



FACULTAD DE CIENCIAS

DEPARTAMENTO DE BIOLOGÍA MOLECULAR

TESIS DOCTORAL

Pathogenic mis-splicing of CPEB4 in schizophrenia

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Madrid, 2023

FACULTAD DE CIENCIAS

DEPARTAMENTO DE BIOLOGÍA MOLECULAR

Memoria de Investigación presentada por

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Graduada en Biología

*Para optar al grado de **Doctora***

*Por la **Universidad Autónoma de Madrid***

Pathogenic mis-splicing of CPEB4 in schizophrenia

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*La presente tesis ha sido realizada en el Centro de Biología Molecular “Severo Ochoa”,
centro mixto CSIC y UAM.*

*El laboratorio del Dr. Lucas también forma parte del Centro de Investigación Biomédica en Red
para Enfermedades Neurodegenerativas (CIBERNED).*



“L’amor che move il sole e l’altre stelle”

Paradiso, XXXIII, v. 145

Dante Alighieri

AKNOWLEDGEMENTS

En primer lugar, me gustaría agradecer a Jose por haberme dado la oportunidad de hacer esta tesis bajo su supervisión, y por haberme acompañado en este periodo de intenso crecimiento científico y personal. Gracias por todo lo que me has enseñado. Cuando llegué, supe que no iba a ser fácil empezar algo nuevo, y aun así me tiré a la piscina, porque sabía que contigo hubiera salido de esta trayectoria mejor de como llegué. Asimismo, quiero dar las gracias a mis compañeros de laboratorio por compartir conmigo logros y fracasos, alegrías y hundimientos. Gracias Parras por haberme enseñado la gran mayoría de técnicas cuando era un patito recién llegado al lab y palabrotas andaluzas (sin que fuera consciente de ello). Gracias Ivó por haberme siempre contagiado con tu humor y con tus constancia y dedicación a la ciencia. Ainara, sabes que eres una de mis personas favoritas en el mundo y mi constante fuente de inspiración en la vida, me has enseñado muchísimo. Sara, en tu caso podría escribir un libro entero, porque has sido como una hermana en estos años. Sabes que eres una de las pocas personas que me ve, muchas veces ni siquiera nos hace falta hablar, ya nos entendemos con una mirada. Gracias por llevarme a la Feria, por nuestro viaje a Tenerife, por nuestra FENS en Paris. A ver cuando vienes a Italia...ahí lo dejo (una vez más). Gracias por compartir conmigo cumpleaños, libros y reflexiones sobre uno mismo, familia, vínculos afectivos y feminismo. Gracias Claudia por haberte convertido en una de las personas de la que más me fio y por nuestras charlas constructivas en coche de vuelta a casa del labo, y Marta por tu risa contagiosa. Gracias a las dos por vuestro apoyo a la hora de cuidar de mi hija perruna durante mis viajes a Italia o de congreso (sé, Marta, que tu foto de perfil de Telegram es todavía con Dina). David, siempre te voy a recordar por sus chistes malos y por no haber querido aprovechar de mi contacto para ir a Italia. Mal, muy mal. Miriam, eres una pieza fundamental de este laboratorio, gracias por inspirarme con tu inteligencia práctica, yo he intentado hacerlo con mi inteligencia emocional, aunque dudes de la existencia de este tipo de inteligencia; gracias por compartir conmigo tus outfits de ceremonias y fiestas. María, nuestra técnica pro, ¡cuántas veces nos has salvado de protocolos imposibles! Las barbacoas anuales en tu casa con Iñi han sido de los pocos eventos sociales de este labo y donde más rico comemos.

Gracias al Profesor Javier Díaz Nido por haberme hecho de tutor a lo largo del doctorado y Andrés, por haber estado cada vez que lo necesitase. Gracias a Claudio Toma por ser excelente colaborador y compañero de viajes en el 714.

Quisiera agradecer el Profesor Jesús Ávila, por haber contestado al correo que le mandé aquel verano de 2017. Era una chica de un pueblo de Italia, llena de sueños, que no hablaba nada de español, pero con muchas ganas de crecer, aprender e investigar. Mi vida ha cambiado para siempre gracias a usted, que reconoció mi potencial y me dió una oportunidad. Después de cinco años, sigo aquí, amo este país, que no me hace sentir tan lejos de casa, y que me ha dado una vida llena y feliz. Asimismo, de mi primer laboratorio español, el 208, quiero agradecer el Profesor Félix Hernández, por haberme dirigido en mi primera instancia y haberme hecho conocer a Jose. Raquel y Esther, hermosas personas, me acogisteis recién llegada y me hicisteis sentir como en casa. Raquel, sigues siendo un punto de refree en tema de historia española, libros y tips para los peores western blots. Gracias a Vega, Nuria, Alberto y Juan por ser parte de mi magnífico entorno. Gracias Alberto por haberme llevado al museo del Prado y contarme la historia de cada cuadro y Juan por nuestras charlas y el tour de Aranjuez en pleno agosto con un calor tremendo. Gracias también a los antiguos miembros del 208, ahora 206. A María Llorens por contarme siempre con entusiasmo su trabajo.

Julia, cuantas cosas hemos compartido: nuestro viaje a Tailandia, nuestra escapada a Alicante con Adri, vuestra boda, tu tesis, solo para mencionar algunas cosas. Y a los demás del grupo Llorens, Miguel, Elena, Bere y Carla, por las charlas en los pasillos para desconectar entre un experimento y el otro y las fiestas de boda.

Del Consorcio PurinesDX, quiero dar las gracias a nuestra coordinadora Isabela, a los IP y a mis compañeros, por todo lo que hemos compartido estudiando el sistema purinergico en las enfermedades neurológicas: Fran, Cris, Caro, Cate, Paula, Lumei, Giorgia, James, Lidia, Linda, Mónica, Antonio y Martina.

Gracias a mi mejor amiga Bea, mi Ferrero Rocher, la primera compañera de piso y amiga española. Eres pilar y refugio, y sabes que, aunque ahora estemos a la distancia, nada cambiará entre las dos. Es maravilloso ver como nuestras vidas han evolucionado, pero nosotras sigamos juntas. La persona que soy es gracias a ti también. ¡Te quiero tanto!

Gracias a mis amigos y compañeros de gym, entre ellos Alex, Sari, Pame, Ire e Kike, por ser grupo deportivo top, por nuestras cañas post-entrenamiento y por ser compañeros de vacaciones en verano.

Grazie famiglia, per essere sempre presente anche se alla distanza. In pochi capiscono cosa significhi non avere i propri cari vicino, ed è molto tempo che vi vedo solo due o tre volte all'anno. Nonna, mamma, papà, Dani ed Eli, grazie per i sacrifici e l'appoggio. Spero che tutto questo ci ripagherà. Grazie a Dina per essere la pet-therapy che mi ha salvata dall'abisso profondo.

Grazie amore mio Guille per essere arrivato all'improvviso e per avermi fatto credere che l'amore esiste. Grazie per le tue braccia, che sono i pilastri della mia nuova casa, per il tuo ottimismo, a ogni costo, per farmi tornare a sognare quando ormai mi ero rassegnata. Grazie per le conchiglie che collezioni per me e per le foto del tramonto al mare ogni sera.

E grazie a me, per aver affrontato, nel bene e nel male, ogni aspetto di questo percorso con dignità, costruendo giorno dopo giorno la migliore versione di me.

Questa tesi è per tutti noi.

I. ABBREVIATIONS.....	15
II. ABSTRACT.....	21
II. RESUMEN.....	25
III. INTRODUCTION.....	29
1. SCHIZOPHRENIA	
1.1 Epidemiology and relevance.....	31
1.2 Symptoms.....	31
1.3 The schizophrenic brain.....	32
1.4 Antipsychotic treatment.....	34
2. COMPLEX AETIOLOGY OF SCHIZOPHRENIA	
2.1 Genetic architecture of SCZ.....	35
2.2 Environmental factors.....	36
2.3 Neurodevelopmental hypothesis of SCZ.....	36
2.4 Mouse models of SCZ.....	38
2.4.1 Pharmacological models.....	38
2.4.2 Genetic models.....	38
2.4.3 Neurodevelopmental models.....	39
3. CYTOPLASMIC POLYADENYLATION ELEMENT BINDING PROTEINS (CPEBS)	
3.1 The family of CPEBs	40
3.2 CPEB-mediated functions.....	41
3.3 CPEB involvement in biological processes.....	43
3.4 CPEBs in the nervous system	44
3.4.1 CPEBs in learning and memory.....	44
3.4.2 CPEBs in neurodegenerative diseases.....	45
3.4.3 CPEBs in glioblastoma.....	46
4. CPEB4 ROLE IN NEURODEVELOPMENTAL DISEASES	
4.1 CPEB4 in Epilepsy	47
4.2 CPEB4 in Autism Spectrum Disorder	47
IV. OBJECTIVES.....	49

V. MATERIALS AND METHODS	53
1. CPEB-related genes enrichment analysis among SCZ risk genes.....	55
2. RNA-seq data analysis.....	55
3. Human brain tissue samples.....	57
4. Mice.....	57
5. RNA isolation and cDNA synthesis.....	60
6. Quantification of CPEB4 transcript splicing and differential splicing analysis.....	60
7. Western Blot.....	60
8. Mouse behaviour tests.....	61
9. Statistical analysis.....	62
VI. RESULTS.....	63
1. Enrichment analysis of CPE-harboured and CPEB4-binder transcripts in SCZ-risk genes.....	65
2. Assessment of CPEB4 splicing in RNA-seq data from individuals with SCZ.....	66
3. Exploration of transcriptomic signatures correlating with CPEB4 microexon inclusion levels.....	67
4. Analysis of CPEB4 splicing in human SCZ post-mortem brains by RT-PCR.....	70
5. Analysis of CPEB4 microexon inclusion in RNA-seq data from cells and model animals treated with antipsychotics.....	72
6. Evaluation of protein levels of CPEB4-related SCZ-risk genes in SCZ-post-mortem brains.....	74
7. Analysis of protein levels of SCZ-risk genes in a mouse model overexpressing the microexon-lacking isoform of CPEB4 (Tg-PN-CPEB4Δ4).....	78
8. Behavioural characterization of Tg-PN-CPEB4Δ4 mice.....	79
VII. DISCUSSION.....	83
VIII. CONCLUSIONS.....	89
VIII. CONCLUSIONES.....	93
IX. REFERENCES.....	97
X. ANNEX I: SUPPLEMENTARY FILES.....	109
XI. ANNEX II: Ph.D. PUBLICATIONS.....	113

