. Treatment-resistant schizophrenia. *Psychiatr Clin North Am* 2007; **30**(3)**:** 511-533.

8. Gillespie AL, Samanaite R, Mill J, Egerton A, MacCabe JH. Is treatment-resistant schizophrenia categorically distinct from treatment-responsive schizophrenia? a systematic review. *BMC Psychiatry* 2017; **17**(1)**:** 12.

9. Stone JM. Glutamatergic antipsychotic drugs: a new dawn in the treatment of schizophrenia? *Ther Adv Psychopharmacol* 2011; **1**(1)**:** 5-18.

10. Nordstrom AL, Farde L, Nyberg S, Karlsson P, Halldin C, Sedvall G. D1, D2, and 5-HT2 receptor occupancy in relation to clozapine serum concentration: a PET study of schizophrenic patients. *Am J Psychiatry* 1995; **152**(10)**:** 1444-1449.

11. Miller R. Mechanisms of action of antipsychotic drugs of different classes, refractoriness to therapeutic effects of classical neuroleptics, and individual variation in sensitivity to their actions: Part I. *Curr Neuropharmacol* 2009; **7**(4)**:** 302-314.

12. Wisler JW, Xiao K, Thomsen AR, Lefkowitz RJ. Recent developments in biased agonism. *Curr Opin Cell Biol* 2014; **27:** 18-24.

13. Wang H, Farhan M, Xu J, Lazarovici P, Zheng W. The involvement of DARPP-32 in the pathophysiology of schizophrenia. *Oncotarget* 2017; **8**(32)**:** 53791-53803.

14. Nishi A, Matamales M, Musante V, Valjent E, Kuroiwa M, Kitahara Y, *et al.* Glutamate Counteracts Dopamine/PKA Signaling via Dephosphorylation of DARPP-32 Ser-97 and Alteration of Its Cytonuclear Distribution. *J Biol Chem* 2017; **292**(4)**:** 1462-1476.

15. Nicoletti F, Bruno V, Ngomba RT, Gradini R, Battaglia G. Metabotropic glutamate receptors as drug targets: what's new? *Curr Opin Pharmacol* 2015; **20:** 89-94.

16. Sainz J, Mata I, Barrera J, Perez-Iglesias R, Varela I, Arranz MJ, *et al.* Inflammatory and immune response genes have significantly altered expression in schizophrenia. *Mol Psychiatry*, vol. 18: England, 2013, pp 1056-1057.

17. Crespo-Facorro B, Prieto C, Sainz J. Schizophrenia gene expression profile reverted to normal levels by antipsychotics. *Int J Neuropsychopharmacol* 2014; **18**(4).

18. Pelayo-Teran JM, Perez-Iglesias R, Ramirez-Bonilla M, Gonzalez-Blanch C, Martinez-Garcia O, Pardo-Garcia G, *et al.* Epidemiological factors associated with treated incidence of first-episode non-affective psychosis in Cantabria: insights from the Clinical Programme on Early Phases of Psychosis. *Early Interv Psychiatry* 2008; **2**(3)**:** 178-187.

19. Llerena S, Garcia-Diaz N, Curiel-Olmo S, Agraz-Doblas A, Garcia-Blanco A, Pisonero H, *et al.* Applied diagnostics in liver cancer. Efficient combinations of sorafenib with targeted inhibitors blocking AKT/mTOR. *Oncotarget* 2018; **9**(56)**:** 30869-30882.

20. Zhang H, Nielsen AL, Stromgaard K. Recent achievements in developing selective Gq inhibitors. *Med Res Rev* 2019.

21. Phillipson OT. Afferent projections to the ventral tegmental area of Tsai and interfascicular nucleus: a horseradish peroxidase study in the rat. *J Comp Neurol* 1979; **187**(1)**:** 117-143.

22. Khlghatyan J, Quintana C, Parent M, Beaulieu JM. High Sensitivity Mapping of Cortical Dopamine D2 Receptor Expressing Neurons. *Cereb Cortex* 2018.

23. Lopez-Gimenez JF, de la Fuente Revenga M, Ruso-Julve F, Saunders JM, Moreno JL, Crespo-Facorro B, *et al.* Validation of schizophrenia gene expression profile in a preclinical model of maternal infection during pregnancy. *Schizophr Res* 2017; **189:** 217-218.

24. Khokhar JY, Henricks AM, Sullivan EDK, Green AI. Unique Effects of Clozapine: A Pharmacological Perspective. *Adv Pharmacol* 2018; **82:** 137-162.

25. Rice MW, Roberts RC, Melendez-Ferro M, Perez-Costas E. Mapping dopaminergic deficiencies in the substantia nigra/ventral tegmental area in schizophrenia. *Brain Struct Funct* 2016; **221**(1)**:** 185-201.

26. Howes OD, Kapur S. The dopamine hypothesis of schizophrenia: version III--the final common pathway. *Schizophr Bull* 2009; **35**(3)**:** 549-562.

27. Kapur S, Zipursky R, Jones C, Remington G, Houle S. Relationship between dopamine D(2) occupancy, clinical response, and side effects: a double-blind PET study of first-episode schizophrenia. *Am J Psychiatry* 2000; **157**(4)**:** 514-520.

28. Mead TJ, Apte SS. ADAMTS proteins in human disorders. *Matrix Biol* 2018; **71-72:** 225-239.

29. Kelwick R, Desanlis I, Wheeler GN, Edwards DR. The ADAMTS (A Disintegrin and Metalloproteinase with Thrombospondin motifs) family. *Genome Biol* 2015; **16:** 113.

30. Colige A, Ruggiero F, Vandenberghe I, Dubail J, Kesteloot F, Van Beeumen J, *et al*. Domains and maturation processes that regulate the activity of ADAMTS-2, a metalloproteinase cleaving the aminopropeptide of fibrillar procollagens types I-III and V. *J Biol Chem* 2005; **280**(41)**:** 34397-34408.

31. Bekhouche M, Leduc C, Dupont L, Janssen L, Delolme F, Vadon-Le Goff S, *et al*. Determination of the substrate repertoire of ADAMTS2, 3, and 14 significantly broadens their functions and identifies extracellular matrix organization and TGF-beta signaling as primary targets. *Faseb j* 2016; **30**(5)**:** 1741-1756.

32. Berretta S. Extracellular matrix abnormalities in schizophrenia. *Neuropharmacology* 2012; **62**(3)**:** 1584-1597.

33. Benes FM, Lim B, Matzilevich D, Walsh JP, Subburaju S, Minns M. Regulation of the GABA cell phenotype in hippocampus of schizophrenics and bipolars. 2007.

34. Hoseth EZ, Krull F, Dieset I, Mørch RH, Hope S, Gardsjord ES, *et al*. Exploring the Wnt signaling pathway in schizophrenia and bipolar disorder. *Translational Psychiatry* 2018; **8**(1)**:** 55.

35. Pietersen CY, Mauney SA, Kim SS, Passeri E, Lim MP, Rooney RJ, *et al*. Molecular profiles of parvalbumin-immunoreactive neurons in the superior temporal cortex in schizophrenia. *J Neurogenet* 2014; **28**(1-2)**:** 70-85.

36. Pietersen CY, Mauney SA, Kim SS, Lim MP, Rooney RJ, Goldstein JM, *et al.* Molecular profiles of pyramidal neurons in the superior temporal cortex in schizophrenia. *J Neurogenet* 2014; **28**(1-2)**:** 53-69.

37. Miller R. Mechanisms of action of antipsychotic drugs of different classes, refractoriness to therapeutic effects of classical neuroleptics, and individual variation in sensitivity to their actions: Part II. *Curr Neuropharmacol* 2009; **7**(4)**:** 315-330.

38. Paul ML, Graybiel AM, David JC, Robertson HA. D1-like and D2-like dopamine receptors synergistically activate rotation and c-fos expression in the dopamine-depleted striatum in a rat model of Parkinson's disease. *J Neurosci* 1992; **12**(10)**:** 3729-3742.

39. Belgacem YH, Borodinsky LN. CREB at the Crossroads of Activity-Dependent Regulation of Nervous System Development and Function. *Adv Exp Med Biol* 2017; **1015:** 19-39.

40. Carlezon WA, Jr., Duman RS, Nestler EJ. The many faces of CREB. *Trends Neurosci* 2005; **28**(8)**:** 436-445.

41. Lonze BE, Ginty DD. Function and regulation of CREB family transcription factors in the nervous system. *Neuron* 2002; **35**(4)**:** 605-623.

42. Aringhieri S, Kolachalam S, Gerace C, Carli M, Verdesca V, Brunacci MG, *et al.* Clozapine as the most efficacious antipsychotic for activating ERK 1/2 kinases: Role of 5-HT2A receptor agonism. *Eur Neuropsychopharmacol* 2017; **27**(4)**:** 383-398.

43. Schmid CL, Streicher JM, Meltzer HY, Bohn LM. Clozapine acts as an agonist at serotonin 2A receptors to counter MK-801-induced behaviors through a betaarrestin2-independent activation of Akt. *Neuropsychopharmacology* 2014; **39**(8)**:** 1902-1913.

44. Lukasiewicz S, Faron-Gorecka A, Kedracka-Krok S, Dziedzicka-Wasylewska M. Effect of clozapine on the dimerization of serotonin 5-HT(2A) receptor and its genetic variant 5-HT(2A)H425Y with dopamine D(2) receptor. *Eur J Pharmacol* 2011; **659**(2-3)**:** 114-123.

45. Wang H, Xu J, Lazarovici P, Quirion R, Zheng W. cAMP Response Element-Binding Protein (CREB): A Possible Signaling Molecule Link in the Pathophysiology of Schizophrenia. *Front Mol Neurosci* 2018; **11:** 255.

46. Seamon KB, Padgett W, Daly JW. Forskolin: unique diterpene activator of adenylate cyclase in membranes and in intact cells. *Proc Natl Acad Sci U S A* 1981; **78**(6)**:** 3363-3367.

47. Zhang G, Liu Y, Ruoho AE, Hurley JH. Structure of the adenylyl cyclase catalytic core. *Nature* 1997; **386**(6622)**:** 247-253.

48. Del'guidice T, Lemasson M, Beaulieu JM. Role of Beta-arrestin 2 downstream of dopamine receptors in the Basal Ganglia. *Front Neuroanat* 2011; **5:** 58.

FIGURE LEGENDS

Table 1. Psychopathological characteristics at baseline, at 3-months and clinical changes during the follow up period. Comparison between aripiprazole and risperidone. BPRS: Brief Psychiatric Rating Scale; CDSS: Calgary Depression Rating Scale for Schizophrenia; CGI: Clinical Global Impression; SANS: Scale for the Assessment of Negative Symptoms; SAPS: Scale for the Assessment of Positive Symptoms; YMRS: Young Mania Rating Scale. [(a)] Wilcoxon Matched-Pairs Signed-Rank test, [(b)] paired Student's t-test; [(c)] comparison between aripiprazole and risperidone at baseline, unpaired Student's t-test; and [(d)] comparison between aripiprazole and risperidone following the antipsychotic treatment, using the total score of the clinical scales at baseline as covariate, analysis of covariance (ANCOVA).

Figure 1. Transcriptional control of ADAMTS2 in SCZ patients at onset and after 3-month of antipsychotic treatment. Relative mRNA expression level of ADAMTS2 gene in PBMCs from peripheral blood samples of an independent cohort of SCZ patients: healthy controls (basal; grey bar), untreated FEP patients (basal drug-naïve; black bar) and 3-months treated patients (striped bar). FEP: first episode of psychosis. All patients were treated with risperidone or aripiprazole drugs and shown clinic response to treatment (Cohort: patients N=30 and controls N=10). Data are mean ± S.E.M; unpaired Wilcoxon Signed-Rank test: ***p < 0.001 compares patients at baseline vs. healthy controls; paired Wilcoxon Signed-Rank test: ###p < 0.001 compares patients at baseline vs. those after 3-month AP treatment.

Figure 2. Transcriptional control of ADAMTS2 using antipsychotic drugs. Relative ADAMTS2 mRNA expression level in SK-N-SH cells incubated with clozapine (1 μM) (N=4), haloperidol (1 μM) (N=5), paliperidone (1 μM) (N=3) and aripiprazole (1 μM) (N=5) for the indicated times. Data are mean ± S.E.M; One-way ANOVA for multiple

comparations: *p < 0.05, **p < 0.01, ***p < 0.001 shows significance with respect to control (C; vehicle, grey bars).

Figure 3. Neurotransmitter receptors and associated signalling pathways involved in the control of ADAMTS2 gene expression. (A) ADAMTS2 mRNA levels by qRT-PCR in SK-N-SH cells incubated 1h with the indicated selective agonist (red bars): SKF 83822 (D_1-class receptors) (N=5), 7-OH-DPAT (D_2-class receptors) (N=3), 8-OH-DPAT (5-HT_{1A} receptor) (N=3) and TCB-2 (5-$HT_{2A/2C}$ receptors) (N=5); and selective antagonist (blue bars): SCH 39165 (D_1-class receptors) (N=3), L 741,626 (D_2-class receptors) (N=4), WAY 100635 (5-HT_{1A} receptor) (N=3) and MDL 100907 (5-HT_{2A} receptors) (N=3) (Drug concentration 1 μM). (B) ADAMTS2 mRNA levels by qRT-PCR in cells incubated for 1 h with SKF 83822 (N=4) and pre-incubated also for 30 min with SCH 39166 (N=4), clozapine (N=3), haloperidol (N=4) , paliperidone (N=4) or aripiprazole (N=4) (Drug concentration 1 μM). (C) CREB activity in cells transfected with CRE-Luc alongside pRL-Null: cells were pre-incubated for 1 h with the indicated APDs and then, incubated for 24 h with SKF 82833 (10 μM) (N=4). (D) Shows ADAMTS2 mRNA levels by qRT-PCR: SK-N-SH cells were pre-incubated for 30 min with MAPK/ERK and cAMP-PKA inhibitors (selumetinib 1 μM N=6 and H89 10 μM N=4, respectively) and then, incubated for 1 h with SKF 82833 (1 μM) (N=5). (E) Shows CREB activity in cells transfected with CRE-Luc alongside pRL-Null (N=4): SK-N-SH cells were pre-incubated for 1 h with the indicated inhibitors and then, incubated for 24 h with SKF 82833 (10 μM). And, (F) western-blots showing relative phosphorylation levels of CREB and ERK1/2: SK-N-SH cells were pre-incubated for 1 h with the indicated inhibitors and then, incubated for 15 min with SKF 82833 (1 μM) (N=3). Blots are representative images of each western-blot. Data are mean ± S.E.M; Student's t-test: *p < 0.05 and ***p < 0.001 vs control condition (vehicle), and #p < 0.05, ##p < 0.01, ###p < 0.001 vs SKF 83822 condition.

Figure 4. Transcriptional mechanisms that control ADAMTS2 gene expression downstream of dopamine D1-class receptors. (A) qRT-PCR showing ADAMTS2 and C-FOS mRNA expression level in SK-N-SH cells incubated for 1 hour with SKF 83822 (1 μM; blue bars) (N=6 and N=4, respectively), forskolin (10 μM; red bars) (N=3 and N= 4, respectively) and TPA (10 ng/ml; green bars) (N=3). (B) CREB1 knock-down SK-N-SH cells by lentiviral inducible shRNA and GFP reporter construct (yellow bars), incubated with doxycycline (1 μg/ml) for 72 h, CREB1 mRNA level (up) and CREB protein total expression (down) in non-targeted control (NTC) or shCREB1 cells (N=3). Inducible CREB1 Knock-down SK-N-SH cells were incubated for 1 h with SKF 83822 (1 μM, blue bars), forskolin (10 μM, red bars) and TPA (10 ng/ml, green bars): qRT-PCR showing ADAMTS2 (C) and C-FOS (D) mRNA expression in inducible shCREB1 or non-targeted control (NTC) cells incubated with doxycycline (1 μg/ml) for 72 h (N=3-4). And (E) shows western-blots using anti-phospho-CREB and anti-phospho-ERK as well as anti-CREB and anti-ERK antibodies: inducible shCREB1 or non-targeted control (NTC) SK-N-SH cells incubated with doxycycline (1 μg/ml) for 72 h and then, incubated for 15 min with SKF 83822 (1 μM), forskolin (10 μM) and TPA (10 ng/ml) (N=3). Inducible knock-down cells were selected with puromycin (1 μg/ml) at least 7 days. Blots images are representative of independent experiments. Data are mean ± S.E.M; Student's t-test: *p < 0.05, **p < 0.01, ***p < 0.001 vs vehicle or doxycycline (-); and #p < 0.05, ###p < 0.001 vs each condition in NTC cells.

Figure 5. Schematic representation of the mechanisms that control ADAMTS2 gene expression. Selective stimulation of D_1 receptors by SKF 83822 (selective D_1 agonist) triggers the expression of ADAMTS2. Two main pathways seem to be

involved: (1) Gαs/AC/cAMP/PKA signalling (dark red), and (2) MEK/ERK1/2 signalling (light red). Downstream of D_1 both PKA and ERK can phosphorylate CREB at Ser133 and activate transcription of ADAMTS2. Specific activators of PKA (Forskolin) and MEK (TPA) are highlighted in blue. Specific inhibitors of PKA (H89) and MEK (selumetinib) are coloured in red. DA D1 (dopamine D1 receptor), Gsα (G-protein α-subunit), Gβγ (G-protein βγ-subunits), AC (adenyl cyclase), PKA (protein kinase A), CRE (cyclic AMP-responsive element) site, PM (plasmatic membrane). Arrows: direct interaction, dashed arrows: indirect interaction.

Table 1. Psychopathological characteristics at baseline, at 3-months and clinical changes during the follow up period comparison between aripiprazole and risperidone.

	Total (N=30)		Statistics	p	Aripiprazole (n=16)		Risperidone (n=14)		Statistics	p
	Mean	SD			Mean	SD	Mean	SD		
CGI										
Baseline	6.5	0.6			6.56	0.51	6.43	0.65	t= 0.63 [c]	0.532
3 months	1.8	1.2			1.63	1.20	2.00	1.24		
3-month change from baseline	-4.7	1.2	z= 4.83 [a]	0.000	-4.90	0.30	-4.47	0.33	F= 0.96 [d]	0.336
YMRS										
Baseline	13.8	6.2			14.25	7.24	13.29	5.01	t= 0.42 [c]	0.679
3 months	1.2	2.3			1.56	2.63	0.79	1.93		
3-month change from baseline	-12.6	6.5	z= 4.78 [a]	0.000	-12.25	0.59	-13.00	0.63	F= 0.75 [d]	0.394
CDSS										
Baseline	1.5	3.0			2.06	3.82	0.86	1.75	t= 1.08 [c]	0.288
3 months	1.2	2.3			1.19	2.34	1.14	2.25		
3-month change from baseline	-0.3	3.7	z= 0.35 [a]	0.724	-0.34	0.59	-0.32	0.63	F= 0.00 [d]	0.985
BPRS										
Baseline	70.3	15.0			69.06	16.08	71.85	13.95	t= -0.49 [c]	0.627
3 months	29.9	7.1			29.06	7.07	30.92	7.48		
3-month change from baseline	-40.4	15.2	t= 14.32 [b]	0.000	-41.13	1.81	-39.53	2.01	F= 0.35 [d]	0.561
SAPS										
Baseline	15.9	4.2			15.63	3.50	16.07	4.94	t= -0.29 [c]	0.775
3 months	0.6	1.7			0.69	1.89	0.71	1.49		
3-month change from baseline	-15.4	4.3	t= 19.28 [b]	0.000	-15.14	0.44	-15.12	0.47	F= 0.00 [d]	0.975
SANS										
Baseline	4.8	6.9			3.47	6.56	5.93	7.43	t= -0.95 [c]	0.352
3 months	3.9	6.6			4.00	6.47	4.93	7.04		
3-month change from baseline	-0.9	5.6	z= 1.14 [a]	0.255	-0.07	1.54	-0.36	1.59	F= 0.02 [d]	0.899

BPRS: Brief Psychiatric Rating Scale; CDSS: Calgary Depression Rating Scale for Schizophrenia; CGI: Clinical Global Impression; SANS: Scale for the Assessment of Negative Symptoms; SAPS: Scale for the Assessment of Positive Symptoms; YMRS: Young Mania Rating Scale.

[a] Wilcoxon Matched-Pairs Signed-Rank test

[b] Paired T-Student test

[c] Comparison between aripiprazole and risperidone at baseline; unpaired Student's t-test.

[d] Comparison between aripiprazole and risperidone following the antipsychotic treatment, using the total score of the clinical scales at baseline as covariate; analysis of covariance (ANCOVA).

Figure 1

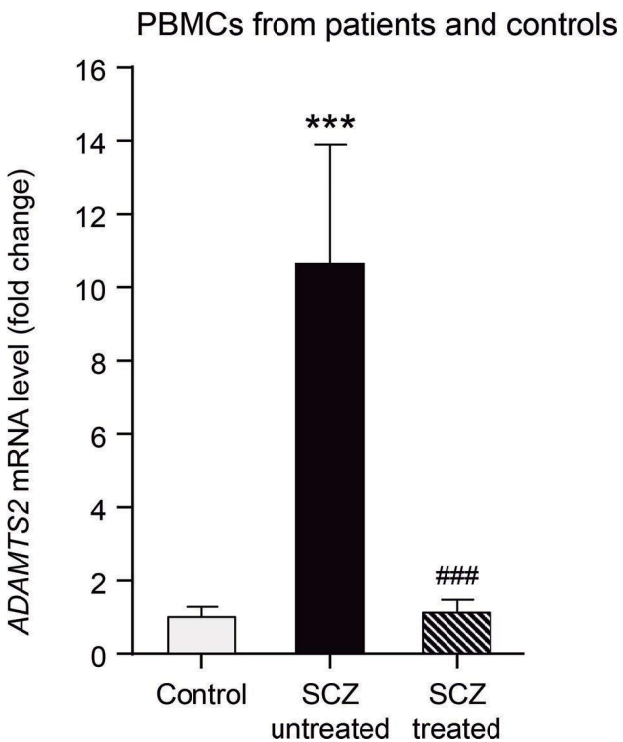


Figure 2

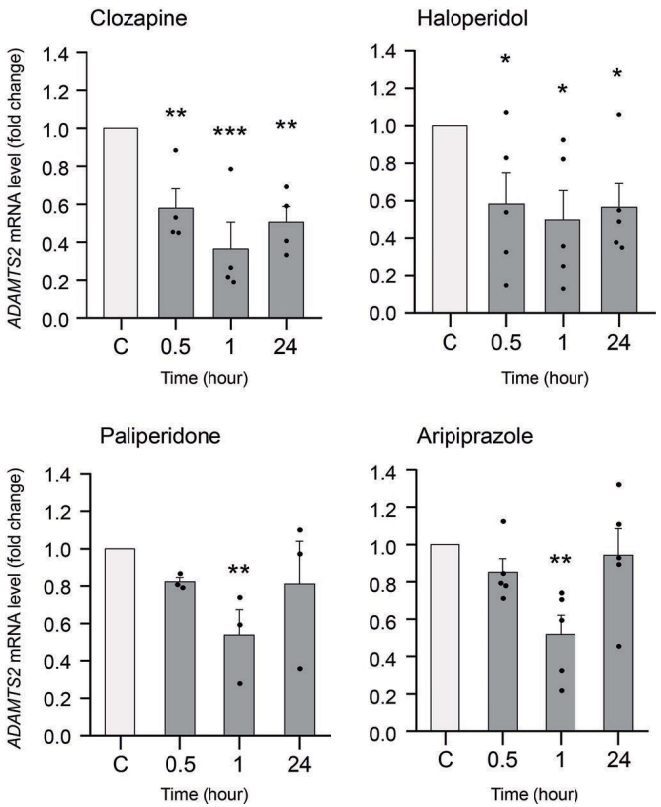


Figure 3

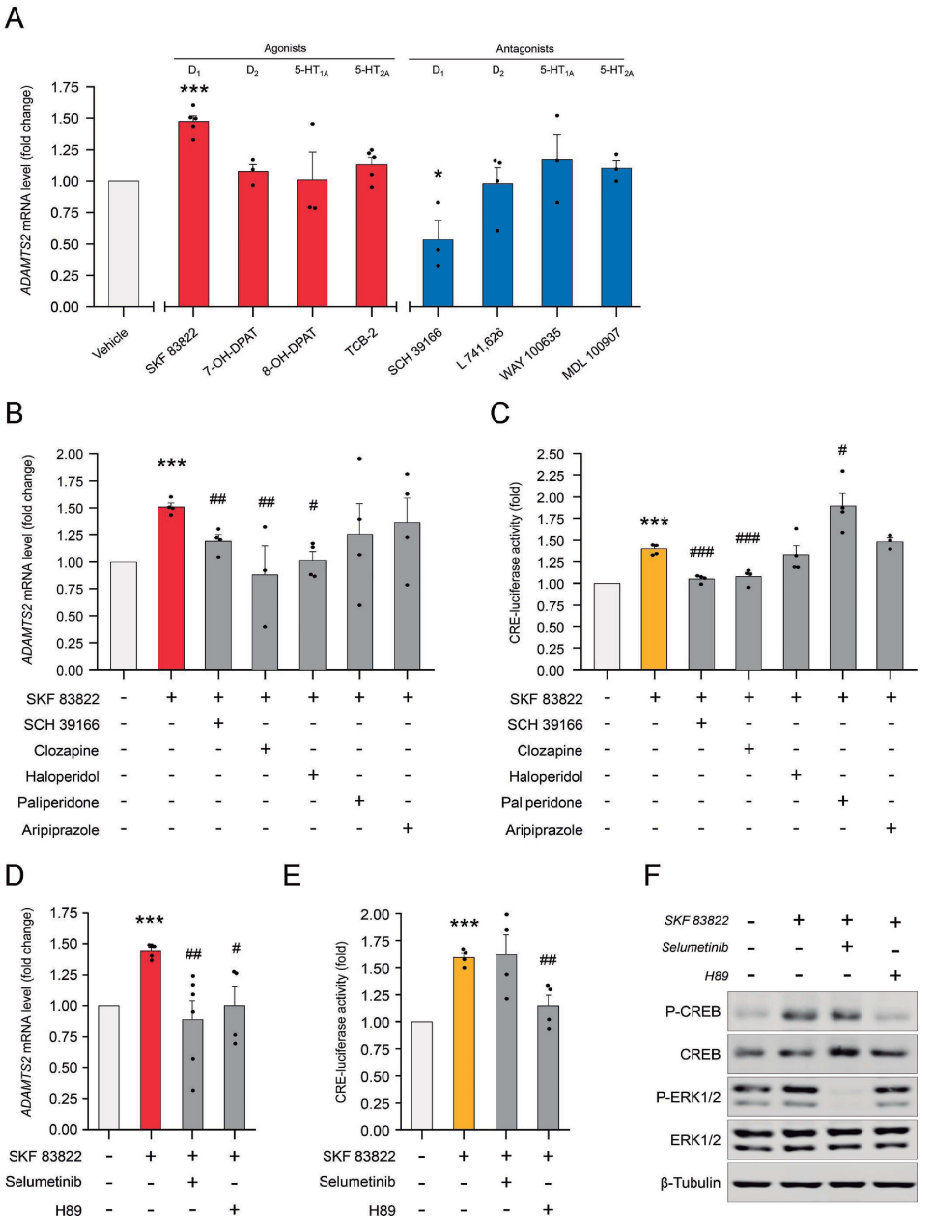


Figure 4

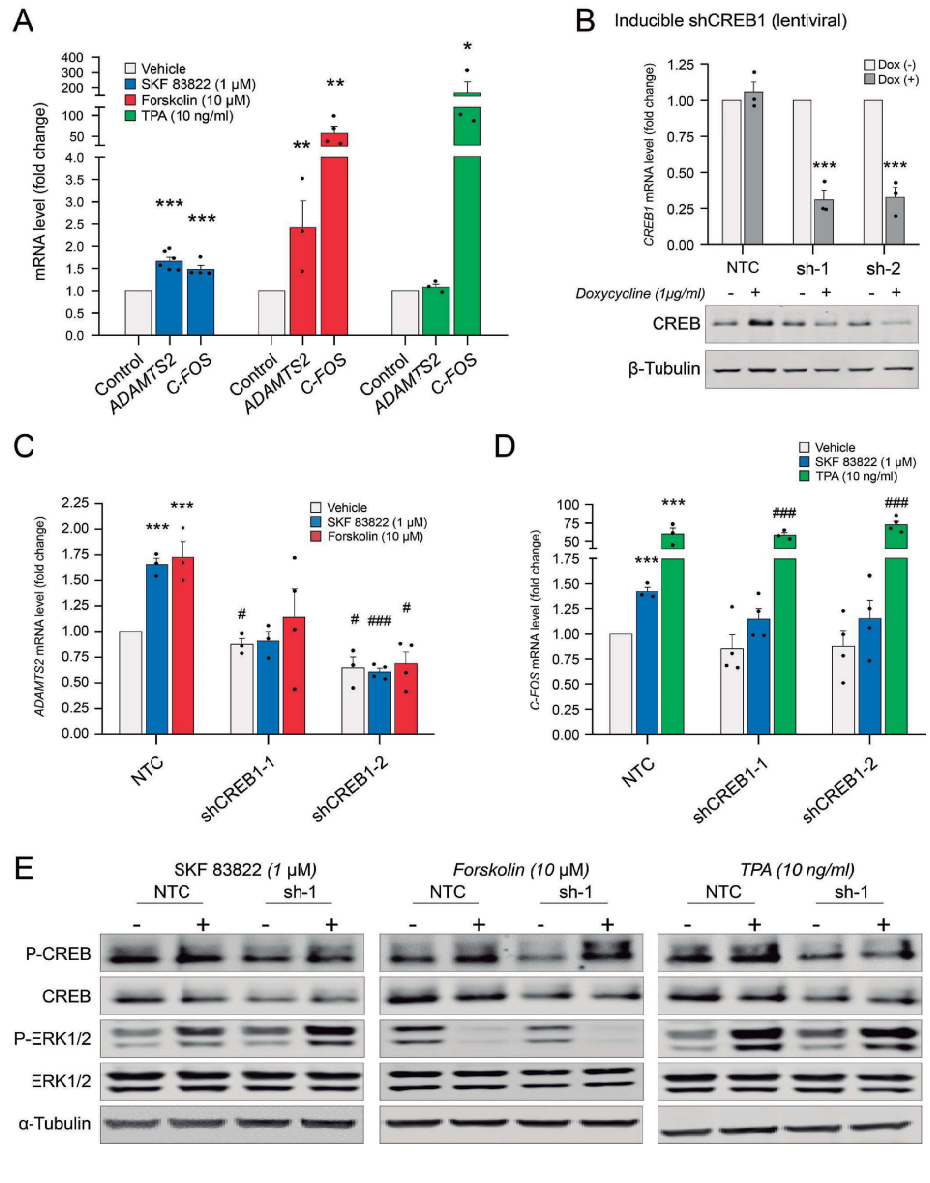
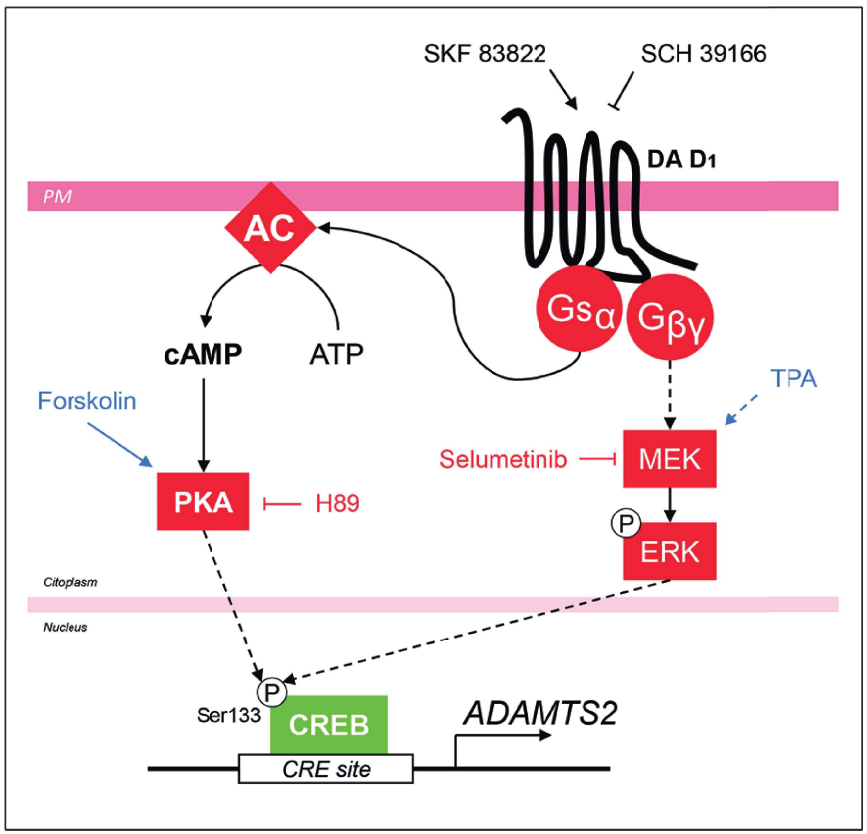