I.ABBREVIATIONS

3'UTR: 3'-Untranslated Region

5-HT2: Type 2 Serotonin Receptor

6-OHDA: 6-Hydroxydopamine

ADHD: Attention-Deficit/Hyperactivity Disorder

APDs: Antipsychotic Drugs

ASD: Autism Spectrum Disorder

ASOs: Antisense Oligonucleotides

ATXN3: Ataxin-3

BA: Brodmann Area

BDNF: Brain-Derived Neurotrophic Factor

CAMKII: Ca²⁺/Calmodulin-Dependent Protein Kinase II

cCPE: Canonical CPE

CNV: Copy Number Variant

CPE: Cytoplasmatic Polyadenylation Element

CPEB: Cytoplasmatic Polyadenylation Element Binding Protein

CPEB4FL: Full Length Transcript of CPEB4

CPEB4Δ3: CPEB4 Transcript Lacking of Exon 3

CPEB4Δ3Δ4: CPEB4 Transcript Lacking of Exon 3 And Exon 4

CPEB4Δ4: CPEB4 Transcript Lacking of Exon 4

CX: Cerebral Cortex

DA: Dopamine

DEGs: Differentially Expressed Genes

DISC1: Disrupted-In-Schizophrenia-1

DLPFC: Dorsolateral Prefrontal Cortex

DMSO: Dimethyl Sulfoxide

DOI: 2,5-Dimethoxy-4-Iodoamphetamine

DOPAC: 3,4-Dihydroxyphenylacetic Acid

dST: Dorsal Striatum

E/I: Excitatory/Inhibitory Imbalance

EPS: Extrapyramidal Symptoms

ERBB4: Epidermal Growth Factor Receptor-4

ES: Extrapyramidal System

FC: Fold Change

FMRP: Fragile X Mental Retardation Protein

FXS: Fragile X Syndrome

GABA: γ -Aminobutyric Acid

GAD67: Glutamate Decarboxylase 67

GWAS: Genome-Wide Association Study

HD: Huntington's Disease

Hex: Hexanucleotide

KA: Kainic Acid

KO: Knock Out

LPS: Lipopolysaccharide

MAF: Minor Allele Frequency

MIA: Maternal Immune Activation

MK-801: Dizocilpine

MRI: Magnetic Resonance Image

mRNA: Messenger RNA

mRNP: Messenger Ribonucleoprotein

NGR1: Neurotrophic Factor Neuregulin-1

NMDA: N-methyl-D-aspartate

NPY: Neuropeptide Y

PFC: Prefrontal Cortex

PGC: Psychiatric Genomic Consortium

POLY(I:C): Polyinosinic:Polycytidylic Acid

PPI: Pre-Pulse Inhibition

PSI: Percent Spliced in Index

RIN: RNA Integrity Number

RRM: RNA Recognition Motif

SCA3: Spinocerebellar Ataxia type 3

SCZ: Schizophrenia

SE: Skipped Exon

SNP: Single Nucleotide Polymorphism

SR: Startle Response

SST: Somatostatin

ST: Striatum

Tg-PN-CPEB4Δ4: Mouse Model Overexpressing CPEB4Δ4 Isoform

TLR3: Toll-Like Receptor-3

TLR4: Toll-Like Receptor-4

URV: Ultra-Rare Variant

vST: Ventral Striatum

VTA: Ventral Tegmental Area

WT: Wild-Type

ZF: Zinc Finger

ΔPSI: Difference in PSI

II. ABSTRACT

Schizophrenia (SCZ) is caused by a complex interplay of polygenic risk and environmental factors, which might alter regulators of gene expression leading to pathogenic mis-expression of SCZ risk genes. The RNA binding protein family CPEB (CPEB1, CPEB2, CPEB3, CPEB4) regulates the translation of target RNAs bearing CPE sequences in their 3'UTR (approximately 40% of overall genes). We previously identified CPEB4 as a key dysregulated translational regulator in autism spectrum disorder (ASD), proving that its neuronal-specific microexon (exon 4) is mis-spliced in brains of ASD probands, leading to concerted underexpression of a plethora of high confidence ASD-risk genes. The genetic and pathogenic mechanisms shared between SCZ and ASD make it plausible that mis-splicing of CPEB4 might occur also in SCZ patients, leading to downstream altered brain expression of multiple SCZ-related genes. In this study, we first analysed Psychiatric Genomics Consortium GWAS data and found significant enrichment of SCZ-associated genes for CPEB4-binder transcripts. We also found decreased inclusion of CPEB4 microexon in post-mortem prefrontal cortex of SCZ probands. This mis-splicing is associated with decreased protein levels of SCZ-associated genes that are targets of CPEB4. Interestingly, this happens specifically in individuals with low exposure to antipsychotic medication. Finally, we show that mild overexpression of a CPEB4 transcript lacking exon 4 (CPEB4 Δ 4) in mice suffices to induce decreased protein levels of SCZ genes targeted by CPEB4; these mice are also characterized by SCZ-linked behaviours. In summary, this study identifies aberrant CPEB4 splicing and downstream mis-expression of SCZ-risk genes as a novel etiological mechanism in SCZ.

II. RESUMEN

La esquizofrenia (SCZ) resulta de una interacción compleja de riesgo poligénico y factores ambientales, que, en conjunto, podrían alterar ciertos reguladores de la expresión génica, y, por tanto, inducir una expresión anómala de múltiples genes de riesgo de SCZ. La familia CPEB de proteínas de unión a ARN (CPEB1, CPEB2, CPEB3, CPEB4) regula la traducción de ARNs diana que portan secuencias CPE en su 3'UTR (aproximadamente el 40 % de los genes totales). Anteriormente, en nuestro laboratorio identificamos a CPEB4 como un regulador traduccional desregulado clave en el trastorno del espectro autista (ASD), pues vimos que su microexón neuronal específico (exón 4) está menos incluido en los cerebros de los pacientes con ASD, lo que conduce a una subexpresión concertada de una plétora de genes de riesgo de ASD. Dado que hay muchos mecanismos genéticos y patogénicos compartidos entre SCZ y ASD, pensamos que un splicing incorrecto de CPEB4 también podría estar ocurriendo en pacientes con SCZ, lo cual llevaría a una expresión cerebral alterada de múltiples genes relacionados con SCZ. En el presente estudio, primero analizamos los datos de GWAS del Psychiatric Genomics Consortium y encontramos un enriquecimiento significativo de los genes asociados a SCZ entre los transcritos regulados por CPEB4. También encontramos una disminución de la inclusión del microexón CPEB4 en la corteza prefrontal post-mortem de los probandos SCZ. Este incorrecto splicing se asocia con niveles reducidos de proteína de genes asociados a SCZ que son diana de CPEB4. Curiosamente, esto sucede específicamente en personas con baja exposición a medicación con antipsicóticos. Finalmente, mostramos que una sobreexpresión leve del transcrito de CPEB4 que carece del exón 4 (CPEB4 Δ 4) en ratones es suficiente para inducir una disminución de los niveles de proteína de genes SCZ que son diana de CPEB4; estos ratones también mostraban comportamientos vinculados a SCZ. En resumen, este estudio identifica el splicing aberrante de CPEB4 y la consiguiente expresión anómala de múltiples genes de riesgo SCZ, como un nuevo mecanismo etiológico en SCZ.

III. INTRODUCTION

1. SCHIZOPHRENIA

1.1 Epidemiology and relevance

SCZ is a severe psychiatric disorder affecting one in a hundred people worldwide (McGrath et al., 2008). It usually debuts in early adult life, with the initial manifestation of the psychotic symptoms occurring during the adolescence. Although SCZ has been traditionally considered as equally involving males and females, the most recent meta-analysis report a modestly higher frequency in men (Jongsma et al., 2019). Childhood onset of SCZ is very uncommon, since it affects approximately 1 in 40.000 children, with no relevant sex differences (Driver et al., 2020). The chronic and debilitating outcome of SCZ is responsible for a life expectancy reduced by 13-15 years and a suicide rate of around 5% (Hor & Taylor, 2010). Importantly, beyond their clinical condition, SCZ patients experience stigma, isolation and unemployment. Thus, they require not only medical assistance and hospitalization, but also social and economic support, making SCZ a major socio-economic issue of our society.

1.2 Symptoms

The diagnosis of SCZ is a difficult process, since patients show heterogeneous clinical features. Clinicians historically described SCZ as characterized by positive and negative symptoms, depending on whether they add or reduce/eliminate certain mental phenomena. Delusion, hallucination and paranoia are examples of positive symptoms, while lack of motivation, diminished emotional behaviour and reduced social interaction represent typical negative symptoms. Despite this, many studies (Grube et al., 1998; Shafer & Dazzi, 2019) suggest a tripartite division for SCZ symptoms in: reality distortion (delusion and hallucination), disorganization (disorganized behaviour, disorganized speech, inappropriate affect) and negative symptoms (apathy). It must be remembered that SCZ is also characterized by the impairment of cognitive functions, including sustained attention, working memory, executive function and processing speed (Keefe, 2008) and that it is frequently accompanied by catatonia, an abnormality of movement and behaviour (e.g. repetitive or purposeless movements). Notably, more than half of the patients experience a high-risk state that precedes the transition to SCZ, fixed by the first psychotic episode occurrence, were prodromal symptoms, such as attenuated psychotic, negative and cognitive symptoms, together with anxiety and depression, can be observed (Hafner et al., 1998).