Figure 5


SKF 83822
SCH 39166
DA D1
PM
AC
Gsα
Gβγ
cAMP
ATP
Forskolin
TPA
Selumetinib
MEK
PKA
H89
P
ERK
Citoplasm
Nucleus
P
Ser133
CREB
ADAMTS2
CRE site

Schizophrenia Research 189 (2017) 217–218

Contents lists available at ScienceDirect

Schizophrenia Research

journal homepage: www.elsevier.com/locate/schres

Correspondence

Validation of schizophrenia gene expression profile in a preclinical model of maternal infection during pregnancy

Animal models of schizophrenia constitute at this moment fundamental experimentation tools to further explore on the etiological basis of this pathology, leading to the discovery of new biological targets and innovative therapeutic strategies for its treatment. Any improvement in the translation and validation of biological data between preclinical and clinical models will therefore represent a substantial advance in the research of this mental disease. Recent data from our group obtained by next-generation sequencing from blood transcriptome samples identified, among 22,278 genes analyzed, differential expression of 200 genes in schizophrenia patients related to biological processes associated with immunological responses (Sainz et al., 2013). Further investigations conducted by the same experimental approach and focused on 6 particular genes selected from those 200 previously detected, *i.e.*, *ADAMTS2*, *CD177*, *CNTNAP3*, *ENTPD2*, *RFX2* and *UNC45B*, revealed an overexpression of these particular genes in drug-naïve patients that was reverted to the control expression levels after antipsychotic treatment (Crespo-Facorro et al., 2014). Pre-clinical models of schizophrenia present important limitations due to the complexity of emulating and/or translating a human mental disease into a particular animal phenotype. We have extensively characterized a neurochemical alteration as a possible molecular explanation for some of the symptoms associated with schizophrenia. This consists in the dysregulation of the elements that constitute the heteroreceptor complex formed by 5-HT_{2A} serotonin and mGlu2 glutamate receptors (Gonzalez-Maeso et al., 2008). Particularly, the up-regulation of 5-HT_{2A} accompanied by the down-regulation of mGlu2 receptors results in a final signaling balance at the cellular level that predispose mice to a "psychotic" phenotype according to behavioral experimental models (Fribourg et al., 2011). This behavioral phenotype resides essentially in an increase in the head-twitch response evoked by hallucinogenic serotonergic agonists accompanied by locomotor hyperactivity induced by dissociative glutamate antagonists. Additionally, we have also described in mouse models of both maternal influenza viral infection (Moreno et al., 2011) and maternal variable stress during pregnancy (Holloway et al., 2013) that descendants presented neurochemical and behavioral changes in line with the dysregulation of the expression level of 5-HT_{2A}-mGlu2 receptor heteromer causing schizophrenia-like alterations in mice only in post-pubertal ages. These models of alterations during pregnancy are useful to explore those hypotheses maintaining that there are environmental events occurring during pre- or perinatal phases of development determinant for the onset of clinical symptoms (Rapoport et al., 2005). One of the hypothesis supporting this concept proposes that exposure to adverse factors during gestation such as certain infections, maternal stress, malnutrition or maternal medical complications would predispose individuals to a latent immune vulnerability that will be manifested at puberty or early adulthood by affecting different processes in the central nervous system (Kinney et al., 2010).

In order to preclinically validate our previous data obtained from blood human samples, we proceeded to evaluate the expression of the orthologous version of these genes in adult mice born to mothers submitted to influenza viral infection during gestation. Pregnant CD1 mice were subjected to influenza viral infection according to the protocol previously described (Moreno et al., 2011) and adult offspring were assessed for behavioral and neurochemical traits corresponding to schizophrenia-like phenotype, essentially increased head-twitch response elicited by treatment with the hallucinogenic drug DOI and changes in the relative 5-HT_{2A} and mGlu2 receptor expression levels detected by *ex vivo* radioligand binding assays. A group of eight animals that statistically presented these alterations were pair matched with control mice. In both experimental groups, we tested animals originating from at least two different litters. Tissue samples from mouse frontal cortex were obtained and mRNA was extracted to conduct subsequent quantitative real-time PCR (qRT-PCR) experiments as previously described with minor modifications (Gonzalez-Maeso et al., 2003) (primer sequences are included in Supplemental material 1). qRT-PCR results demonstrated, first, that these six genes are expressed in cortical tissue from mouse brain. Second and more importantly, statistical analysis comparing control and influenza-infected descendant mice (Fig. 1), performed by correcting α value as described before (Kurita et al., 2012), revealed a significant overexpression of at least three of the six total genes considered in this study, in particular *CD177*, *CNTNAP3* and *ENTPD2* ($t_{14} = 3.85$, $P = 0.0018$; $t_{14} = 3.31$, $P = 0.0052$; $t_{14} = 3.19$, $P = 0.0065$ respectively. Significance at $P < 0.01$). Other two genes such as *ADAMTS2*, and *RFX2* although not reaching statistical significance also presented a tendency to be overexpressed when compared with control values.

In conclusion, these data represent one of the first preclinical validations of alterations in gene expression previously observed in clinical samples. This set of genes, whose expression is regulated in blood samples by exposure to antipsychotic drugs in schizophrenia patients and dysregulated in the frontal cortex by prenatal insults in mouse models, might provide new and interesting information about mechanisms of effective action of antipsychotics. These findings may ultimate lead to the identification of new therapeutic targets for treatment of schizophrenia and other neurodevelopemental psychiatric conditions.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.schres.2017.02.005.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

This study was funded by NIH R01 MH084894 and R01 MH111940 grants and SAF2010-20840-C02-01/02 and SAF2013-46292-R from MINECO (Spain).

http://dx.doi.org/10.1016/j.schres.2017.02.005

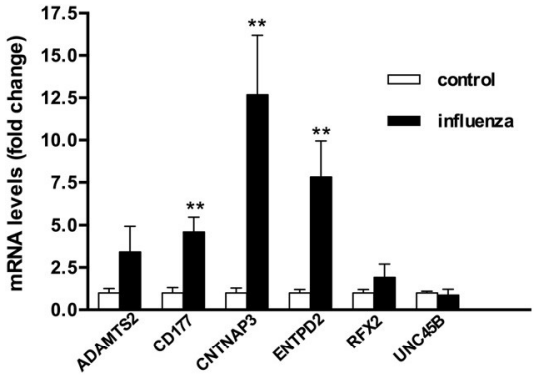


Fig. 1. Expression of ADAMTS2, CD177, CNTNAP3, ENTPD2, RFX2 and UNC45B genes in frontal cortex of adult mice born to influenza virus-infected mothers (filled bars) and controls (opened bars) determined by qRT-PCR assays ($n = 8$ animals per group). Data are mean ± SEM ($^{**}p < 0.01$). Student's *t*-test.

References

Crespo-Facorro, B., Prieto, C., Sainz, J., 2014. Schizophrenia gene expression profile reverted to normal levels by antipsychotics. J Neuropsychopharmacol]–>Int. J. Neuropsychopharmacol. 18 (4).

Fribourg, M., Moreno, J.L., Holloway, T., Provasi, D., Baki, L., Mahajan, R., Park, G., Adney, S.K., Hatcher, C., Eltit, J.M., Ruta, J.D., Albizu, L., Li, Z., Umali, A., Shim, J., Fabiato, A., MacKerell Jr., A.D., Brezina, V., Sealfon, S.C., Filizola, M., Gonzalez-Maeso, J., Logothetis, D.E., 2011. Decoding the signaling of a GPCR heteromeric complex reveals a unifying mechanism of action of antipsychotic drugs. Cell 147 (5), 1011–1023.

Gonzalez-Maeso, J., Yuen, T., Ebersole, B.J., Wurmbach, E., Lira, A., Zhou, M., Weisstaub, N., Hen, R., Gingrich, J.A., Sealfon, S.C., 2003. Transcriptome fingerprints distinguish hallucinogenic and nonhallucinogenic 5-hydroxytryptamine 2A receptor agonist effects in mouse somatosensory cortex. J. Neurosci. 23 (26), 8836–8843.

Gonzalez-Maeso, J., Ang, R.L., Yuen, T., Chan, P., Weisstaub, N.V., Lopez-Gimenez, J.F., Zhou, M., Okawa, Y., Callado, L.F., Milligan, G., Gingrich, J.A., Filizola, M., Meana, J.J., Sealfon, S.C., 2008. Identification of a serotonin/glutamate receptor complex implicated in psychosis. Nature 452 (7183), 93–97.

Holloway, T., Moreno, J.L., Umali, A., Rayannavar, V., Hodes, G.E., Russo, S.J., Gonzalez-Maeso, J., 2013. Prenatal stress induces schizophrenia-like alterations of serotonin 2A and metabotropic glutamate 2 receptors in the adult offspring: role of maternal immune system. J. Neurosci. 33 (3), 1088–1098.

Kinney, D.K., Hintz, K., Shearer, E.M., Barch, D.H., Riffin, C., Whitley, K., Butler, R., 2010. A unifying hypothesis of schizophrenia: abnormal immune system development may help explain roles of prenatal hazards, post-pubertal onset, stress, genes, climate, infections, and brain dysfunction. Med. Hypotheses 74 (3), 555–563.

Kurita, M., Holloway, T., Garcia-Bea, A., Kozlenkov, A., Friedman, A.K., Moreno, J.L., Heshmati, M., Golden, S.A., Kennedy, P.J., Takahashi, N., Dietz, D.M., Mocci, G., Gabilondo, A.M., Hanks, J., Umali, A., Callado, L.F., Gallitano, A.L., Neve, R.L., Shen, L., Buxbaum, J.D., Han, M.H., Nestler, E.J., Meana, J.J., Russo, S.J., Gonzalez-Maeso, J., 2012. HDAC2 regulates atypical antipsychotic responses through the modulation of mGlu2 promoter activity. Nat. Neurosci. 15 (9), 1245–1254.

Moreno, J.L., Kurita, M., Holloway, T., Lopez, J., Cadagan, R., Martinez-Sobrido, L., Garcia-Sastre, A., Gonzalez-Maeso, J., 2011. Maternal influenza viral infection causes schizophrenia-like alterations of 5-HT(2)A and mGlu(2) receptors in the adult offspring. J. Neurosci. 31 (5), 1863–1872.

Rapoport, J.L., Addington, A.M., Frangou, S., Psych, M.R., 2005. The neurodevelopmental model of schizophrenia: update 2005. Mol. Psychiatry 10 (5), 434–449.

Sainz, J., Mata, I., Barrera, J., Perez-Iglesias, R., Varela, I., Arranz, M.J., Rodriguez, M.C., Crespo-Facorro, B., 2013. Inflammatory and immune response genes have significantly altered expression in schizophrenia. Mol. Psychiatry 18 (10), 1056–1057.

Juan F. Lopez-Gimenez[1]
Institute of Biomedicine and Biotechnology of Cantabria (IBBTEC-CSIC), Santander, Spain
Department of Physiology and Biophysics, Virginia Commonwealth University School of Medicine, Richmond, Virginia 23298, USA
Correspondence to: J.F. Lopez-Gimenez, Institute of Biomedicine and Biotechnology of Cantabria (IBBTEC-CSIC), Santander, Spain.
E-mail address: jf.lopez.gimenez@csic.es.

Mario de la Fuente Revenga
Department of Physiology and Biophysics, Virginia Commonwealth University School of Medicine, Richmond, Virginia 23298, USA

Fulgencio Ruso-Julve
University Hospital Marqués de Valdecilla, IDIVAL, Department of Psychiatry, School of Medicine, University of Cantabria, Santander, Spain

Justin M. Saunders
Department of Physiology and Biophysics, Virginia Commonwealth University School of Medicine, Richmond, Virginia 23298, USA

José L. Moreno
Department of Physiology and Biophysics, Virginia Commonwealth University School of Medicine, Richmond, Virginia 23298, USA

Benedicto Crespo-Facorro[1]
University Hospital Marqués de Valdecilla, IDIVAL, Department of Psychiatry, School of Medicine, University of Cantabria, Santander, Spain
CIBERSAM, Centro Investigación Biomédica en Red Salud Mental, Santander, Spain

Javier González-Maeso[1]
Department of Physiology and Biophysics, Virginia Commonwealth University School of Medicine, Richmond, Virginia 23298, USA
Corresponding author.
E-mail address: javier.maeso@vcuhealth.org.

23 January 2017

[1] These authors supervised equally this work.