1.3 The schizophrenic brain

More than 300 magnetic resonance image (MRI) studies support the notion that SCZ is characterized by a structural enlargement of the cerebral lateral ventricles. Such expansion is the consequence of a slight reduction in brain volume- mainly of the frontal lobe (Haijma et al., 2013)-, which in turn is linked to decreased grey matter level and aberrant network organization (Kambeitz et al., 2016). From a functional perspective, multiple brain circuits are disrupted in SCZ brains. The negative and cognitive symptoms, for example, have been associated with an imbalanced communication between cortical excitatory and inhibitory neurons (Uhlhaas & Singer, 2010). The neocortical circuits normally show a synchronized activity through neural potential oscillations occurring at high frequency (the γ -oscillations). Such fluctuations in neural activity are the result of the γ -aminobutyric acid (GABA)-ergic interneurons activity, particularly the parvalbumin positive ones, that act as a pacemaker inhibiting the excitatory firing of the glutamatergic pyramidal neurons (Aleksichuk et al., 2016). This connection is reciprocal, thus generating a functional loop modulating neural function. Electrophysiological studies demonstrated that in SCZ such oscillations are disrupted (Sheffield & Barch, 2016), leading to an excitatory/inhibitory imbalance (E/I) (**Figure 1**) (Gao & Penzes, 2015). Indeed, lower dendritic spines on pyramidal neurons, lower levels of parvalbumin messenger RNA (mRNA), and reduced mRNA and protein levels of the glutamate decarboxylase 67 (GAD67), an enzyme involved in GABA synthesis, have been found in SCZ (Lewis et al., 2012).

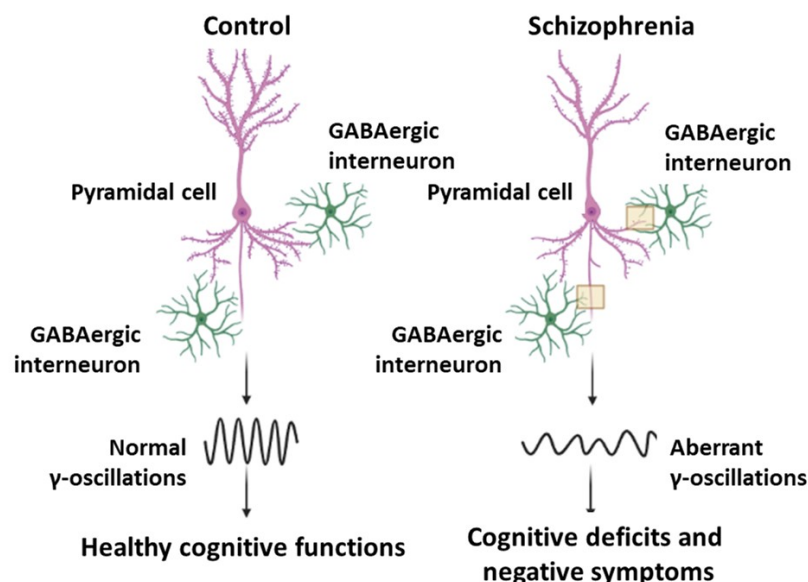


Figure 1: In control, pyramidal cells activity is regulated by GABAergic interneurons through reciprocal interactions, generating the γ -oscillations, which orchestrate many cognitive functions. In SCZ this communication is imbalanced (yellow squares), γ -oscillations result aberrant, leading to cognitive and negative symptoms. Adapted from McCutcheon R.A. *et al.*, "Schizophrenia-An Overview", *JAMA Psychiatry*, 2019.

Negative and cognitive symptoms have also been associated to the hypoactivity of the mesocortical pathway (Brozoski et al., 1979; Goldman-Rakic, 1994)(**Figure 2A**). This circuit includes dopaminergic neurons projecting from the ventral tegmental area (VTA) to the prefrontal cortex (PFC) and is involved in multiple processes (e.g. executive functions, motivation and emotional responses). On the other hand, positive symptoms have been linked to the hyperactivity of the mesostriatal pathway (McCutcheon et al., 2019) (**Figure 2A**). In this case, the dopaminergic neurons of the VTA project to the ventral striatum (vST); their activity follows the exposure to pleasant/reward-related stimuli, as well as to aversive/non-rewarding ones, thus mediating positive reinforcement or aversive behaviours (Schultz et al., 1997). Higher striatal dopamine (DA) synthesis has been detected in individual in prodromal phase (Howes et al., 2009), which gets worse with the beginning of the psychosis (Howes et al., 2011). When the DA signalling is decoupled from a relevant, triggering stimulus, the assignment of salience becomes clueless. This could explain, for example, the effort by the patient to make sense of aberrantly salient experiences, such as delusion or hallucinations, where an aberrant salience is associated to internal representations (Kapur, 2003). SCZ symptoms have also been associated with the disturbance of the serotonergic system (Breier, 1995). Its neurons are located in the Raphe nuclei and reach the cerebral cortex (CX), the striatum (ST) and other limbic regions (Jacobs & Azmitia, 1992) (**Figure 2B**). It is usually involved in mood changes, appetite and sleep regulation (Graeff, 1997). Other than its own functions, the serotonergic system modulates the dopaminergic signalling, as well, both at cortical and striatal level (Kapur & Remington, 1996). Altogether, this findings highlighting a profound interrelation among neurochemical disruptions in SCZ brains.

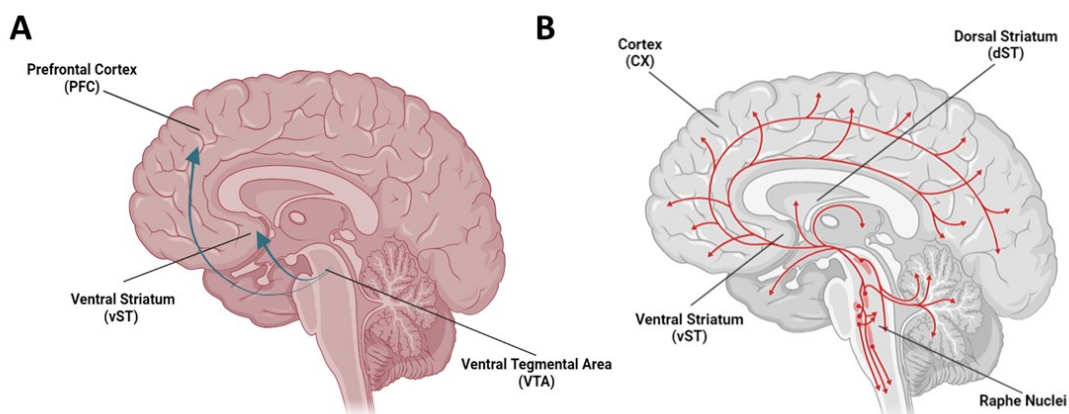


Figure 2: **A)** Dopaminergic mesocortical pathway (from the VTA to the PFC) and mesostriatal pathway (from the VTA to the vST) are represented as green arrows. **B)** Serotonergic system symbolised through red arrows.

1.4 Antipsychotic treatment

Antipsychotic drugs (APDs) for the treatment of SCZ greatly vary in efficacy and side effects, as well as in their mechanism of action. They are classified as typical or atypical depending on their ability to provoke or not extrapyramidal symptoms (EPS), such as parkinsonism, acute dyskinesia and dystonia. Typical APDs, also named “of first generation” for being the first to be discovered, act antagonizing the dopaminergic D₂-receptors of the mesostriatal system. Since, as mentioned above, this pathway is hyperactive in SCZ, the blockage of DA signalling ameliorates the psychosis and the positive symptoms in general. However, D₂-receptors are also expressed in the nigrostriatal pathway, whose dopaminergic neurons project from the *Substantia Nigra* to the dorsal striatum (dST), in order to intervene in postural control, locomotion and complex movements. This motor pathway is also termed as extrapyramidal system (ES), since it does not include the motor tracts coming from the CX and forming the pyramids of the *Medulla Oblongata*. Thus, the DA depletion at this level is responsible for the EPS typical of these APDs (Blair & Dauner, 1992). The side effects, the lack of effectiveness in some patients and the little improvement of the negative and cognitive symptoms of typical APDs gave impulse to the development of new compounds. The atypical APDs, also termed “of second generation” for being introduced in a subsequent period, lack of EPS at therapeutic doses, displaying a relatively weak binding affinity for D₂-receptor. Such APDs are mainly distinguished in two subclasses, although such distinction is not always absolute. One class include drugs with a binding preference for the serotonin type 2 receptors (5-HT₂), over the D₂ ones. This antagonism on the serotonergic system virtually reduce the mesostriatal hyperactivity and compensate the mesocortical hypofunction, ameliorating both positive and negative/cognitive symptoms (Worrel et al., 2000). The second class is represented by drugs that show a binding preference for D₃-dopaminergic receptors, another dopaminergic receptor subtype expressed in the brain, over the D₂ ones, but many of them also have effect on the serotonergic system (Meltzer, 2013). The most recent APDs are designed as “of third generation” and function through a partial agonism or functional selectivity of D₂-receptors (Mailman & Murthy, 2010). Although APDs represent the gold standard treatment for SCZ (and are also used for other mental diseases, including depression, bipolar disorder and ASD), they are still largely unsuccessful against negative and cognitive impairments (Kahn & Keefe, 2013). Moreover, patients response is frequently incomplete: 20-30% of SCZ patients are treatment resistant (Elkis, 2007). Hence, the development of further therapeutic approaches is still required.

2. COMPLEX AETIOLOGY OF SCHIZOPHRENIA

2.1 Genetic architecture of SCZ

Multiple twin, family and adoption studies agree on a heritability estimation at around 80% for SCZ (Sullivan et al., 2003), thus underlying a significant involvement of genetic factors in the aetiology of SCZ. Soon in the 60s, emerged the hypothesis of SCZ as a highly polygenic disease, with more than a single genetic locus contributing to risk (Gottesman & Shields, 1967). However, this was demonstrated only in 2014, when a first large-scale GWAS was published. This study identified multiple common genetic variants with very small individual effect sizes, the single nucleotide polymorphisms (SNPs), in 108 genetic loci in SCZ (Schizophrenia Working Group of the Psychiatric Genomics, 2014). A second (Pardinas et al., 2018) and a third (Trubetskoy et al., 2022) GWAS study managed to cover a progressively larger sample size, expanding the number of imputed loci to 145 and to 287, respectively. A number of publications (Malhotra & Sebat, 2012; Marshall et al., 2017; Purcell et al., 2014; T. Singh et al., 2016) documented the involvement of rare genetic variants, both *de novo* and inherited, including rare SNPs and copy number variants (CNVs), in the aetiology of SCZ. Finally, thanks to exome sequencing studies (Genovese et al., 2016; Singh et al., 2022) ultra-rare variants (URVs) have been related to SCZ, as well (Figure 3).

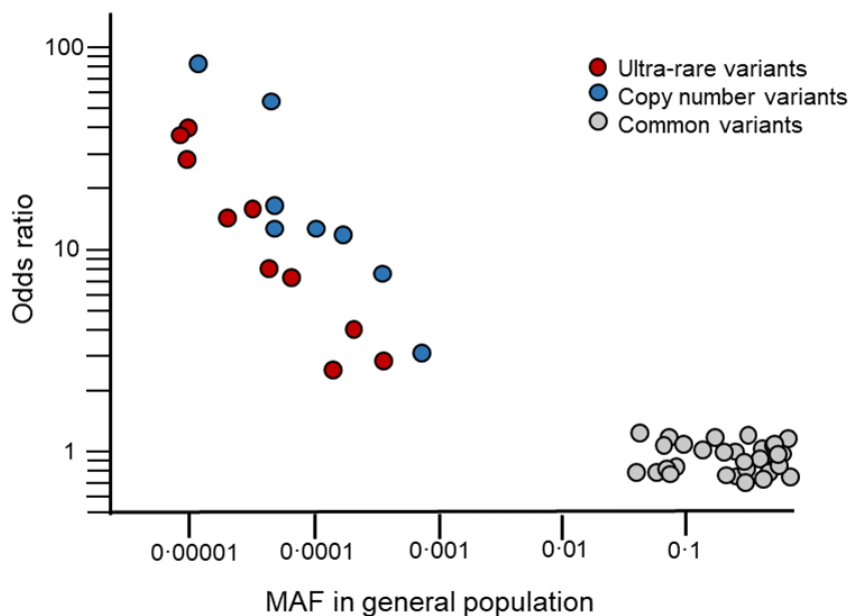


Figure 3: Representation of common (Trubetskoy et al., 2022), rare (Malhotra & Sebat, 2012) and ultra-rare (Singh et al., 2022) variants through their odds ratio (Y-axis, $-\log_{10}$) and minor allele frequency in population (MAF; X-axis, $-\log_{10}$) values, derived from milestone studies. Adapted from Jauhar S. *et al.*, “Schizophrenia”, *Lancet*, 2022.

The gene-set analyses of SCZ risk genes revealed an enrichment in brain-expressed genes, especially the ones involved in brain development and differentiation (including the targets of the fragile X mental retardation protein, FMRP), synaptic plasticity and neurotransmission (e.g. DRD2, the D₂-receptor encoding gene), as well as genes related to immunity (Pocklington et al., 2015; Trubetskoy et al., 2022; Walsh et al., 2008). Remarkably, genes affected by common and rare/ultra-rare variants overlap (Akingbuwa et al., 2022), suggesting convergent biological mechanisms implicated in SCZ origin. Taken together, these studies highlight how genetic factors contribute significantly, but not uniquely to SCZ. Indeed, as it happens in the case of other psychiatric disorders, SCZ is a “complex disease”, where genetic factors, environmental factors and their interplay contribute to the aetiology.

2.2 Environmental factors

The environmental component-and its interaction with the genetic background-contribute to SCZ risk (van Os et al., 2010). A large body of research reports a variety of social stressors as SCZ risk factors: being an immigrant (Cantor-Graae & Selten, 2005), being part of an ethnic minority (Akdeniz et al., 2014), being raised in a urban environment (Krabbendam & van Os, 2005) and having low parental socio-economic status (Byrne et al., 2004). Childhood trauma (e.g. loss of a parent or abuse) (Varese et al., 2012) has been associated to an augmented risk, as well. On the other hand, many biological insults occurring during pregnancy and birth contribute to the risk, including maternal immune activation (MIA) (Brown & Derkits, 2010), maternal starvation (Xu et al., 2009) and stress (Khashan et al., 2008), and obstetric complications (Cannon et al., 2002). Finally, drugs and cannabis abuse in adolescence (Marconi et al., 2016), head injury (Orlovska et al., 2014), epilepsy (Clancy et al., 2014), autoimmune disease and severe infections (Benros et al., 2011) are also considered among SCZ environmental risk factors.

2.3 Neurodevelopmental hypothesis of SCZ

The neurodevelopmental hypothesis for SCZ supports the notion that SCZ aetiology relies on the disturbance of the developmental processes occurring in the brain, especially the synapse formation and connectivity (Birnbaum & Weinberger, 2017; Murray & Lewis, 1987; Weinberger, 1987). More precisely, together with intellectual disability, ASD, attention-deficit/hyperactivity disorder (ADHD), bipolar disorder and epilepsy, SCZ is thought to be part of a neurodevelopmental continuum (Owen & O'Donovan, 2017). Indeed, depending on the timing of the disturbance, different brain developmental processes would be disrupted, eventually producing different pathological outcomes (**Figure 4**).

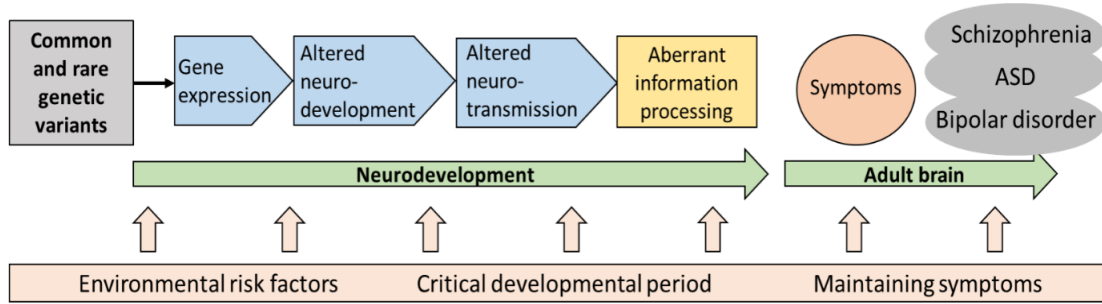


Figure 4: Common and rare genetic variants, other than environmental factors, interfere with neurodevelopment. From Smeland *et al.* “The polygenic architecture of schizophrenia-rethinking pathogenesis and nosology”, *Nature Reviews Neurology*, 2020.

Such hypothesis is supported by various considerations. First, the diagnostic criteria for all these syndromes are approximate, since many clinical features and phenotypes are shared and a significant comorbidity can be observed (Owen & O'Donovan, 2017). Second, several environmental insults associated with SCZ, especially the ones occurring during the neurodevelopment, have also been associated to other neurodevelopmental disorders (Brown, 2012). Third, a genetic overlap exists. A SNPs-based genetic correlation takes place between SCZ and ASD, ADHD and bipolar disorder (Brainstorm *et al.*, 2018; Cross-Disorder Group of the Psychiatric Genomics *et al.*, 2013). This is further supported by the statistical tool named MiXeR (Frei *et al.*, 2019), which estimates the proportion of unique and shared causal variants in compared phenotypes: there is a considerable genetic overlap between SCZ and other psychiatric disorders, including ASD, bipolar disorder and ADHD (**Figure 5**).

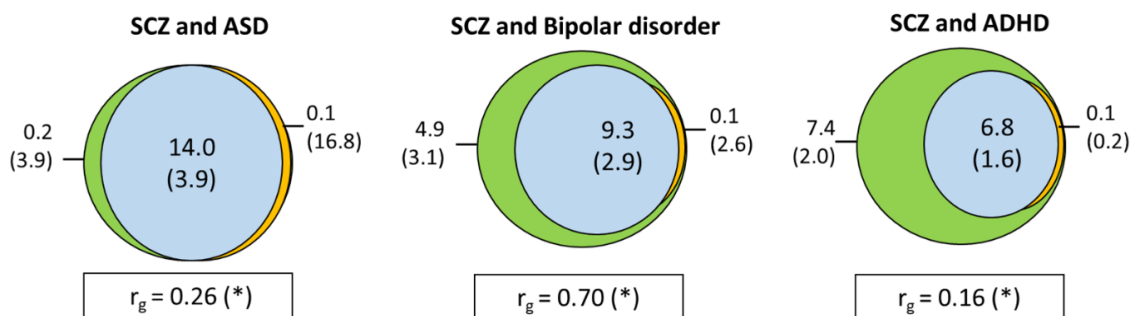


Figure 5: Venn diagrams representing unique (green/yellow) and shared (light blue) polygenic components at the causal level following MiXeR application. The numbers indicate the estimated quantity of causal variants (in thousands) per component, explaining 90% of SNPs heritability in each phenotype, followed by the standard error. The size of the circles mirrors the degree of polygenicity. The estimates of significant (*) and genetic correlation (r_g) are reported. Adapted from Smeland *et al.* “The polygenic architecture of schizophrenia-rethinking pathogenesis and nosology”, *Nature Reviews Neurology*, 2020.