Brain, Behavior, and Immunity 80 (2019) 909–915

Contents lists available at ScienceDirect

Brain, Behavior, and Immunity

journal homepage: www.elsevier.com/locate/ybrbi

Time dependent expression of the blood biomarkers *EIF2D* and *TOX* in patients with schizophrenia

Javier Gilabert-Juan[a,b,c,d,*], Guillermo López-Campos[e], Noelia Sebastiá-Ortega[a,c,d], Sonia Guara-Ciurana[a], Fulgencio Ruso-Julve[c,f], Carlos Prieto[g], Benedicto Crespo-Facorro[c,f], Julio Sanjuán[c,d,h], María Dolores Moltó[a,c,d]

[a] Department of Genetics, Universitat de València, Valencia, Spain
[b] Neurobiology Unit, Cell Biology Department, Interdisciplinary Research Structure for Biotechnology and Biomedicine (BIOTECMED), Universitat de València, Valencia, Spain
[c] Centro de Investigación Biomédica en Red de Salud Mental (CIBERSAM), Spain
[d] INCLIVA, Valencia, Spain
[e] Centre for Experimental Medicine, Queen's University Belfast, Belfast, UK
[f] University Hospital Marqués de Valdecilla, IDIVAL, Department of Psychiatry, School of Medicine, University of Cantabria, Santander, Spain
[g] Servicio de Bioinformática, Nucleus, Universidad de Salamanca, Salamanca, Spain
[h] Unit of Psychiatry, Universitat de València, Valencia, Spain

A B S T R A C T

Background: During last years, there has been an intensive search for blood biomarkers in schizophrenia to assist in diagnosis, prognosis and clinical management of the disease.

Methods: In this study, we first conducted a weighted gene coexpression network analysis to address differentially expressed genes in peripheral blood from patients with chronic schizophrenia (n = 30) and healthy controls (n = 15). The discriminating performance of the candidate genes was further tested in an independent cohort of patients with first-episode schizophrenia (n = 124) and healthy controls (n = 54), and in postmortem brain samples (cingulate and prefrontal cortices) from patients with schizophrenia (n = 34) and healthy controls (n = 35).

Results: The expression of the *Eukaryotic Translation Initiation Factor 2D* (*EIF2D*) gene, which is involved in protein synthesis regulation, was increased in the chronic patients of schizophrenia. On the contrary, the expression of the *Thymocyte Selection-Associated High Mobility Group Box* (*TOX*) gene, involved in immune function, was reduced. *EIF2D* expression was also altered in first-episode schizophrenia patients, but showing reduced levels. Any of the postmortem brain areas studied did not show differences of expression of both genes.

Conclusions: EIF2D and TOX are putative blood markers of chronic patients of schizophrenia, which expression change from the onset to the chronic disease, unraveling new biological pathways that can be used for the development of new intervention strategies in the diagnosis and prognosis of schizophrenia disease.

1. Introduction

During recent decades, great efforts have been made to understand the molecular basis of schizophrenia (SZ). As a result, a growing list of genetic polymorphisms, rare genetic variants, *de novo* mutations, epigenetic changes and other genomic alterations showing association with SZ has been provided (Chen et al., 2015; Modai and Shomron, 2016; Bustamante et al., 2017). However, these findings have not been translated into suitable biomarkers for clinical use because most markers account for low disease risk and those based on gene expression in postmortem brain tissues cannot be analyzed since brain biopsies are not acceptable for clinical evaluation.

Blood is a good source of samples for disease assessment in living patients because this tissue is easily accessible and is in contact with tissues and cells throughout the body. Many studies have showed that alteration of metabolism and cellular functions in the central nervous system (CNS), as well as disturbances in neurotransmitter and hormonal systems are related with altered function and metabolism of blood cells (Gladkevich et al., 2004; Marques-Deak et al., 2005). This connection results from a complex network involving the nervous, endocrine and immune systems and has been investigated in several neuropsychiatric disorders. Different studies have compared gene expression in peripheral blood (PB) and brain to evaluate the usefulness of PB as a source of biomarkers that mirror the state of disease in the brain (Glatt et al., 2005; Sullivan et al., 2006; Marazziti et al., 2010). Results from transcriptomic, proteomic and epigenomic analysis support the view that some genes, which expression is dysregulated in blood, might reflect specific changes that characterizes the abnormal biological

* Corresponding author at: Centre for Interdisciplinary Research in Biology (CIRB), Collège de France, CNRS/UMR 7241 – INSERM U1050, Paris, France.
E-mail address: javier.gilabert@uv.es (J. Gilabert-Juan).

https://doi.org/10.1016/j.bbi.2019.05.015
Received 31 March 2019; Received in revised form 5 May 2019; Accepted 8 May 2019
Available online 09 May 2019
0889-1591/

processes in CNS (Tylee et al., 2013; Hess et al., 2016; Wojsiat et al., 2017; Xu et al., 2018). Therefore, in recent years, there has been an intensive search for this type of biomarker as a useful and cheaper alternative for SZ (Lai et al., 2016).

Here, we sought to identify gene expression markers in PB that may be useful as biomarkers of development and disease evolution in SZ. We first performed an expression microarray analysis in blood samples from male SZ patients with several years of illness duration for comparison to controls. To prioritize biomarkers, a systems biology approach was used based on weighted gene coexpression network analysis (WGCNA), and the discriminating performance of candidate genes was further tested in an independent cohort of patients with first-episode SZ.

2. Methods

2.1. Peripheral sample cohorts

Thirty unrelated male patients with SZ were recruited at the Psychiatric Out-patient Unit of the Clinic Hospital of Valencia University, Spain (cohort 1). The control group (CNT) consisted of 15 age-matched healthy males without a history of drug abuse or familial background of mental disorders. A second sample of 124 patients with first-episode SZ (62 males) and 54 healthy controls (30 males) were recruited from the University Hospital Marqués de Valdecilla in Santander, Spain (cohort 2). Detailed description of these cohorts is shown in the supplementary material and supplementary Table 1. This study was performed according to the guidelines of each institution involved and was approved by the Local Ethics Committee of each participating center.

2.2. Postmortem sample

Frozen RNA from the postmortem frontal cortex and cingulate cortex was obtained from the Stanley Medical Research Institute Array Collection (http://www.stanleyresearch.org/brain-research/array-collection). Postmortem brain samples were from donor subjects with SZ (n = 35) and controls with no history of psychosis (n = 34). The distributions of age and gender in the two groups remained similar. The tissue characteristics of this sample are detailed in Supplementary Table 2.

2.3. Expression microarray analysis

PB of subjects from cohort 1 was collected in RNA-stabilizing PAXgene tubes (Qiagen, Izasa, Spain). Total RNA was extracted from the obtained cells using the PAXgene blood RNA Kit (Qiagen, Izasa, Spain) according to the manufacturer's protocol (supplementary Table 1). Gene expression analysis was performed using the GeneChip Human Gene 2.0 ST Array (Affymetrix, Santa Clara, CA, USA) covering more than 750,000 unique 25-mer oligonucleotide features constituting over 40,000 RefSeq transcripts. Hybridization experiments and microarray data generation were conducted in the Central Unit for Research in Medicine Faculty-INCLIVA, University of Valencia (Spain). A gene expression profile was obtained for each subject in cohort 1.

2.4. Gene coexpression network analysis

WGCNA package in R (Langfelder and Horvath, 2008) was used to calculate the coexpression network from the reduced gene set (24618 probes). We initially identified the value that transformed the data into an approximate scale-free topology. Next, we used Pearson's correlation coefficient, a minimum module size of 30 genes and a minimum height value of 0.25 to merge modules (groups of highly correlated genes). For the functional annotation of the network modules associated with the different traits, we used the Database for Annotation, Visualization and Integrated Discovery (DAVID) (http://david.abcc.ncifcrf.gov/home.jsp). We considered only those results with a false discovery rate of 0.05 to be significantly enriched. DAVID analysis results were visualized using the EnrichmentMap 2.0.1 (Merico et al., 2010) plugin for Cytoscape 3.3.0 (http://cytoscape.org). For detailed description see supplementary material.

2.5. Reverse transcription quantitative PCR (RT-qPCR)

Total RNA of subjects from cohort 1 was converted into cDNA with Expand Reverse Transcriptase and hexanucleotide mix (Roche Applied Science, Indianapolis, USA), following the experimental conditions as previously described (Gilabert-Juan et al., 2015).

Amplification was conducted with an ABI PRISM 7700 Sequence Detector (Applied Biosystems) as previously described (16), using 5X HOT FIREPol EvaGreen qPCR Mix Plus (Solis BioDyne, Estonia) and gene-specific primers (Supplementary Table 3).

2.6. RNA sequencing (RNA-seq)

Total RNA was extracted from the PB of individuals from cohort 2. mRNA was isolated from the total RNA and was fragmented once transformed into cDNA. Fragments of 300 bp on average were selected to construct the libraries for sequencing. Pair-end sequences of 70 nucleotides for each end were produced. Then it was sequenced using Illumina HiSeq instruments (San Diego, CA, USA).

RT-qPCR and RNA-seq analyses are further described in the supplementary material.

2.7. Statistical analyses

Demographic, clinical and tissue features of cohorts were compared using ANOVA and *t*-test. Statistical significance was indicated at $p < 0.05$. To identify potential blood biomarkers for SZ contributing to the differentiation between control individuals and patients, a binary logistic regression was carried out with the gene expression data. A significance test of the Wald statistic was conducted to investigate genes whose expression is significantly associated in the final equation ($p < 0.05$). All statistical analyses were performed using SPSS v14.0 software (SPSS, Chicago, IL).

3. Results

3.1. Gene expression array analysis

Whole transcriptome analysis in PB samples of cohort 1 was performed to identify potential biomarkers in SZ. We focused on male subjects to minimize sex differences in gene expression. We identified 10 modules of correlated genes (genes with similar expression attending to the different groups studied and variables of the sample), each one of them was coded with an arbitrary color, and an eleventh gray module containing the genes that were not part of any particular module. The functional annotation of each module is shown in Supplementary Table 4.

The main purpose of the analysis was to identify genes whose expression was associated with the clinical condition (CNT *versus* SZ). We specified a significance threshold of p-value ≤ 0.05 for the correlation between connected-gene modules and traits. According to this threshold, four modules (turquoise, purple, yellow and blue) significantly correlated with the clinical condition (Fig. 1), which means that there are differences in gene expression levels among CNT *versus* SZ. In addition, two modules (magenta and blue) were correlated with the patient condition, which accounts for differences in gene expression levels between SZ patients with and without auditory hallucinations. Three modules (turquoise, purple and yellow) were associated with medication (supplementary Table 5); two modules (black and pink)

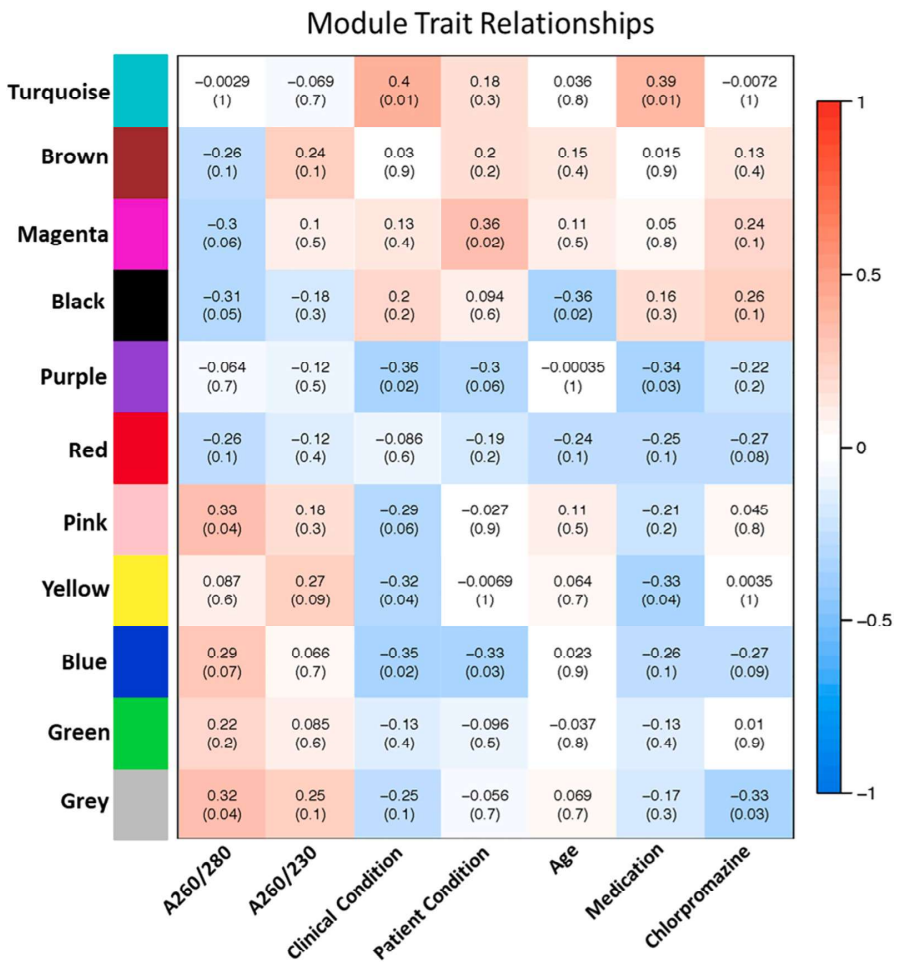


Fig. 1. Relationship between the different traits analyzed (RNA purity measured as A260/A280 and A260/230; Clinical condition; Patient condition; Age; Medication; Chlorpromazine equivalent) and gene modules using the WGCNA approach. Gene modules are designated by different colors on the left, color scheme on the right (blue-red) denotes the correlation (−1 to 1) between module and trait, and the *p*-value of the correlation in each interaction is indicated in brackets. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

correlated with RNA purity and one module (black) with the subject' age. Importantly, we did not identify any highly connected gene module specifically related to chlorpromazine dosage under the thresholds applied (Fig. 1).

3.2. Selection of candidate genes as potential blood biomarkers in schizophrenia

We selected modules that were significantly correlated with the clinical or the patient conditions (with a cutoff value of $p \leq 0.02$ at least in one of these conditions) but that showed no correlation with the other traits analyzed. Gene expression in the blue module (Fig. 1) is associated with the clinical condition ($p = 0.02$) and patient condition ($p = 0.03$). Purple and turquoise modules showed correlations with the clinical condition ($p = 0.02$ and 0.01, respectively). In these two modules, medication might have an effect on the expression of the included genes ($p = 0.03$ and $p = 0.01$, respectively). No significant effect was observed for the remaining confounding factors. Finally, the magenta module is associated with the patient condition ($p = 0.02$).

Next, we chose genes from the selected modules (Supplementary Figs. 1–4), whose expression differed in the clinical or patient condition traits with high statistical significance ($p < 0.001$ in the blue and turquoise modules because they included a high number of genes; $p < 0.01$ for purple and magenta modules, with fewer genes). A total of 14 genes were selected; *CPQ*, *MSL1*, *NRD1*, *SRPK1*, *CMTM5*, *APOBEC3G*, *NKG7*, *SLAMF6*, *TOX*, *C12orf73*, *EIF2D*, *RNPS1*, *KPL29P2* and *SIGIRR* (supplementary Table 6). These genes are involved in different biochemical pathways, including proteolysis, chromatin organization and RNA splicing (turquoise module); chemotaxis (magenta module); cytidine deamination, inflammatory process, protein binding, regulation of immune response and DNA regulation (purple module); and gene expression, translation initiation, signal transduction and unknown functions (blue module). Expression of these genes was measured by RT-qPCR and compared between the control group and the SZ patients in cohort 1 (Supplementary Fig. 5). We found differences in two of them. The expression of the *Eukaryotic Translation Initiation Factor 2D* (*EIF2D*) gene was increased in SZ patients *versus* controls (fold change = 2.05, $p = 0.008$, Fig. 2A). On the contrary, the expression of the *Thymocyte Selection-Associated High Mobility Group Box* (*TOX*) gene was reduced in the SZ group (fold change = 0.6, $p = 0.017$, Fig. 2A).