Also, the rare inherited CNVs that confer risk to SCZ are also found in ASD (Stefansson et al., 2014), intellectual disability (Rees et al., 2016), ADHD (Hamshire et al., 2013) and epilepsy (Mefford et al., 2010), and genes affected by rare *de novo* CNVs in SCZ significantly overlap with genes affected by the same class of mutation in ASD and intellectual disability (Deciphering Developmental Disorders, 2017). Finally, there is a convergence between genes hit by common and rare variants (Trubetskoy et al., 2022), and the gene set enrichment analyses reveals that all these genes are functionally related, being implicated in synaptic neurodevelopment and function (Fromer et al., 2014; Tarjinder Singh et al., 2016; Trubetskoy et al., 2022). All together, these findings support the idea of SCZ as part of a spectrum.

2.4 Mouse models of SCZ

Various SCZ mouse models have been generated through a variety of approaches.

2.4.1 Pharmacological models

A number of molecules are administered to mice to alter certain SCZ-related neurotransmitter pathways in order to model SCZ. Amphetamine is used to produce a dopaminergic hyperfunction model (Tenn et al., 2003; Tenn et al., 2005), while the injection of 6-hydroxydopamine (6-OHDA) in animals' PFC represents a dopaminergic hypofunction model (Fernandez Espejo, 2003). On the other hand, 2,5-dimethoxy-4-iodoamphetamine (DOI), acting on the frontal serotonergic functions, induces head-twitch responses, mimicking hallucinations (Canal & Morgan, 2012). Finally, among glutamatergic antagonists there are: phencyclidine (Dutra-Tavares et al., 2021), ketamine (Damazio Pacheco et al., 2019) and dizocilpine (MK-801) (Sawahata et al., 2021). All these mice exhibit alterations in the following behaviours in different combinations: locomotion, memory, social behaviour and pre-pulse inhibition (PPI).

2.4.2 Genetic models

Although the exact contribution of a SCZ risk gene to the development of the illness is uncertain, various SCZ genetic models have been proposed:

- Disrupted-in-Schizophrenia-1 (DISC1) gene encodes for a protein regulating CX development (Kamiya et al., 2005). A chromosomal translocation in DISC1 is associated with a rare familial SCZ, where the truncated protein acts as a dominant-negative (Millar et al., 2000). Mice recapitulating this condition display memory deficits and altered PPI (Segal-Gavish et al., 2017). Mice with DISC1 knock down (Tripathi et al., 2018), *in utero* knock out (KO) (Niwa et al., 2010)

- or with a naturally occurring deletion in the gene (Gomez-Sintes et al., 2014) are other SCZ models.
- The 22q11.2 region contains many brain developmental genes. Interestingly, its deletion in humans is associated with a 25% risk of developing SCZ symptoms (Qin et al., 2020). Mice with such deletion (named as Df(16)A+/- mice) exhibit impairments in synaptic function and plasticity, and increased locomotor activity, disrupted social memory and PPI deficits (Ellegood et al., 2014).
 - Dysbindin-1 protein, encoded by DTNBP1 gene, regulates neurite outgrowth, spine morphology and synaptic activity (Ma et al., 2011). Mutations in DTNBP1 are recognized as risk factors in SCZ pathogenesis. Also, SCZ post-mortem tissue from multiple cerebral areas are characterized by a reduced level of its protein (Weickert et al., 2008). Mice carrying deletion in DTNBP1 (known as *sandy* mice) show impaired social behavior, working memory and fear conditioning processes (Bhardwaj et al., 2009), other than altered dopaminergic, GABAergic and glutamatergic functions (Hattori et al., 2008; Papaleo et al., 2012; Trantham-Davidson & Lavin, 2019).
 - The epidermal growth factor receptor-4 (ERBB4) and its ligand, the neurotrophic factor neuregulin-1 (NGR1), increases susceptibility to SCZ (Norton et al., 2006). Indeed, NGR1 mRNA and protein are diminished in SCZ post-mortem brains (Marballi et al., 2012), and the ligand-receptor signalling is disturbed in patients (Joshi et al., 2014). The viable heterozygous mutant for NGR1 displays an E/I imbalance via reduced presynaptic excitatory terminal on cortical interneurons (Navarro-Gonzalez et al., 2021), similarly to what is observed in the case of ERBB4 deletion (Wang et al., 2018), as well as typical SCZ-like behaviours.

2.4.3 Neurodevelopmental models

Based on the neurodevelopmental hypothesis of SCZ, MIA models have been proposed to mimic SCZ in mice (Estes & McAllister, 2016). Pregnant females are injected with polyinosinic:polycytidylic acid (poly(I:C)) at different points during the gestation, in order to activate the toll-like receptor-3 (TLR3) dependent innate immune system (Haddad et al., 2020). Another MIA model is obtained through maternal immunization via lipopolysaccharide (LPS), which activates the toll-like receptor 4 (TLR4)-dependent innate immune response (Ashdown et al., 2006). The offspring is characterized by increased DA and its metabolite 3,4-dihydroxyphenylacetic acid (DOPAC) concentrations in the PFC and ST (Ozawa et al., 2006). Also, glutamatergic (Rahman et al., 2017), GABAergic (Labouesse et al., 2015) and serotonergic (MacDowell et al., 2021) transmissions are perturbed. Offspring are also characterized by changes in a panel of SCZ-related behaviours (Mueller et al., 2021).

3. CYTOPLASMIC POLYADENYLATION ELEMENT BINDING PROTEINS (CPEBS)

3.1 The family of CPEBs

The cytoplasmatic polyadenylation element binding proteins (CPEBs) are a family of RNA-binding proteins regulating the translation of specific transcripts. CPEBs are widespread in the animal kingdom. In invertebrates, they are present in different numbers: in *Drosophila melanogaster* Orb1 and Orb2 have been described (Lantz et al., 1994), while in *Caenorhabditis elegans* they are called Fog-1 and cpb1-3 (Nousch & Eckmann, 2013), and other have been found in clam (Minshall et al., 1999) and in *Aplysia californica* (Minshall et al., 1999). In vertebrates, the family is composed by four paralogues, CPEB1-4. From zebrafish to human, the orthologues comparison unveils that they are highly conserved across species. Despite this, the across-paralog comparison demonstrated that CPEB2-3-4 are more closely related to each other, while CPEB1 is a distant member of the family (Wang & Cooper, 2010) (**Figure 6**).

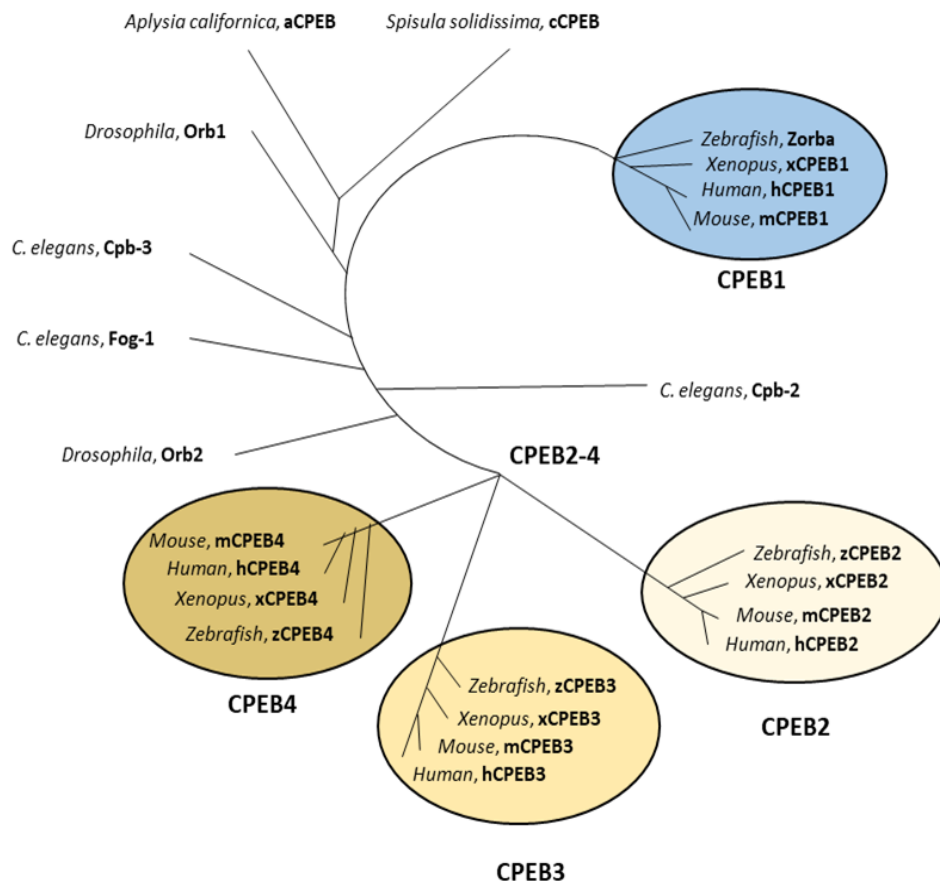


Figure 6: Phylogenetic tree of the CPEB proteins based on a complete protein sequences alignment. In vertebrate, CPEB1 is the most distant member of the family, while CPEB2-4 are closely related. Adapted from Fernández-Miranda and Méndez, "The CPEB-family of proteins, translational control in senescence and cancer", *Ageing Research Reviews*, 2012.