Next, we studied the expression of both genes in prefrontal and cingulated post-mortem brain regions, which have been largely

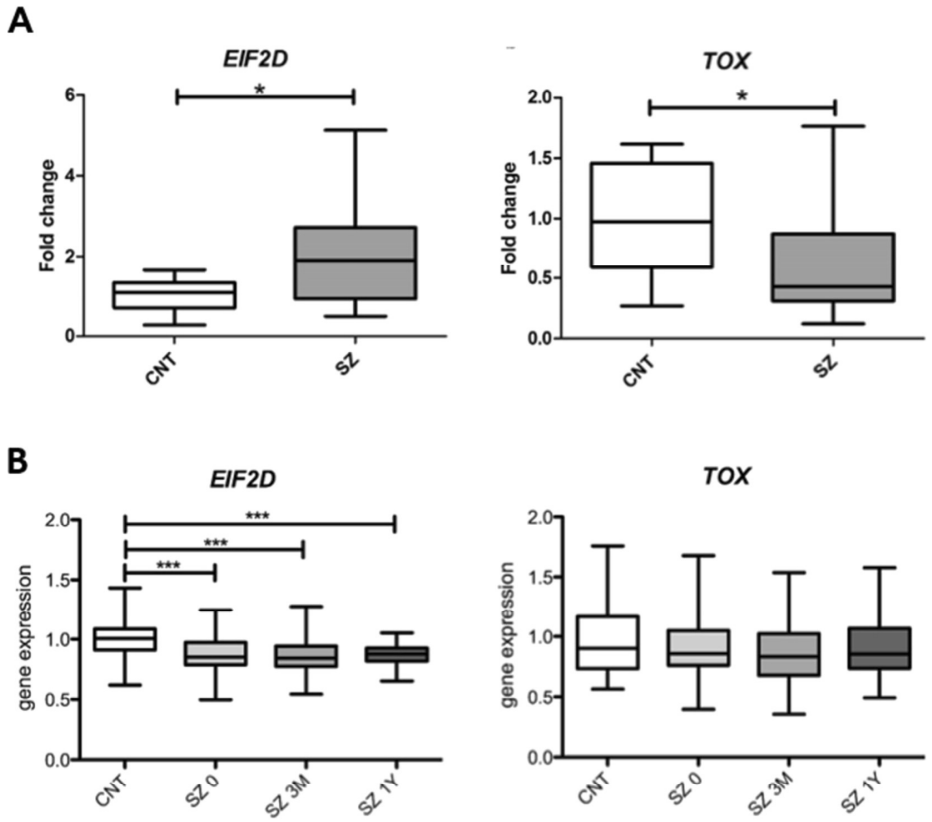


Fig. 2. A) Boxplots representing the quartiles of the relative gene expression values for the *EIF2D* and *TOX* in cohort 1; controls (CNT) are compared with schizophrenia patients (SZ). * $p < 0.05$. B) Boxplots in cohort 2, CNT are compared with the SZ patients at the onset of the disease (SZ 0), after 3 months (SZ 3 M) and 1 year of disease onset (SZ 1Y), *** $p < 0.001$.

associated with SZ symptoms, and no significant changes were observed (Supplementary Fig. 6).

3.3. Predictive model equation

Using binary logistic regression, we obtained a predictive equation in which *EIF2D* and *TOX* gene expression could differentiate the SZ patients *versus* controls in cohort 1. The final equation model is $-0.106 + [2.957 \times EIF2D] + [-4.198 \times TOX]$, where *EIF2D* and *TOX* respectively indicate the expression levels of these genes. Applying this equation to subjects in cohort 1, SZ patients were distinguished from healthy controls with 86.5% accuracy, with 0.5 as a cutoff value (Supplementary Table 7).

3.4. Gene expression biomarkers in a new cohort

We assessed the predictive value of the 14 selected genes by measuring their expression in a new cohort (cohort 2). Gene expression was analyzed with RNA-seq data at disease onset (0), after 3 months (3 M) and after 1 year (1Y) of disease onset. In this case, we also found significant differences in *EIF2D* expression between controls and patients, but *EIF2D* mRNA levels were lower in patients than controls, contrary to what we observed in cohort 1. This reduction was found at the 3 time points tested (fold change at disease onset = 0.88; after 3 months = 0.87; after 1 year = 0.87, $p < 0.0001$ in all cases (Fig. 2B). *TOX* expression was reduced in the SZ patients as in cohort 1, although this change showed a trend at time point 0 and was not significant at 3 M or 1Y (Fig. 2B).

Next, we investigated a new predictive model equation using the expression data of the 14 selected genes in the cohort 2. We again found that *EIF2D* is included in a predictive model at each of 3 time points, but not *TOX* (Supplementary Table 8). Nevertheless, the predictive values of *EIF2D* expression in cohort 2 were found to be in opposite directions relative to those in cohort 1.

3.5. EIF2D changes with disease duration

We found that *EIF2D* expression increased in SZ patients in cohort 1 but decreased in SZ patients in cohort 2 regarding to the corresponding controls. Cohort 1 includes men with chronic SZ, while cohort 2 is composed of first-episode patients of both sexes evaluated over 1 year from the disease onset (Supplementary Table 1). To examine the potential effect of sex on *EIF2D* expression, we compared *EIF2D* mRNA levels only in men from cohort 2. We found a result similar to that described before for all samples, including men and women. Next, we compared *EIF2D* expression with respect to disease duration in SZ patients in cohort 1 and observed a positive correlation trend between *EIF2D* mRNA levels and years of disease ($p = 0.0794$; $r = 0.35$) (Fig. 3A). Then, we grouped the SZ patients into those with a disease duration of less (n = 17) and more (n = 9) than 10 years. When comparing the *EIF2D* expression between both subgroups of SZ patients, we found a trend of an increase in the expression levels of *EIF2D* ($p = 0.0634$) in patients suffering from the disease more than 10 years compared with the SZ patients from the other subgroup (Fig. 3B).

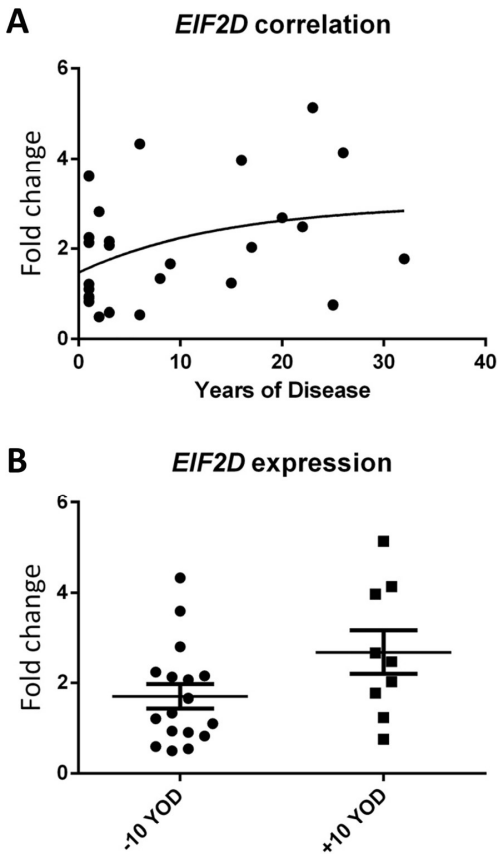


Fig. 3. *EIF2D* expression with disease duration in SZ patients in cohort 1. A) Correlation between *EIF2D* mRNA level and the years of the disease in the SZ patients in cohort 1. B) Differences in *EIF2D* expression in SZ patients with more than 10 years of the disease compared with SZ patients with more than 10 years of the disease.

4. Discussion

The identification of PB biomarkers for SZ risk assessment has become a new and promising area of translational investigation in psychiatry. Our results revealed that the expression levels of *EIF2D* and *TOX* could discriminate between SZ patients and controls with 86.5% accuracy in the cohort 1.

EIF2D encodes a factor involved in a noncanonical translation initiation mechanism of protein synthesis that is believed to operate mainly in cells under stress (Dmitriev et al., 2010). The expression of the yeast orthologue gene rises under some stresses; such as anoxia, oxidative stress and accumulation of unfolded proteins in the endoplasmic reticulum. In these situations, the activity of eIF2, which participates in the canonical eukaryotic translation initiation pathway, is suppressed (Dmitriev et al., 2010). In mammalian cells, eIF2 function is also blocked by different stress conditions (Wek et al., 2006). Our results of a two-fold increase in *EIF2D* mRNA levels in SZ patients relative to the levels in controls suggest the presence of cellular stressors in SZ patients. A growing body of data supports the implication of oxidative and endoplasmic reticulum stress in SZ (reviewed in (Patel et al., 2017)). Most studies analyzing markers of oxidative status, such as glutathione, in blood and in neural tissues point out oxidative imbalance in this disease (Gawryluk et al., 2011; Flatow et al., 2013).

On the other hand, EIF2D has been involved in translation control of cellular mRNAs having short regulatory upstream open reading frames in the 5′UTR (Schleich et al., 2014; Weisser et al., 2017). A large number of mammalian proteins are regulated by this mechanism, including many proteins needed for cell growth and proliferation (Schleich et al., 2014). Other noncanonical initiation factors, DENR (density regulated protein) and MCT-1 (product of malignant T cell-amplified sequence 1 oncogene), also promote the translation reinitiation process through the DENR-MCTS1 complex (Schleich et al., 2014; Weisser et al., 2017). Interestingly, missense mutations in DENR have been reported in unrelated patients with autism spectrum disorder (Haas et al., 2016). These results highlight the importance of proper mRNA translation reinitiation processes for brain development. We can speculate that higher EIF2D doses above its physiological level could also interfere with the translation reinitiation mechanism. In this line, increasing the levels of eIF4E in mice resulted in exaggerated cap-dependent translation and autistic-like behaviors (Santini et al., 2013). Finally, *EIF2D* is located on 1q32.1, a chromosomal locus in which several studies have reported genetic linkage with SZ (Jang et al., 2007; Ryu et al., 2013).

TOX is an evolutionarily conserved member of the HMG-box family of transcription factors that has been studied mainly in the immune system and hematopoiesis (Aliahmad et al., 2010; Yun et al., 2011). TOX is necessary for the development of some cells of the adaptive and innate immune systems including $CD4^+$ T cells, natural killer cells and lymphoid tissue inducer cells (Aliahmad et al., 2012; Seehus and Kaye, 2015). Interestingly, a high number of studies related dysregulation of immune and inflammatory function with SZ (Lai et al., 2016; Schiavone and Trabace, 2017). Remarkably, inflammation is closely interconnected with oxidative stress, being both a cause and a consequence, and vice versa (Barron et al., 2017). In addition, *Tox* expression in the mouse thymus is calcineurin dependent (Aliahmad et al., 2004); and the calcineurin pathway has been proposed to be implicated in the pathogenesis of SZ (Wada et al., 2017). In neural tissues, *Tox* functions as an important regulator of neural stem cell proliferation and dendritogenesis, and its expression in the brain is also controlled by calcineurin (Artegiani et al., 2015). However, the changes in expression of *EIF2D* and *TOX* that we found in PB were not observed in the prefrontal and cingulated regions analyzed when comparing SZ patients with controls (Supplementary Fig. 6), but these genes might be dysregulated in specific cell types and/or other different brain areas. In line, regionally specific changes in gene expression in SZ have been found in postmortem brain tissue samples (Scarr et al., 2018).

To further assess the predictive value of the 14 selected genes for SZ risks, we analyzed their expression in a second independent cohort of patients and controls. This sample comprised first-episode SZ patients and then, its comparison with the chronic SZ subjects from cohort 1 could also reveal potential PB biomarkers dependent on the disease evolution. In cohort 2, *TOX* expression was also reduced with respect to controls, but it was not significant. In this cohort, we also found significantly altered expression of *EIF2D* between the first-episode SZ patients and control subjects; however, *EIF2D* mRNA levels were reduced in this case. This finding contrasts with the increased *EIF2D* mRNA levels in the chronic patients of cohort 1. The reduction of *EIF2D* expression in patients of cohort 2 was similar in the three time points tested during the first year of disease duration, suggesting that medication probably did not affect *EIF2D* expression. The different results obtained for this gene in the SZ patients of the cohorts 1 and 2 might be explained by the disease state. In line with these findings, we observed a trend toward increased *EIF2D* expression in patients suffering from the disease for more than 10 years compared with patients with less than 10 years of illness. Therefore, the expression level of this gene may be indicative of redox status in PB revealing higher redox dysregulation after several years of disease progression. This result agrees with lower antioxidant response found in SZ patients with longer duration of illness (Zhang et al., 2012). So, *EIF2D* expression may show a stage of disease progression. Future longitudinal studies in the first-episode SZ patients

are needed to address this question together with molecular approaches to identify the underlying mechanism.

One of the limitations of our work is the sample size, but it is comparable to that used in other studies looking for biomarkers in human tissues (Kurian et al., 2011; Chaumette et al., 2018). Then further analysis should be followed up in larger cohorts at different time points and in different postmortem brain regions to compare the results. In addition, we cannot exclude the possibility that medication may have an effect on gene expression levels. It could be, in part, the cause of the differences found between the two cohorts, since the cohort 1 is composed by long term medicated chronic patients and the cohort 2 is composed by naïve patients. Nevertheless, the significant reduction of *EIF2D* expression in cohort 2 was maintained during the first year of the disease, when the patients are already medicated. Moreover, we tried to reduce the effect of this variable in the first discrimination point, excluding the nodes of genes associated with medication. Finally, different approaches have been used to study gene expression (RNA microarray, RT-qPCR and RNA sequencing), which could result in some variability in the final results. Nevertheless, all the techniques are evaluating the differences in gene expression. Also, appropriate controls were chosen to discard any alteration of the techniques and all methods have been normalized to minimize such technical deviations. In conclusion, we can underline the potential implication of protein synthesis regulation, oxidative stress and immune processes in the development and prognosis of SZ, marked by the expression of *EIF2D* and *TOX* genes in our samples. Our results support the use of antioxidants in SZ as an adjunctive treatment to standard antipsychotic medication.

Acknowledgments

This work was supported by Fondo de Investigación Sanitaria, Ministerio de Economía y Competitividad, Spain (PI10/01399, PI13/00447; PI17/00402, co-financed by FEDER) to J. Sanjuan and M.D. Moltó; Generalitat Valenciana PROMETEO Excellence Program, Spain (PROMETEO2016/082) to J Sanjuán. J Gilabert-Juan and N. Sebastiá-Ortega were recipients of research contracts from CIBERSAM, Spain. The RNA samples donated by the Stanley Medical Research Institute Brain Collection were courtesy of Drs. Michael B. Knable, E. Fuller Torrey, Maree J. Webster, and Robert H. Yolken. The authors also thank the collaboration of the staff members of the hospitals.

Conflict of interest

The authors have declared that there are no conflicts of interest in relation to the subject of this study.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbi.2019.05.015.