CPEB1-4 have similar protein structures. The carboxy-terminal region, which harbours two RNA recognition motifs (RRMs) and two zinc-fingers-like motifs (ZFs), is highly conserved (Tsuda et al., 2014). On the contrary, the amino-terminal region is highly variable (Fernandez-Miranda & Mendez, 2012); one or more polyglutamine stretches are present in some CPEBs (**Figure 7**). Expression of CPEBs can be detected in multiple tissues, with patterns partially overlapping (Theis et al., 2003; Wang & Cooper, 2010).

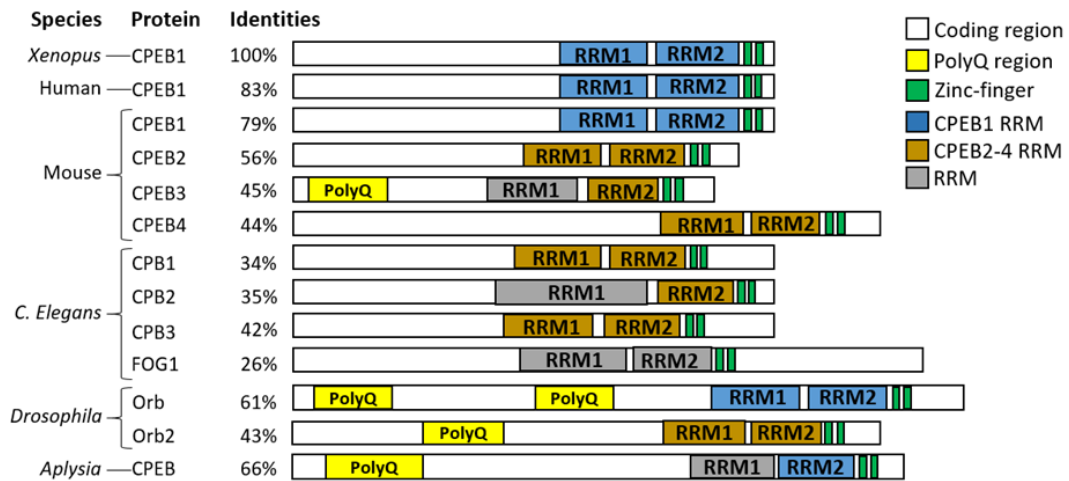


Figure 7: CPEBs protein structures in multiple species. The C-terminus RRM1 and RRM2 are shared among the CPEBs. Polyglutamine rich stretches are indicated as PolyQ. The percent identities refer to comparisons relative to Xenopus CPEB1. Modified from Ivshina *et al.*, “Cytoplasmic polyadenylation element binding proteins in development, health, and disease”, *Annu Rev Cell Dev Biol*, 2014.

3.2 CPEB-mediated functions

In eukaryotes, gene transcription takes place in the nucleus. The enzyme RNA polymerase II, starting from a DNA template, synthesizes an immature mRNA, the pre-mRNA. In the meantime, the conversion of the pre-mRNA to a mature mRNA occurs (Huang et al., 2015). First, the transcript is capped at its 5' end with a 7-methylguanosine cap (Ramanathan et al., 2016). Second, introns are removed and exons are ligated by splicing (Alpert et al., 2017). Interestingly, exons can be joined in different arrangements by alternative splicing: generating multiple transcripts from a single gene, diverse but related proteins can be obtained, something that adds complexity to gene expression (Wang et al., 2015). Third, the 3' end is cleaved and extended with a stretch of adenine residues, the poly(A) tail, by nuclear polyadenylation (Di Giammartino et al., 2011). The poly(A) tail confers stability to the mRNA and guarantees its export from the nucleus. Indeed, the mature mRNA, associated with multiple proteins in a messenger ribonucleoprotein (mRNP) (Bentley, 2014), is exported to the cytoplasm across the nuclear pore complexes (Katahira, 2015). If mRNA quality control fails or a defect in mRNP assembly occurs, the poly(A) tail adenine residues are removed by nuclear deadenylation and the transcript is

decapped, to prevent its export and favour the degradation (Moore, 2002). Otherwise, translation takes place in the cytoplasm, where a number of RNA binding proteins present the mRNA in a circularized conformation, ribosomes are recruited and the polypeptide chain is synthesized (Sonnenberg & Hinnebusch, 2009). In this phase, the poly(A) tail plays a pivotal role in the translational initiation by interacting with the 5' cap for the circular conformation (Weill et al., 2012). mRNA deadenylation also occurs in the cytoplasm, but is not uniquely responsible for the elimination of aberrant or useless transcripts. Rather, deadenylated mRNAs assembled in mRNPs can be stored as stable but translationally silent entities, which are frequently located in specific parts of the cell. A variety of environmental stimuli, both internal and external, can reactivate such mRNAs by cytoplasmic polyadenylation, allowing the synthesis at the precise time and specific place of the needed proteins. Notably, the process is very quick, being exempt from all the previous steps of gene expression (transcription, maturation, export) (Weill et al., 2012).

The CPEBs are involved in cytoplasmic polyadenylation. Around 30-40% of mRNAs harbour the cytoplasmic polyadenylation elements (CPEs), regulatory sequences consisting of UUUUA₁₋₃U in the 3'-untranslated region (3'UTR) of the transcript, which represent the binding sites for the CPEBs (Villalba et al., 2011). Importantly, CPEBs recruitment requires a 3'UTR carrying not only CPEs, but also the conserved hexanucleotide (Hex), a near sequence consisting of AAUAAA or AUUAAA (Pique et al., 2008). Although all CPEBs bind to these elements similarly, they show different affinities. Consequently, even partially sharing their mRNA targets, CPEBs act differently on their spatiotemporal translation patterns (Huang et al., 2006; Igea & Mendez, 2010; Novoa et al., 2010; Ortiz-Zapater et al., 2011; Pavlopoulos et al., 2011). Also, all CPEBs exist in multiple isoforms generated by alternative splicing, which are determined by varying biological features (e.g. activity, tissue specificity etc.) (Johnson et al., 2015; Kaczmarczyk et al., 2016), what increases the complexity of their functions (Theis et al., 2003; Wang & Cooper, 2010). Importantly, the fate of a transcript depends not only on the CPEBs and on the combinatorial arrangement of the regulatory sequences in the 3'UTR of the transcripts, but also on other RBPs and noncoding RNAs, which are part of this machinery (Weill et al., 2012).

Regarding the mechanism by which CPEBs regulate cytoplasmic polyadenylation, they can assemble two kind of complexes. When a CPEB binds to the CPEs of a transcript with a short poly(a) tail, a closed loop conformation is acquired and translation is repressed. Following activation by environmental stimuli, such conformation is lost, polyadenylation is favoured and translation initiation factors recruited, allowing an efficient translation (Pique et al., 2008) (**Figure 8**).

Multiple signals regulate CPEB activity. CPEB1 is controlled by phosphorylation by Aurora kinase A, Cdc2 (Mendez et al., 2002; Mendez et al., 2000) and Ca^{2+} /calmodulin-dependent protein kinase II (CAMKII) (Atkins et al., 2005; Atkins et al., 2004). CPEB3 is regulated by phosphorylation (Kaczmarczyk et al., 2016), SUMOylation (Driscaldi et al., 2015) and monoubiquitination (Pavlopoulos et al., 2011). On the other hand, CPEB4 is phosphorylated by ERK2/Cdk1 (Guillen-Boixet et al., 2016). Finally, CPEBs undergo autoregulation, and they also influence each other's activity: as an example, CPEB1 translationally activates CPEB4 (Igea & Mendez, 2010).

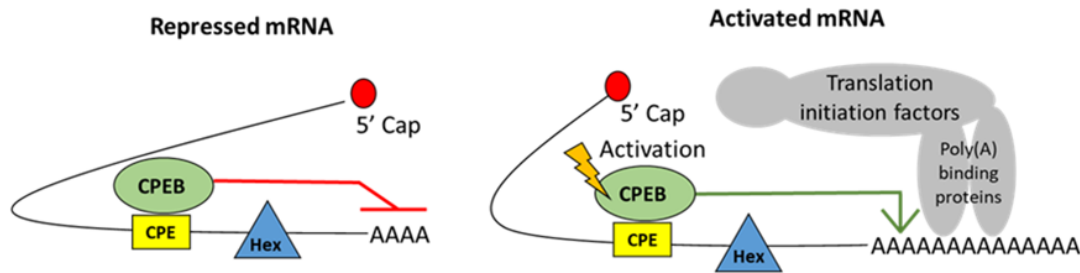


Figure 8: Schematic representation of the two kind of complexes CPEBs can form to regulate mRNA translation. A CPEB proteins is shown as a green circle, CPE and the Hex sequences are indicated as a rectangle and triangle, respectively. CPEB activation by a stimulus is represented as a lightning.

Remarkably, other than cytoplasmatic polyadenylation, CPEBs also exert functions in the nucleus. Indeed, all CPEBs are nucleus-cytoplasm shuttling proteins. Nuclear CPEB1, for example, affects pre-mRNA alternative polyadenylation and alternative splicing (Bava et al., 2013), while CPEB4 becomes nuclear following endoplasmic reticulum stress associated to focal ischemia contributing to reduce programmed cell death (Kan et al., 2010).