References

Chen, J., Cao, F., Liu, L., Wang, L., Chen, X., 2015. Genetic studies of schizophrenia: an update. Neurosci. Bull. 31 (1), 87–98.
Modai, S., Shomron, N., 2016. Molecular risk factors for schizophrenia. Trends Mol. Med. 22 (3), 242–253.
Bustamante, M.L., Herrera, L., Gaspar, P.A., Nieto, R., Maturana, A., Villar, M.J., et al., 2017. Shifting the focus toward rare variants in schizophrenia to close the gap from genotype to phenotype. Am. J. Med. Genet. B Neuropsychiatr. Genet. 174 (7), 663–670.
Gladkevich, A., Kauffman, H.F., Korf, J., 2004. Lymphocytes as a neural probe: potential for studying psychiatric disorders. Prog. Neuro-Psychopharmacol. Biol. Psychiatry 28 (3), 559–576.
Marques-Deak, A., Cizza, G., Sternberg, E., 2005. Brain-immune interactions and disease susceptibility. Mol. Psychiatry 10 (3), 239–250.
Glatt, S.J., Everall, I.P., Kremen, W.S., Corbeil, J., Sasik, R., Khanlou, N., et al., 2005. Comparative gene expression analysis of blood and brain provides concurrent validation of SELENBP1 up-regulation in schizophrenia. Proc. Natl. Acad. Sci. U.S.A. 102 (43), 15533–15538.
Sullivan, P.F., Fan, C., Perou, C.M., 2006. Evaluating the comparability of gene expression in blood and brain. Am. J. Med. Genet. B Neuropsychiatr. Genet. 141B (3), 261–268.
Marazziti, D., Catena Dell'osso, M., Baroni, S., Masala, I., Dell'Osso, B., Consoli, G., et al., 2010. Alterations of the dopamine transporter in resting lymphocytes of patients with different psychotic disorders. Psychiatry Res. 175 (1–2), 54–57.
Wojsiat, J., Laskowska-Kaszub, K., Mietelska-Porowska, A., Wojda, U., 2017. Search for Alzheimer's disease biomarkers in blood cells: hypotheses-driven approach. Biomark Med. 11 (10), 917–931.
Tylee, D.S., Kawaguchi, D.M., Glatt, S.J., 2013. On the outside, looking in: a review and evaluation of the comparability of blood and brain "-omes". Am. J. Med. Genet. B Neuropsychiatr. Genet. 162B (7), 595–603.
Hess, J.L., Tylee, D.S., Barve, R., de Jong, S., Ophoff, R.A., Kumarasinghe, N., et al., 2016. Transcriptome-wide mega-analyses reveal joint dysregulation of immunologic genes and transcription regulators in brain and blood in schizophrenia. Schizophr. Res. 176 (2–3), 114–124.
Xu, L., Qi, X., Zhu, C., Wan, L., 2018. Activation of IL-8 and its participation in cancer in schizophrenia patients: new evidence for the autoimmune hypothesis of schizophrenia. Neuropsychiatr. Dis. Treat. 14, 3393–3403.
Lai, C.Y., Scarr, E., Udawela, M., Everall, I., Chen, W.J., Dean, B., 2016. Biomarkers in schizophrenia: a focus on blood based diagnostics and theranostics. World J. Psychiatry 6 (1), 102–117.
Langfelder, P., Horvath, S., 2008. WGCNA: an R package for weighted correlation network analysis. BMC Bioinf. 9, 559.
Merico, D., Isserlin, R., Stueker, O., Emili, A., Bader, G.D., 2010. Enrichment map: a network-based method for gene-set enrichment visualization and interpretation. PLoS One 5 (11), e13984.
Gilabert-Juan, J., Saez, A.R., Lopez-Campos, G., Sebastia-Ortega, N., Gonzalez-Martinez, R., Costa, J., et al., 2015. Semaphorin and plexin gene expression is altered in the prefrontal cortex of schizophrenia patients with and without auditory hallucinations. Psychiatry Res. 229 (3), 850–857.
Dmitriev, S.E., Terenin, I.M., Andreev, D.E., Ivanov, P.A., Dunaevsky, J.E., Merrick, W.C., et al., 2010. GTP-independent tRNA delivery to the ribosomal P-site by a novel eukaryotic translation factor. J. Biol. Chem. 285 (35), 26779–26787.
Wek, R.C., Jiang, H.Y., Anthony, T.G., 2006. Coping with stress: eIF2 kinases and translational control. Biochem. Soc. Trans. 34 (Pt 1), 7–11.
Patel, S., Sharma, D., Kalia, K., Tiwari, V., 2017. Crosstalk between endoplasmic reticulum stress and oxidative stress in schizophrenia: the dawn of new therapeutic approaches. Neurosci. Biobehav. Rev. 83, 589–603.
Flatow, J., Buckley, P., Miller, B.J., 2013. Meta-analysis of oxidative stress in schizophrenia. Biol. Psychiatry 74 (6), 400–409.
Gawryluk, J.W., Wang, J.F., Andreazza, A.C., Shao, L., Young, L.T., 2011. Decreased levels of glutathione, the major brain antioxidant, in post-mortem prefrontal cortex from patients with psychiatric disorders. Int. J. Neuropsychopharmacol. 14 (1), 123–130.
Schleich, S., Strassburger, K., Janiesch, P.C., Koledachkina, T., Miller, K.K., Haneke, K., et al., 2014. DENR-MCT-1 promotes translation re-initiation downstream of uORFs to control tissue growth. Nature 512 (7513), 208–212.
Weisser, M., Schafer, T., Leibundgut, M., Bohringer, D., Aylett, C.H.S., Ban, N., 2017. Structural and functional insights into human re-initiation complexes. Mol Cell 67 (3) 447–56 e7.
Haas, M.A., Ngo, L., Li, S.S., Schleich, S., Qu, Z., Vanyai, H.K., et al., 2016. De Novo mutations in DENR disrupt neuronal development and link congenital neurological disorders to faulty mRNA translation re-initiation. Cell Rep. 15 (10), 2251–2265.
Santini, E., Huynh, T.N., MacAskill, A.F., Carter, A.G., Pierre, P., Ruggero, D., et al., 2013. Exaggerated translation causes synaptic and behavioural aberrations associated with autism. Nature 493 (7432), 411–415.
Jang, Y.L., Kim, J.W., Lee, Y.S., Park, D.Y., Cho, E.Y., Jeun, H.O., et al., 2007. Linkage of schizophrenia with chromosome 1q32 in Korean multiplex families. Am. J. Med. Genet. B Neuropsychiatr. Genet. 144B (3), 279–284.
Ryu, S., Won, H.H., Oh, S., Kim, J.W., Park, T., Cho, E.Y., et al., 2013. Genome-wide linkage scan of quantitative traits representing symptom dimensions in multiplex schizophrenia families. Psychiatry Res. 210 (3), 756–760.
Aliahmad, P., de la Torre, B., Kaye, J., 2010. Shared dependence on the DNA-binding factor TOX for the development of lymphoid tissue-inducer cell and NK cell lineages. Nat. Immunol. 11 (10), 945–952.
Yun, S., Lee, S.H., Yoon, S.R., Kim, M.S., Piao, Z.H., Myung, P.K., et al., 2011. TOX regulates the differentiation of human natural killer cells from hematopoietic stem cells in vitro. Immunol. Lett. 136 (1), 29–36.
Aliahmad, P., Seksenyan, A., Kaye, J., 2012. The many roles of TOX in the immune system. Curr. Opin. Immunol. 24 (2), 173–177.
Seehus, C.R., Kaye, J., 2015. The role of TOX in the development of innate lymphoid cells. Mediators Inflamm. 2015, 243868.
Schiavone, S., Trabace, L., 2017. Inflammation, stress response, and redox dysregulation biomarkers: clinical outcomes and pharmacological implications for psychosis. Front. Psychiatry 8, 203.
Barron, H., Hafizi, S., Mizrahi, R., 2017. Towards an integrated view of early molecular changes underlying vulnerability to social stress in psychosis. Mod. Trends Pharmacopsychiatry 31, 96–106.
Aliahmad, P., O'Flaherty, E., Han, P., Goularte, O.D., Wilkinson, B., Satake, M., et al., 2004. TOX provides a link between calcineurin activation and CD8 lineage commitment. J. Exp. Med. 199 (8), 1089–1099.
Wada, A., Kunii, Y., Matsumoto, J., Hino, M., Yang, Q., Niwa, S.I., et al., 2017. Prominent increased calcineurin immunoreactivity in the superior temporal gyrus in schizophrenia: a postmortem study. Psychiatry Res. 247, 79–83.
Artegiani, B., de Jesus Domingues, A.M., Bragado Alonso, S., Brandl, E., Massalini, S.,

Dahl, A., et al., 2015. Tox: a multifunctional transcription factor and novel regulator of mammalian corticogenesis. EMBO J. 34 (7), 896–910.
Scarr, E., Udawela, M., Dean, B., 2018. Changed frontal pole gene expression suggest altered interplay between neurotransmitter, developmental, and inflammatory pathways in schizophrenia. NPJ Schizophr. 4 (1), 4.
Zhang, X.Y., Chen, D.C., Xiu, M.H., Tang, W., Zhang, F., Liu, L., et al., 2012. Plasma total antioxidant status and cognitive impairments in schizophrenia. Schizophr. Res. 139 (1–3), 66–72.
Chaumette, B., Kebir, O., Pouch, J., Ducos, B., Selimi, F., ICAAR Study Group, et al., 2018. Longitudinal analyses of blood transcriptome during conversion to psychosis. Schizophr. Bull.
Kurian, S.M., Le-Niculescu, H., Patel, S.D., Bertram, D., Davis, J., Dike, C., et al., 2011. Identification of blood biomarkers for psychosis using convergent functional genomics. Mol. Psychiatry 16 (1), 37–58.

frontiers in Molecular Neuroscience

ORIGINAL RESEARCH
published: 06 March 2018
doi: 10.3389/fnmol.2018.00073

Blood Gene Expression Profile Predicts Response to Antipsychotics

Jesus Sainz[1], Carlos Prieto[2], Fulgencio Ruso-Julve[3] and Benedicto Crespo-Facorro[3,4]**

[1]Spanish National Research Council (CSIC), Institute of Biomedicine and Biotechnology of Cantabria (IBBTEC), Santander, Spain, [2]Bioinformatics Service, Nucleus, University of Salamanca (USAL), Salamanca, Spain, [3]School of Medicine, Department of Psychiatry, University Hospital Marqués de Valdecilla, IDIVAL, University of Cantabria, Santander, Spain, [4]Centro Investigación Biomédica en Red Salud Mental, CIBERSAM, Santander, Spain

Antipsychotic drugs are one of the largest types of prescribed drugs and have large inter-individual differences in efficacy, but there is no methodology to predict their clinical effect. Here we show a four-gene blood expression profile to predict the response to antipsychotics in schizophrenia patients before treatment. We sequenced total mRNA from blood samples of antipsychotic naïve patients who, after 3 months of treatment, were in the top 40% with the best response (15 patients) and in the bottom 40% with the worst response (15 patients) according to the Brief Psychiatric Rating Scale (BPRS). We characterized the transcriptome before treatment of these 30 patients and found 130 genes with significant differential expression (P_{adj} value < 0.01) associated with clinical response. Then, we used Random Forests, an ensemble learning method for classification and regression, to obtain a list of predictor genes. The expression of four genes can predict the response to antipsychotic medication with a cross-validation accuracy estimation of 0.83 and an area under the curve of 0.97 using a logistic regression. We anticipate that this approach is a gateway to select the specific antipsychotic that will produce the best response to treatment for each specific patient.

Keywords: psychosis, schizophrenia, gene expression profiling, clinical response, prediction test

OPEN ACCESS

Edited by:
Detlev Boison,
Legacy Health, United States

Reviewed by:
Gianluca Serafini,
Ospedale San Martino (IRCCS), Italy
Jitao Li,
Peking University Sixth Hospital, China

****Correspondence:***
Jesus Sainz
sainzjv@unican.es
Benedicto Crespo-Facorro
benedicto.crespo@unican.es

Received: *05 December 2017*
Accepted: *21 February 2018*
Published: *06 March 2018*

Citation:
Sainz J, Prieto C, Ruso-Julve F and Crespo-Facorro B (2018) Blood Gene Expression Profile Predicts Response to Antipsychotics. Front. Mol. Neurosci. 11:73. doi: 10.3389/fnmol.2018.00073

INTRODUCTION

Antipsychotic medications are the mainstay of schizophrenia treatment (Insel, 2010). They are also used for several other clinical conditions (i.e., other psychoses, bipolar disorder, delirium, depression, personality disorders, dementia and autism; Alexander et al., 2011; Carton et al., 2015; Roberts et al., 2016) and are therefore one of the most widely used and costly types of drugs having experienced a significant increase in overall prescription in recent years (Kantor et al., 2015). Unfortunately, only 55%–60% of first episode patients will have significantly reduced the severity of their psychopathology with adequate doses of antipsychotic drugs (Crespo-Facorro et al., 2007) and 30% of patients will fail to respond to two antipsychotics after adequate trials (Meltzer, 1997; Zhang and Malhotra, 2011; Pouget et al., 2014). Research to find predictors of the response to antipsychotic treatment is an old field of psychiatry; however, despite decades of research to find clinical biomarkers, there is not a useful molecular test available to predict the response to treatment (Prata et al., 2014). In a previous study (Crespo-Facorro et al., 2015), we analyzed the blood transcriptome of 22 schizophrenia patients before and after medication with atypical antipsychotics and we found that 17 genes had significantly altered expression after medication (P value adjusted < 0.05). With the goal of generating an expression profile that could predict the outcome of treatment, we characterized the transcriptome of a drug-naïve (before any dose of

TABLE 1 | Sociodemographic and clinical characteristics of study individuals.

Characteristics	Total (n = 30)		Best-Responders (n = 15)		Worst-Responders (n = 15)	
	Mean	SD	Mean	SD	Mean	SD
Age at admission (years)	29.6	10.5	29.3	11.7	29.9	9.6
Age at psychosis onset (years)	28.6	10.4	28.7	11.7	28.5	9.4
Duration of untreated psychosis (months)	16.4	23.8	14.9	25.5	18.0	22.9
Duration of untreated illness (months)	12.0	17.8	7.9	10.9	16.1	22.3
BPRS at admission	74.1	15.4	83.5	8.9	64.8	15.0
BPRS at 3 months of treatment	34.6	11.8	29.7	5.2	39.5	14.4

antipsychotic medication was taken) cohort of 37 first episode schizophrenia patients. Patients were then divided into two groups according to their response after 3 months of antipsychotic treatment according to their Brief Psychiatric Rating Scale (BPRS; Lukoff et al., 1986) total scores: the first group included the top 40% of patients who had the best response to treatment (highest absolute decrease of BPRS score) further referred to as "best-responders", and the other group included the bottom 40% of patients with the worst response (lower absolute decrease of BPRS score) further referred to as "worst-responders" (**Table 1**). The transcriptomes at baseline of both groups were compared using the program Deseq (Anders and Huber, 2010) to define the genes with significant differential expression.

MATERIALS AND METHODS

Study Setting and Subjects

The cohort analyzed in this study was obtained from an ongoing epidemiological and 3-year longitudinal intervention program of first-episode psychosis (PAFIP) conducted at the outpatient clinic and the inpatient unit at the University Hospital Marques de Valdecilla (Cantabria, Spain). Conforming to international standards for research ethics, this study was approved by the Cantabria Ethics Institutional Review Board (IRB). Patients meeting inclusion criteria and their families provided written informed consent to be included in the PAFIP. The biological samples of patients included in the study were provided by the Valdecilla biobank.

All referrals to PAFIP were screened for patients who met the following criteria: (1) 15–60 years old; (2) living in the catchment area (Cantabria); (3) experiencing a first episode of psychosis; (4) having received no prior treatment with antipsychotic medication; (5) DSM-IV criteria for schizophrenia, schizophreniform disorder, schizoaffective disorder, or brief psychotic disorder. Patients were excluded for any of the following reasons: (1) meeting DSM-IV criteria for drug dependence; (2) meeting DSM-IV criteria for mental retardation; and (3) having a history of neurological disease or head injury. Only patients with a history of drug dependence (DSM-IV diagnosis; but not drug abuse) during the last 12 months were excluded of the study. The diagnoses were confirmed using the Structured Clinical Interview for DSM-IV (SCID–I) carried out by an experienced psychiatrist 6 months on from the baseline visit. Our operational definition for a "first episode of psychosis" includes individuals with a non-affective psychosis (meeting the inclusion criteria defined above) who have not previously received antipsychotic treatment regardless the duration of psychosis. The 37 individuals who gave written consent to their participation in the program, who fulfilled inclusion criteria at 6 months, and had mRNA samples at baseline and at 3 months, were included in our analyses.