3.3 CPEB involvement in biological processes

CPEBs have been implicated in a variety of physiological and pathological pathways:

- **Development:** CPEBs play a role in germ line maturation in multiple species, including *D. melanogaster* (Lantz et al., 1994), *C. elegans* (Kimble & Crittenden, 2007), *X. laevis* (Hake & Richter, 1994) and mammals (Reyes & Ross, 2016). They are also involved in developmental processes of the nervous system in *D. melanogaster* (Hafer et al., 2011), and mouse, with CPEB4 as a main player (Shin et al., 2016; Theis et al., 2003).
- **Metabolism:** CPEB1 KO mice display aberrant glucose metabolism (Alexandrov et al., 2012) and defects in brain mitochondrial ATP production (Oruganty-Das et al., 2012), while CPEB1-deficient human fibroblasts show augmented rates of glycolysis (Burns & Richter, 2008). On

the other hand, CPEB4 is essential for a high-fat-diet adaptation and endoplasmic reticulum stress induced ageing in mice (Maillo et al., 2017).

- **Cell proliferation, senescence and cancer:** CPEBs are implicated in proliferative processes such as meiotic and mitotic divisions (Kim et al., 2011; Novoa et al., 2010). However, they can also induce senescence (Burns et al., 2011; Xiaoping et al., 2013) and have been related to a variety of cancers (Chen et al., 2018; Johnson et al., 2015; Ortiz-Zapater et al., 2011).

3.4 CPEBs in the nervous system

The link between CPEBs and brain functions is clear when exploring their KO mouse models. CPEB1 KO mice show altered long-term potentiation (Alarcon et al., 2004) and the inability to extinguish memories (Berger-Sweeney et al., 2006). Cholinergic neurons from CPEB2 KO mice are faulty (Lai et al., 2016), CPEB3 KO mice hippocampal-dependent short-term memory is enhanced (Chao et al., 2013) and CPEB4 KO pyramidal neurons have elongated dendritic spines (Tsai et al., 2013). Thus, their functions in the nervous system have been explored in depth.

3.4.1 CPEBs in learning and memory

Following stimulation, synapses change morphologically and biochemically. This is known as synaptic plasticity and is a mechanism underlying learning and memory (Kandel, 2001; Mayford et al., 2012; Richter & Klann, 2009; Sutton & Schuman, 2006). The modification of the synapses is driven by the synthesis of specific proteins in dendrites. Indeed, the corresponding mRNAs are allocated and accumulated at this level in a dormant form as mRNPs and their translation is triggered by the activation of the synapse (Kang & Schuman, 1996). Proteins are therefore quickly produced where and when they are needed. CPEBs participate in the transport of the required mRNAs along microtubules, in their localization in dendrites, and, following synapse activation, in the positive regulation of their translation by cytoplasmatic polyadenylation (Huang et al., 2006; Kozlov et al., 2021; Pavlopoulos et al., 2011; Wu et al., 1998). It has been demonstrated that CPEB1 binds to kinesin and dynein to allow the transport of particular mRNAs to the dendrites (Huang et al., 2003). As an example, CPEB1 mediates the transport of the transcript of the brain-derived neurotrophic factor (BDNF), one of the components of tagged synapses and essential player in long-term memory (Oe & Yoneda, 2010). Once in dendrites, CPEB1-containing mRNPs and other components of the cytoplasmatic polyadenylation machinery are accumulated as large complexes to be ready for usage (Udagawa et al., 2012; Wu et al., 1998). One of the mRNA undergoing activity-dependent CPEB1-mediated polyadenylation is the one encoding NR2A, one of the subunits of the N-methyl-D-aspartate (NMDA) glutamatergic receptor, which is essential for synaptic plasticity (Swanger et al., 2016).

However, CPEB1 interacts with many other transcripts, involved in long-term potentiation, memory formation and synapse morphogenesis (Ohashi & Shiina, 2020). Similarly, it has been demonstrated that CPEB3 is able to interact with actin cytoskeleton (Stephan et al., 2015). It behaves as monomer when located in P-bodies, together with particular mRNA targets whose translation is therefore kept silent (Drisaldi et al., 2015). This is the case of the mRNAs for NR1, NR2A and NR2B subunits of the NMDA receptors, and PSD95, a scaffolding protein implicated in synaptic plasticity (Chao et al., 2013). Following neuronal stimulation, CPEB3 aggregates and the targeted mRNAs are translated (Fioriti et al., 2015). Another point is that the long-term retention of the memory must overcome the timing of protein decay. In this regard, a prion theory of memory has been proposed (Tomba & Friedrich, 1998): prions involved in memory have a non-toxic conformation and can renew themselves indefinitely transforming the newly synthesized proteins into prions. Remarkably, CPEBs emerged as regulator of synaptic tagging behaving as prions. Indeed, *Aplysia californica* CPEB (Si et al., 2010), *Drosophila melanogaster* Orb2 (Majumdar et al., 2012) and mammalian CPEB3 (Fioriti et al., 2015) contain a prion-like domain and form amyloid-like aggregates that would act as prions.

3.4.2 CPEBs in neurodegenerative diseases

A large body of evidence supports the implication of CPEBs in neurodegenerative pathologies. One of the most common types of inherited ataxias, the spinocerebellar ataxia type 3 (SCA3), is caused by the expansion of a CAG triplet repeats in the ataxin-3 (ATXN3) gene (Ikeda et al., 1996). A *Drosophila* SCA3 model, characterized by abnormal wing posture, locomotor defects and retinal degeneration, is rescued by overexpressing Orb2 (Bilen & Bonini, 2007; Shieh & Bonini, 2011). On the other hand, various studies support the link between CPEBs and Huntington's disease (HD), another inherited neurodegenerative disorder caused by an expanded CAG triplet affecting the basal ganglia and leading to motor and cognitive impairments (Bates et al., 2015). A first evidence emerged from the comparison of the polysome-associated mRNAs in wild-type (WT) and CPEB1 KO mice embryonic fibroblasts: the mistranslations affects HD-related genes only in the absence of CPEB1 (Alexandrov et al., 2012). Later, alterations in CPEBs have been reported in patients and mouse models with HD: the protein levels of CPEB1 are augmented, while the ones of CPEB4 decreased (Pico et al., 2021). These changes in CPEBs correlate with altered polyadenylation of transcripts related not only to HD, but also to Alzheimer's disease and Parkinson's disease. Moreover, the overexpression of CPEB4 levels in R6/1 mice attenuates many behavioural features of this HD mouse model (Pico et al., 2021). Altogether, these results not only demonstrate a role of CPEBs in HD, but also suggests their possible implication in many other neurodegenerative conditions.

3.4.3 CPEBs in glioblastoma

Glioblastoma multiform, one of the most common brain tumors, is characterized by altered protein levels of CPEBs. In particular, CPEB1 is decreased. Coherently, experiments in glioblastoma cell cultures demonstrated that CPEB1 overexpression drastically reduce tumor cell proliferation (Galardi et al., 2016). On the contrary, CPEB4 protein levels are increased and a reduction of CPEB4 levels diminishes tumor size, proliferation rate and micro vessel density (Ortiz-Zapater et al., 2011).

4. CPEB4 ROLE IN NEURODEVELOPMENTAL DISEASES

The notion that CPEBs play a role of in neurodevelopmental disorders is nowadays commonly accepted. One of the first studies to be published in this regard reports that a mouse expressing a truncated version of CPEB4 protein lacking the RRM and the ZFs and consisting only of the unstructured low complexity N-terminal domain exhibits an altered neuronal development, with reduced motor axon branching and abnormal neuro-muscular formation (Shin et al., 2016). However, more recent publications directly relate physiological CPEB4 isoforms to specific neurodevelopmental disorders.

4.1 CPEB4 in Epilepsy

A recent study elucidates the role of CPEB4 in temporal lobe epilepsy, the most frequent form of epilepsy in adults (Nearing et al., 2007). Mice injected via intra-amygdala with kainic acid (KA) are used to model status epilepticus and epilepsy, taking as a reference two different time points subsequent to the injection. Hippocampal RNAs from both mouse models are characterized by changes in poly(A) tail length. An analysis revealed that not only poly(A) altered genes are enriched in CPEB4 binders at both time points, but also that such binders are enriched in epilepsy-related genes. Furthermore, CPEB4 brain expression increases in these mice and in patients affected by temporal lobe epilepsy, possibly as an adaptive mechanism, since CPB4 KO mice display increased susceptibility to seizures and hippocampal damage. Thus, these data support the notion of CPEB4 as a key regulator in brain excitability and seizure-induced damage (Parras et al., 2020).

4.2 CPEB4 in Autism spectrum disorder

ASD was initially linked to CPEBs, an in particular to CPEB1, via FMRP. FMRP is the gene whose inactivation is responsible for a monogenic form of ASD: the fragile X syndrome (FXS) (Verkerk et al., 1991). In FMRP KO mice, other than a FXS-like phenotype, a 15-20% increase in protein synthesis is observed, and it is what has been considered as contributing to the disease (Darnell & Klann, 2013). Interestingly, the FMRP KO mouse phenotype is rescued in a FMRP/CPEB1 double KO mouse and the protein synthesis homeostasis is restored (Udagawa et al., 2013).