After informed consent was signed, patients were included in a prospective, randomized, flexible-dose, open-label study (EudraCT number 2013-005399-16). We used a simple randomization procedure. At study intake, all patients were antipsychotic naïve and were randomized to aripiprazole (N = 17), risperidone (N = 20). Initial dose ranges were 5–10 mg/day of aripiprazole and 1–2 mg/day of risperidone. Rapid titration schedule (5-day), until optimal dose was reached, was used as a rule unless severe side effects occurred. Dose range was 5–30 mg/day aripiprazole and 2–6 mg risperidone. The dose and type of antipsychotic medication could be changed, at the physician's discretion, based on clinical efficacy and the profile of side effects during the follow-up period. Initial prescribed medications should be switched to olanzapine (range dose 5–20 mg) due to lack of clinical response at 3 weeks (less than 30% of total BPRS score reduction and CGI $\geq$ 5) or persisting distressing adverse drug reactions. For patients who fail to reach a clinical response after two anti-psychotic agents, clozapine should be started. However, despite this recommendation, clozapine treatment should not be commenced at clinician's discretion.

For the present investigation, drug-naïve first episode patients were divided into two groups based on their clinical response after 3 months of antipsychotic treatment according to their BPRS (Lukoff et al., 1986) total scores: the first group included the top 40% of patients who had the best response to treatment (highest absolute decrease of BPRS score) and are referred to as "best-responders", and the other group included the bottom 40% of patients with the worst response (lower absolute decrease of BPRS score) and are referred to as "worst-responders".

At 3-month follow-up patients were on: Aripiprazole (7 worst-responders; 3 best-responders), Risperidone (5 worst-responders; 8 best-responders), Olanzapine (3 worst-responders; 4 best-responders).

No statistically significant differences between the two groups (best vs. worst clinical response patients) with respect to percentage of concomitant use of antidepressants, antimuscarinics, hypnotics and benzodiazepines at 3 months were observed (all p's $>$ 0.542). After 6 months follow-up

patients were diagnosed as follows, in the group of patients with the best-response to antipsychotic treatment: nine schizophrenia, four schizophreniform disorder, one brief psychotic disorder, and one schizoaffective disorder; in the group of patients with worst-response to antipsychotic treatment: nine schizophrenia, four schizophreniform disorder and two brief psychotic disorder. No significant differences between groups were either found.

Premorbid and Sociodemographic Variables

Premorbid and sociodemographic information was collected from patients (**Table 1**), relatives and previous medical records. Age of onset of psychosis was defined as the age when the emergence of the first continuous psychotic symptom occurred. Duration of untreated psychosis (DUP) was defined as the time from the first psychotic symptom to the initiation of antipsychotic drug treatment. Duration of untreated illness (DUI) was defined as the time from the first unspecific symptoms related to psychosis to the initiation of antipsychotic drug treatment.

Laboratory Assessments

To minimize the effects of diet and technique, blood samples were obtained from fasting subjects from 8:00 to 10:00 a.m. by the same staff, in the same setting. None of the patients had a chronic inflammation or infection, or were taking medication that could influence the results of blood tests.

RNA Extraction

Total RNA was extracted from blood using the Tempus^{TM} Blood RNA Tube and Tempus^{TM} Spin RNA Isolation Kit (Applied Biosystems, Foster City, CA, USA) following the manufacturer protocols. To define expression profiles, a key factor is that the RNA is intact. To select only high-quality RNA, the RNA Integrity Number (RIN) was characterized with a Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) and samples with a RIN of at least 7.2 were selected. The selected samples have RINs that range from 8 to 10 with an average of 9.11.

RNA Next Generation Sequencing

Total RNA was extracted from peripheral whole blood of each individual. The mRNA obtained from blood was sequenced at the Centro Nacional de Análisis Genómico (CNAG) using Illumina HiSeq instruments (San Diego, CA, USA). The mRNA was isolated from the total RNA and was fragmented once transformed into cDNA. Fragments of 300 bp on average were selected to construct the libraries for sequencing. Pair-end sequences of 70 nucleotides for each end were produced.

Alignment of Reads to the Human Genome Reference

Alignment of the reads was performed in an SLURM HPC server running Tophat 2.0.6 with default options (Trapnell et al., 2009). Tophat aligns RNA-Seq reads to genomes using the Bowtie 2.0.2 alignment program (Langmead et al., 2009), and then analyses the mapping results to identify splice junctions between exons.

Differential Expression Statistical Analyses

Bedtools 2.17.0 (multicov option; Quinlan and Hall, 2010) was used to count the amount of reads mapped to each gene. The Reference Sequence (RefSeq) gene coordinates were defined using the RefFlat file from the UCSC Genome Bioinformatics Site (as February 28th, 2014). DESeq 1.4 package (Anders and Huber, 2010), setting up fit-only as fitting method, was used to test for differential expression using gene-count data. Two sided Fisher tests were carried out to identify functional enrichment of biological annotations.

Prediction Method

Gene selection was performed with the implementation of Random Forest method in the Random Forest 4.6-12 package (Breiman, 2001) of R. Expression values of the 130 genes with significantly different expression between the best-responders and worst-responders was used as input with default parameters. Genes with the best Gini were selected for the predictor. It was trained using Logistic regression (Cox, 1958) with the glm function of R 3.2.3 and the calculation of the estimated cross-validation was performed with the cv.glm function of boot package which implements bootstrapping methods (Hinkley, 1988).

RESULTS

Differential Gene Expression Between Best-Responders and Worst-Responders Before Treatment

We found 130 genes with significant differential expression between the best-responders and the worst-responders (P_{adj} value < 0.01; Supplementary Table S1). These genes were significantly enriched for schizophrenia related genes according to the scientific literature in the Gene Reference into Function (GeneRIF) database (Mitchell et al., 2003). We found 14 schizophrenia differential expression genes between the best-responders and the worst-responders (13.4% observed vs. 6.8% expected; Fisher P value = 0.016).

Differential Gene Expression Between the Best-Responders and the Worst-Responders After Treatment

To obtain more information, we sequenced the transcriptome of the patients after 3 months of treatment with antipsychotics. We defined 219 differential expression genes between the best-responders and the worst-responders after 3 months of medication (Supplementary Table S2). These genes were enriched significantly for schizophrenia (21 genes or 11.3% vs. 6.8%; Fisher P value = 0.027). After 3 months of medication with antipsychotics, 6 out of the 14 schizophrenia-annotated genes with differential expression before medication between the best-responders and the worst-responders no longer had differential expression: *VWF*, a glycoprotein with increased levels in plasma of non-medicated patients and in bipolar disorder and schizophrenia compared to control individuals

(Yri et al., 2012); *UGT1A1*, a gene with promoter variations in patients with schizophrenia that result in lower serum bilirubin levels (Vitek et al., 2010); *HMOX1*, an enzyme that has anti-inflammatory properties and mediates the first step of heme catabolism involved in the production of carbon monoxide, a putative neurotransmitter, is over-expressed in transgenic mice with schizophrenia-like features (Song et al., 2012); *IL8* (also known as *CXCL8*), a chemokine with altered expression in the dorsolateral prefrontal cortex of individuals with schizophrenia (Fillman et al., 2013); *NTNG2*, a gene with haplotypes associated with schizophrenia and isoform expression significantly different in schizophrenic and control brains (Aoki-Suzuki et al., 2005); and *PTGDS*, a prostaglandin that acts as a neuromodulator as well as a trophic factor in the central nervous system and was studied as a schizophrenia candidate (Ruano et al., 2007) and with reduced mRNA expression in peripheral blood of bipolar disorder patients compared with healthy control subjects (Munkholm et al., 2015). The differential expression genes between the best-responders and the worst-responders after 3 months of medication indicates that 5 out the 11 genes involved in "drug processing" with differential expression before medication still had differential expression and similar expression profile after medication. These five genes are: *GSTM1*, a glutathione S-transferase involved in the detoxification of electrophilic compounds, such as therapeutic drugs, by conjugation with glutathione, with genetic variations that affect the toxicity and efficacy of certain drugs (Li et al., 2013); *THBS1*, an adhesive glycoprotein that mediates cell-to-cell and cell-to-matrix interactions and related to drug resistance (Rath et al., 2006); *PRAME*, an antigen related to cytotoxic drug sensitivity (Kewitz and Staege, 2013); *GSTT1*, a glutathione S-transferase that functions as a drug metabolizing enzyme (Yri et al., 2012); and *SLC22A16*, a solute carrier reported to be involved in drug response (Aouida et al., 2010).

Differential Gene Expression of the Best-Responders Before and After Treatment and of the Worst-Responders Before and After Treatment

We also compared the transcriptome of the best-responders before and after medication and characterized 176 genes with differential expression (Supplementary Table S3). These genes were also enriched for schizophrenia annotations (9.4% observed vs. 6.7% expected). When we defined the differential expression genes of the worst-responders, before and after medication, we found only 23 genes (Supplementary Table S4) and these were not enriched for schizophrenia annotations (5.2% vs. 6.7%). These data indicate that the individuals that respond worst to treatment have fewer altered genes (a 7.6 fold decrease) in their expression by antipsychotics than the best-responders.

Predictor Test

To generate a predictor test before medication of response to antipsychotics, we analyzed all 130 genes with significantly different expression between the best-responders and the worst-responders using Random Forests, an ensemble learning method for classification and regression, among other tasks, that operates by constructing a multitude of decision trees (Díaz-Uriarte and Alvarez de Andrés, 2006; Koo et al., 2013). Functional analyses of the 30 genes with the highest predictive power (**Figure 1**; Supplementary Table S5) indicated a significant enrichment of genes related to schizophrenia (29.2% observed vs. 6.9% expected; Fisher P value = 0.0009) and bipolar disorder (16.7% vs. 2.6%; Fisher P value = 0.003). In the complete list of 130 genes, the enrichment for schizophrenia genes was smaller (13.4%).

Using logistic regression we defined the area under the curve (*AUC*), representing the combined predictive power of the genes, and a cross-validation estimate of accuracy for the prediction using the first two, three and four genes. We found that the prediction using two genes (*SLC9A3* and *HMOX1*) had an area under the curve of 0.92 and a cross-validation estimate of accuracy of 0.73; with three genes (adding *SLC22A16*) the values were 0.96 and 0.833 respectively; and with four genes (adding *LOC284581*) they were 0.97 and 0.833 respectively (**Figure 2**). These data indicate that a test with the top four or three of the most predictive genes could be an appropriate choice.

DISCUSSION

We found a significantly different number of schizophrenia differential expression genes between the groups of best-responders and worst-responders, suggesting that the response to treatment could be due, at least partially, to the fact

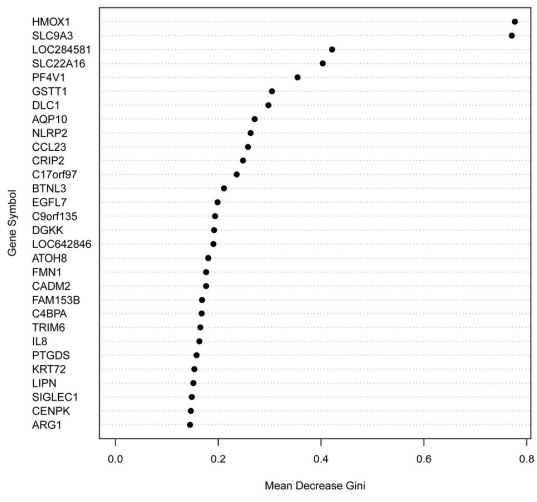


FIGURE 1 | Variable Importance (Gini) for the top 30 predictor genes. Gini variable importance measures reflect the mean decrease in impurity by splits of a given variable in the classification tree, weighted by the proportion of samples reaching that node. A greater "mean decrease Gini" indicates that the gene plays a greater role in partitioning the data into the defined classes.

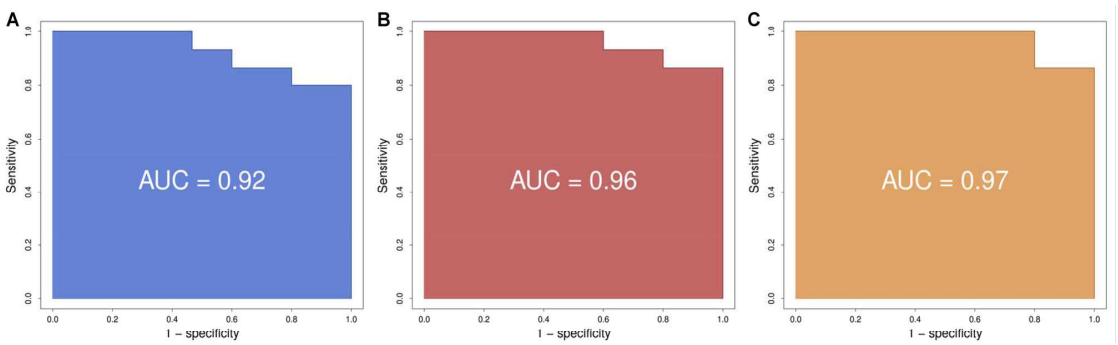


FIGURE 2 | Receiver operating characteristic (ROC) curves. **(A)** Using the best two genes (*HMOX1*, *SCL9A3*) to train the predictor we obtain a ROC with an area under the curve (AUC) of 0.92. **(B)** Using the best three genes (*HMOX1*, *SCL9A3*, *SLC22A16*) to train the predictor we obtain a ROC with an AUC of 0.96. **(C)** Using the best four genes (*HMOX1*, *SCL9A3*, *SLC22A16*, *LOC284581*) to train the predictor we obtain a ROC with an AUC of 0.97.

that the two groups of patients analyzed have different genetic background causing schizophrenia.

Our results indicate that 6 out of the 14 schizophrenia-annotated genes with differential expression before medication between the best and worst-responders had no longer differential expression after 3 months of medication. These six genes are excellent candidates to be the targets used by the drugs to improve the symptoms of schizophrenia. The data show that the group of best-responders has a significant enrichment of schizophrenia-annotated genes with differential expression before and after 3 months of medication. However, we do not observe this significant enrichment of schizophrenia-annotated genes in the group of worst-responders, suggesting that the efficacy of antipsychotics is dependent of the expression profile of the patient before medication and that a predictor could be generated using a gene expression profile of untreated patients.

With the purpose of finding genes that can predict the response to antipsychotics, we characterized 130 genes with differential expression between the best and worst responders before treatment. We found that the 30 genes with the highest predictive power among these 130 genes, had a higher enrichment of schizophrenia-annotated genes, indicating that genes related to schizophrenia tend to have a higher predictive value. This would suggest that schizophrenia genes are involved in the response and that the schizophrenia causative genes tend to be different between the best-responders and the worst-responders.

We tested the predictive power of the top four predictor genes in our patients, and found that the test would accurately predict 93% of the worst-responders and 87% of the best-responders (**Figure 3**). The gene with the highest predictive value, *SLC9A3*, is a Na/H exchanger and belongs to several pathways such as the transmembrane transportation of small molecules and the SLC-mediated transmembrane transport that facilitate the movement of a specific substrate either against or following their concentration gradient. Currently, there are novel drugs being developed targeting *SLC9A3* that could be of interest for the field

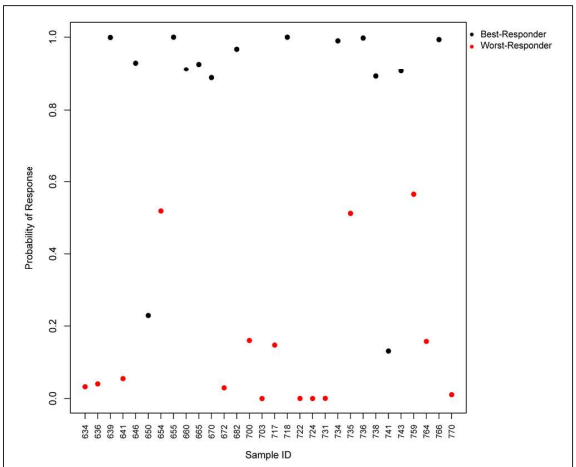


FIGURE 3 | Predicted probability of response to antipsychotics using a 4-gene test. Scatter plot, which represents the predicted probability of response (y-axis) for each input sample (x-axis) in the logistic regression predictor. Red points represent worst-responder patients and black points represent best-responder patients.

of schizophrenia (Dominguez Rieg et al., 2016). The second best predictor is *HMOX1*, or Heme oxygenase, an essential enzyme in heme catabolism, that cleaves heme to form biliverdin, which is subsequently converted to bilirubin by biliverdin reductase, and carbon monoxide, a putative neurotransmitter. The relationship of this gene with the generation of a neurotransmitter could explain the relationship of his expression with the response to antipsychotics and open the gate to possible medical actions. The previous observations are consistent with the fact that the mouse homologous gene has been related to schizophrenia-like features in transgenic mice, what can facilitate new therapeutics for schizophrenia (Song et al., 2012). *HMOX1* has been also related to drug resistance in acute myeloid leukemia (Zhe et al., 2015).

The third predictor, *SLC22A16*, encodes a member of the organic zwitterion transporter protein family which transports carnitine. The encoded protein has also been shown to be involved in the transport of anticancer drugs such as bleomycin (Aouida et al., 2010) and successful treatment has been correlated with the level of activity of this transporter in tumor cells. Variant alleles of SLC22A16 are associated with response and levels of toxicity caused by doxorubicin and cyclophosphamide therapy in the treatment of breast cancer which is consistent with the fact that doxorubicin is a substrate this transporter (Bray et al., 2010). Interestingly, SLC22A16 is located within a schizophrenia susceptibility locus in chromosome 6q (Cao et al., 1997). The fourth gene, *LOC284581*, is not annotated but it maps within a Parkinson's disease locus of 15.8 Mb (Satake et al., 2009). Interestingly, all three annotated genes are involved in drug resistance, toxicity or transportation and offer new possibilities to generate therapeutic actions for schizophrenia.

A potential limitation of the study is the small sample size (n = 30 or n = 15 per group). However, we are not aware of any full transcriptome study on drug-naïve patients (not having received a single dose of antipsychotics) that had studied similar or higher number of samples than our study. Besides, our study sample size is larger than in most studies based on RNASeq data (according to the mean of samples reported by the Gene Expression Omnibus database). A possible confounding factor could be the cannabis use that has been reported to enhance negative outcomes such as suicidal risk (Serafini et al., 2012). However, it is unlikely that this factor could affect the results of our study given that only eight patients reported as cannabis users and were equally divided in both groups, four in the best-responders and four in the worst-responders. The data we are presenting here is a first step to generate a simple test (expression levels of a small number of genes) to predict the response to antipsychotics, one of the most prescribed types of drugs worldwide, and to provide a tool to select the antipsychotic expected to generate the best clinical response.

AUTHOR CONTRIBUTIONS

JS, CP and BC-F designed the study, analyzed the data and wrote the manuscript; JS and BC-F directed and supervised the research; BC-F performed the clinical analysis; CP and JS performed the bioinformatic work; FR-J provided technical assistance and was responsible for sample managing.

FUNDING

This work was supported by the Ministerio de Ciencia e Innovación (MICINN) in a coordinated project (grant SAF2010-20840-C02-01/02) and by the Spanish Ministry of Economy, Industry and Competitiveness (grants SAF2013-46292-R; PTA2015-10483-I); and by the National Institutes of Health (grant 5R01HD056465-07. Sub-award #320793 from The Children's Hospital of Philadelphia). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

ACKNOWLEDGMENTS

Bioinformatics work was partially performed using the Altamira supercomputer (Spanish Supercomputing Network). We thank the Valdecilla Biobank for blood RNA sampling handling and storage and the CNAG for mRNA sequencing. We also wish to thank the participants and their families for enrolling in this study.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnmol.2018.00073/full#supplementary-material

REFERENCES

Alexander, G. C., Gallagher, S. A., Mascola, A., Moloney, R. M., and Stafford, R. S. (2011). Increasing off-label use of antipsychotic medications in the United States, 1995–2008. *Pharmacoepidemiol. Drug Saf.* 20, 177–184. doi: 10.1002/pds.2082

Anders, S., and Huber, W. (2010). Differential expression analysis for sequence count data. *Genome Biol.* 11:R106. doi: 10.1186/gb-2010-11-10-r106

Aoki-Suzuki, M., Yamada, K., Meerabux, J., Iwayama-Shigeno, Y., Ohba, H., Iwamoto, K., et al. (2005). A family-based association study and gene expression analyses of netrin-G1 and -G2 genes in schizophrenia. *Biol. Psychiatry* 57, 382–393. doi: 10.1016/j.biopsych.2004.11.022

Aouida, M., Poulin, R., and Ramotar, D. (2010). The human carnitine transporter SLC22A16 mediates high affinity uptake of the anticancer polyamine analogue bleomycin-A5. *J. Biol. Chem.* 285, 6275–6284. doi: 10.1074/jbc.M109.046151

Bray, J., Sludden, J., Griffin, M. J., Cole, M., Verrill, M., Jamieson, D., et al. (2010). Influence of pharmacogenetics on response and toxicity in breast cancer patients treated with doxorubicin and cyclophosphamide. *Br. J. Cancer* 102, 1003–1009. doi: 10.1038/sj.bjc.6605587

Breiman, L. (2001). Random forests. *Mach. Learn.* 45, 5–32. doi: 10.1023/A:1010933404324

Cao, Q., Martinez, M., Zhang, J., Sanders, A. R., Badner, J. A., Cravchik, A., et al. (1997). Suggestive evidence for a schizophrenia susceptibility locus on chromosome 6q and a confirmation in an independent series of pedigrees. *Genomics* 43, 1–8. doi: 10.1006/geno.1997.4815

Carton, L., Cottencin, O., Lapeyre-Mestre, M., Geoffroy, P. A., Favre, J., Simon, N., et al. (2015). Off-label prescribing of antipsychotics in adults, children and elderly individuals: a systematic review of recent prescription trends. *Curr. Pharm. Des.* 21, 3280–3297. doi: 10.2174/1381612821666150619092903

Cox, D. R. (1958). The regression-analysis of binary sequences. *J. R. Stat. Soc. B Stat. Methodol.* 20, 215–242.

Crespo-Facorro, B., Pelayo-Terán, J. M., Pérez-Iglesias, R., Ramírez-Bonilla, M., Martínez-García, O., Pardo-García, G., et al. (2007). Predictors of acute treatment response in patients with a first episode of non-affective psychosis: sociodemographics, premorbid and clinical variables. *J. Psychiatr. Res.* 41, 659–666. doi: 10.1016/j.jpsychires.2006.05.002

Crespo-Facorro, B., Prieto, C., and Sainz, J. (2015). Schizophrenia gene expression profile reverted to normal levels by antipsychotics. *Int. J. Neuropsychopharmacol.* 18:pyu066. doi: 10.1093/ijnp/pyu066

Díaz-Uriarte, R., and Alvarez de Andrés, S. (2006). Gene selection and classification of microarray data using random forest. *BMC Bioinformatics* 7:3. doi: 10.1186/1471-2105-7-3

Dominguez Rieg, J. A., de la Mora Chavez, S., and Rieg, T. (2016). Novel developments in differentiating the role of renal and intestinal sodium hydrogen exchanger 3. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 311, R1186–R1191. doi: 10.1152/ajpregu.00372.2016

Fillman, S. G., Cloonan, N., Catts, V. S., Miller, L. C., Wong, J., McCrossin, T., et al. (2013). Increased inflammatory markers identified in the dorsolateral prefrontal cortex of individuals with schizophrenia. *Mol. Psychiatry* 18, 206–214. doi: 10.1038/mp.2012.110

Hinkley, D. V. (1988). Bootstrap methods. *J. R. Stat. Soc. B Methodol.* 50, 321–337.

Insel, T. R. (2010). Rethinking schizophrenia. *Nature* 468, 187–193. doi: 10.1038/nature09552

Kantor, E. D., Rehm, C. D., Haas, J. S., Chan, A. T., and Giovannucci, E. L. (2015). Trends in prescription drug use among adults in the united states from 1999–2012. *JAMA* 314, 1818–1831. doi: 10.1001/jama.2015.13766

Kewitz, S., and Staege, M. S. (2013). Knock-down of PRAME increases retinoic acid signaling and cytotoxic drug sensitivity of Hodgkin lymphoma cells. *PLoS One* 8:e55897. doi: 10.1371/journal.pone.0055897

Koo, C. L., Liew, M. J., Mohamad, M. S., and Salleh, A. H. (2013). A review for detecting gene-gene interactions using machine learning methods in genetic epidemiology. *Biomed Res. Int.* 2013:432375. doi: 10.1155/2013/432375

Langmead, B., Trapnell, C., Pop, M., and Salzberg, S. L. (2009). Ultrafast and memory-efficient alignment of short DNA sequences to the human genome. *Genome Biol.* 10:R25. doi: 10.1186/gb-2009-10-3-r25

Li, C., Long, J., Hu, X., and Zhou, Y. (2013). GSTM1 and GSTT1 genetic polymorphisms and risk of anti-tuberculosis drug-induced hepatotoxicity: an updated meta-analysis. *Eur. J. Clin. Microbiol. Infect. Dis.* 32, 859–868. doi: 10.1007/s10096-013-1831-y

Lukoff, D., Liberman, R. P., and Nuechterlein, K. H. (1986). Symptom monitoring in the rehabilitation of schizophrenic patients. *Schizophr. Bull.* 12, 578–602. doi: 10.1093/schbul/12.4.578

Meltzer, H. Y. (1997). Treatment-resistant schizophrenia—the role of clozapine. *Curr. Med. Res. Opin.* 14, 1–20. doi: 10.1185/03007999709113338

Mitchell, J. A., Aronson, A. R., Mork, J. G., Folk, L. C., Humphrey, S. M., and Ward, J. M. (2003). Gene indexing: characterization and analysis of NLM's GeneRIFs. *AMIA Annu. Symp. Proc.* 2003, 460–464.

Munkholm, K., Peijs, L., Kessing, L. V., and Vinberg, M. (2015). Reduced mRNA expression of PTGDS in peripheral blood mononuclear cells of rapid-cycling bipolar disorder patients compared with healthy control subjects. *Int. J. Neuropsychopharmacol.* 18:pyu101. doi: 10.1093/ijnp/pyu101

Pouget, J. G., Shams, T. A., Tiwari, A. K., and Muller, D. J. (2014). Pharmacogenetics and outcome with antipsychotic drugs. *Dialogues Clin. Neurosci.* 16, 555–566.

Prata, D., Mechelli, A., and Kapur, S. (2014). Clinically meaningful biomarkers for psychosis: a systematic and quantitative review. *Neurosci. Biobehav. Rev.* 45, 134–141. doi: 10.1016/j.neubiorev.2014.05.010

Quinlan, A. R., and Hall, I. M. (2010). BEDTools: a flexible suite of utilities for comparing genomic features. *Bioinformatics* 26, 841–842. doi: 10.1093/bioinformatics/btq033

Rath, G. M., Schneider, C., Dedieu, S., Rothhut, B., Soula-Rothhut, M., Ghoneim, C., et al. (2006). The C-terminal CD47/IAP-binding domain of thrombospondin-1 prevents camptothecin- and doxorubicin-induced apoptosis in human thyroid carcinoma cells. *Biochim. Biophys. Acta* 1763, 1125–1134. doi: 10.1016/j.bbamcr.2006.08.001

Roberts, R. J., Lohano, K. K., and El-Mallakh, R. S. (2016). Antipsychotics as antidepressants. *Asia Pac. Psychiatry* 8, 179–188. doi: 10.1111/appy.12186

Ruano, D., Macedo, A., Soares, M. J., Valente, J., Azevedo, M. H., Pato, C., et al. (2007). Family-based and case-control studies reveal no association of lipocalin-type prostaglandin D2 synthase with schizophrenia. *Am. J. Med. Genet. B Neuropsychiatr. Genet.* 144B, 642–646. doi: 10.1002/ajmg.b.30477

Satake, W., Nakabayashi, Y., Mizuta, I., Hirota, Y., Ito, C., Kubo, M., et al. (2009). Genome-wide association study identifies common variants at four loci as genetic risk factors for Parkinson's disease. *Nat. Genet.* 41, 1303–1307. doi: 10.1038/ng.485

Serafini, G., Pompili, M., Innamorati, M., Rihmer, Z., Sher, L., and Girardi, P. (2012). Can cannabis increase the suicide risk in psychosis? A critical review. *Curr. Pharm. Des.* 18, 5165–5187. doi: 10.2174/138161212802884663

Song, W., Zukor, H., Lin, S. H., Hascalovici, J., Liberman, A., Tavitian, A., et al. (2012). Schizophrenia-like features in transgenic mice overexpressing human HO-1 in the astrocytic compartment. *J. Neurosci.* 32, 10841–10853. doi: 10.1523/JNEUROSCI.6469-11.2012

Trapnell, C., Pachter, L., and Salzberg, S. L. (2009). TopHat: discovering splice junctions with RNA-Seq. *Bioinformatics* 25, 1105–1111. doi: 10.1093/bioinformatics/btp120

Vitek, L., Novotna, M., Lenicek, M., Novotny, L., Eberova, J., Petrásek, J., et al. (2010). Serum bilirubin levels and UGT1A1 promoter variations in patients with schizophrenia. *Psychiatry Res.* 178, 449–450. doi: 10.1016/j.psychres.2009.12.008

Yri, O. E., Ekstrøm, P. O., Hilden, V., Gaudernack, G., Liestøl, K., Smeland, E. B., et al. (2012). Polymorphisms in genes encoding interleukin-10 and drug metabolizing enzymes GSTP1, GSTT1, GSTA1 and UGT1A1 influence risk and outcome in Hodgkin lymphoma. *Leuk. Lymphoma* 53, 1934–1944. doi: 10.3109/10428194.2012.682307

Zhang, J. P., and Malhotra, A. K. (2011). Pharmacogenetics and antipsychotics: therapeutic efficacy and side effects prediction. *Expert Opin. Drug Metab. Toxicol.* 7, 9–37. doi: 10.1517/17425255.2011.532787

Zhe, N., Wang, J., Chen, S., Lin, X., Chai, Q., Zhang, Y., et al. (2015). Heme oxygenase-1 plays a crucial role in chemoresistance in acute myeloid leukemia. *Hematology* 20, 384–391. doi: 10.1179/1607845414y.0000000212

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Gracias a Guillaume por dejarme usar su dibujo como cubierta de la Tesis.