However, a more recent publication reports a role of CPEB4 in idiopathic ASD. Indeed, although there are few cases of syndromic ASD, such as FXS, where the disorder can be associated to single highly-penetrant mutation or chromosomal abnormality, the majority of ASD cases are idiopathic, in which multiple small effect-size variants in hundreds of genes and environmental risk-factors are believed to be responsible for the disease (Gaugler et al., 2014; Geschwind & State, 2015; Sztainberg & Zoghbi, 2016; Willsey & State, 2015). The first evidence from this study is that mRNAs of the majority of ASD risk genes are enriched in CPEs sequences and are targets of CPEB4, suggesting a role of the latter in this disorder. Subsequently, the amount of the CPEB4 protein levels have been measured in brain tissue from people with ASD, revealing its decrease. Interestingly, CPEB4 undergoes mis-splicing in ASD. As above mentioned, multiple transcripts are generated from CPEBs genes by alternative splicing (Johnson et al., 2015; Kaczmarczyk et al., 2016; Theis et al., 2003). More precisely, CPEB4 owns two consecutive alternatively spliced exons, the exon3, which has a length of 51 nucleotides, and the exon 4, which is a brain-specific 24 nucleotides long microexon. Depending on their combination, four different isoforms of CPEB4 can be generated in the brain: the one including both exons, known as the full length CPEB4 isoform (CPEB4FL), the ones lacking of only the exon 3 or the microexon (CPEB4 Δ 3 or CPEB4 Δ 4, respectively), and the one lacking both (CPEB4 Δ 3 Δ 4). In ASD, the inclusion of the microexon is reduced, with an altered ratio in the relative abundance of these four CPEB4 transcripts. The altered CPEB4 splicing in ASD correlates with shortened poly(A) length in many high-confidence ASD-risk genes mRNAs, whose protein levels are in turn reduced. Finally, a mouse model with neuron specific overexpression of the CPEB4 Δ 4 isoform starting at late embryonic stages mimics the CPEB4-related changes observed in the brains of individuals with idiopathic ASD and presents an ASD-like phenotype (Parras et al., 2018).

IV. OBJECTIVES

Considering the great number of ASD and SCZ shared features and the recent publication from our research group indicating a key role of CPEB4 in ASD, the hypothesis of a possible implication of CPEB4 in SCZ was formulated. To test this hypothesis, in this thesis we established the following objectives:

1. Enrichment analysis of CPE-harboured and CPEB4-binder transcripts in SCZ-risk genes.
2. Assessment of CPEB4 splicing in RNA-seq data from individuals with SCZ.
3. Exploration of transcriptomic signatures correlating with CPEB4 microexon inclusion levels.
4. Analysis of CPEB4 splicing in human SCZ post-mortem brains by RT-PCR.
5. Analysis of CPEB4 microexon inclusion in RNA-seq data from cellular and animal models treated with antipsychotics.
6. Evaluation of protein levels of CPEB4-related SCZ-risk genes in SCZ-post-mortem brains.
7. Analysis of protein levels of SCZ-risk genes in a mouse model overexpressing the microexon-lacking isoform of CPEB4 (Tg-PN-CPEB4 Δ 4).
8. Behavioural characterization of Tg-PN-CPEB4 Δ 4 mice.

V. MATERIALS AND METHODS

1. CPEB-related genes enrichment analysis among SCZ risk genes

To establish a relationship between SCZ genes and CPEBs, we carried out gene-set enrichment analyses through MAGMA, a bioinformatics tool for gene-level analyses of GWAS data sets (de Leeuw et al., 2015). European and East Asian SCZ GWAS summary statistics were obtained from the latest public multi-stage study of the Psychiatric Genomics Consortium (Trubetskoy et al., 2022), and processed through a gene-level analysis in MAGMA as described therein. Briefly, this involved computing gene-wide mean *P*-values with each ancestry-specific subsample, which were then meta-analysed leading to combined gene-level data from 67,390 SCZ cases and 94,015 controls (CTRL). For these analyses, gene boundaries were retrieved from Refseq (GRCh38.p13; last updated on 23-11-2020), lifted over to GRCh37 coordinates and expanded by 35kb/10kb upstream/downstream flanking regions to encompass potential regulatory elements (Maston et al., 2006). Regarding CPE-containing genes in humans, we considered a previously published database (Pique et al., 2008). CPEB1 and CPEB4 gene lists were derived from an RNA immunoprecipitation (RIP) study performed in mouse striatum (Parras et al., 2018). A list of CPEB3 binders has been recently obtained through a RIP experiment performed in mouse cortical tissue (Lu et al., 2021). Covariate gene sets used to carry out conditional analyses included (i) targets of the FMRP regulon (Darnell et al., 2011), (ii) genes with significant expression in the brain (Fagerberg et al., 2014); as processed (Genovese et al., 2016) and (iii) genes specifically involved in synaptic development and function (Koopmans et al., 2019).

2. RNA-seq data analysis

To explore the alternatively spliced events of CPEBs in SCZ patients, we examined the data in the BrainGVEX RNA-seq study (Psych et al., 2015). Such study comprises well-characterized human post-mortem prefrontal (BA46) cortex samples from SCZ subjects (n=95) and matched CTRL (n=75), originating from the Stanley Medical Research Institute (SMRI). Fastq files were downloaded from the PsychENCODE Consortium (PEC) Knowledge Portal repository (<https://psychencode.synapse.org/>). For quality control analysis, we run FastQC (v0.11.9). Then, the four parameters i) *per base GC content*, ii) *per sequence GC content*, iii) *sequence duplication level* and iv) *overrepresented sequences* were monitored for each strand (forward and reverse), thus resulting in a total of 8 tests. Finally, only samples that overall pass at least four of the latter were considered. Following the quality filter, a total of n=54 CTRL and n=66 SCZ samples got access to the final step of the analysis pipeline. Reads were aligned to human hg19 reference genome and counts and per cent spliced in (PSI) values were computed through complete Vertebrate Alternative Splicing and Transcription Tools (vast-tools) v.2.5.1 program (Irimia et al., 2014; Tapial et al., 2017).

Regarding CPEB4 Ex4 PSI values relative frequency distribution, a bin width of 10 has been considered. Differentially expressed genes between CTRL and SCZ were obtained calculating the mean of the cRPKM values of each gene and computing the fold change (FC) value. *P*-values obtained with a T-test were corrected by false discovery rate (FDR) method for multiple comparison. Downregulated genes in SCZ with a CPEB4 exon 4 PSI<65 compared to CTRL were obtained with a FDR<1%. In the case of the 100 top genes, a FDR<5% has been chosen. Enrichment analysis of already published SCZ-related DEG signature among our DEGs were performed using http://nemates.org/MA/progs/overlap_stats.html. The representation factor (RF) is the number of overlapping genes divided by the expected number of overlapping genes drawn from two independent groups. A RF>1 indicates more overlap than expected of two independent groups, while a RF<1 indicates less overlap than expected.

Three APDs-related RNA-seq studies have been analysed to assess a possible effect of these drugs on CPEB4 exon 4 inclusion levels. i) The GSE149611 study explores the effect of three APDs, volinanserine (n=4), risperidone (n=4) and amisulpride (n=4) versus dimethyl sulfoxide (DMSO) (n=4) as control using SH-SY5Y human neuroblastoma cell line (Malekizadeh et al., 2020). The GSE117174 study is performed on samples from mouse PFC and Nucleus Accumbens 2 hours after peritoneal injection with haloperidol (n=4) or risperidone (n=4) and using saline as a control (n=8) (Zygmunt et al., 2018). Finally, a third RNA-seq data on DLPFC BA46 samples of *Macaca mulatta* animals treated with high doses of haloperidol (4 mg/kg/d) (n=7) or with low doses of haloperidol (0.14 mg/kg/d) (n=10) or with placebo (n=8) (Synapse ID: syn4566233) was obtained from Synapse Storage of PsychENCODE Knowledge Portal at <https://doi.org/10.7303/syn4921369> (Hoffman et al., 2019). In the case of the GSE149611 study, after downloading files, executing the quality control and aligning reads to human hg19 reference genome as mentioned before, PSI have been computed by vast-tools pooling together replicates in order to overcome the poor depth of read coverage. The GSE117174 study was analysed by vast-tools as above described, aligning reads to mouse mmu8 reference genome without merging replicates. Finally, for the syn4566233 study, following the download and quality control of the files, reads were aligned to the rhesus macaque genomes of reference (MMUL-1.85) through STAR aligner (Dobin et al., 2013) and CPEB4 splicing analysed by rMATS (Shen et al., 2012).

3. Human brain tissue samples

Brain specimens from the frontal cortex (BA9/BA46) of individuals with SCZ and matched CTRL used in qRT–PCR and immunoblotting were obtained at autopsies performed in the Basque Institute of Legal Medicine, Bilbao, Spain and by the NIH NeuroBioBank (NBB), Maryland, USA (CTRL n=57 and SCZ n=42). Demographic and clinical feature of individuals are reported in **Supplementary Table 1**. A toxicological screening for a panel of drugs, including antipsychotics, antidepressant, cotinine and ethanol, was performed on both cohorts by the Central Analysis Service at the University of the Basque Country, Spain. A variety of standard procedures including radioimmunoassay, enzymatic immunoassay, high-performance liquid chromatography and gas chromatography–mass spectrometry have been performed. Following toxicological screening, only CTRL who resulted negative to any substance were included in the study (n=45), while schizophrenic individuals were divided in a treatment-free group (FREE-SCZ n=21) and a group of antipsychotic treated patients (APDs-SCZ n=21).

4. Mice

Conditional transgenic mice carrying a cDNA of human CPEB4 lacking exon 4 (CPEB4Δ4) under control of the inducible TetO promoter were previously generated (Parras et al., 2018) and maintained in a C57BL/6J background. CPEB4Δ4 mice were crossed with a driver mouse line with low expression of the transactivator tTA (Tet-Off) under the CamkII promoter (Low-CamkII-tTA mice) to obtain conditional double transgenic mice with low forebrain neuronal expression of CPEB4Δ4 (Tg-PN-CPEB4Δ4 mice). Also, conditional double transgenic mice with strong forebrain neuronal expression of CPEB4Δ4 during the embryonic stage (Tg-E-CPEB4Δ4) which represent an ASD model, were used as control (Parras et al., 2018). All mice were housed in the CBMSO animal facility. Mice were housed four per cage with food and water available *ad libitum* and maintained in a temperature-controlled environment on a 12 h–12 h light–dark cycle with light onset at 08:00. Animal housing and maintenance protocols followed local authority guidelines. Animal experiments were performed under protocols approved by the Centro de Biología Molecular Severo Ochoa Institutional Animal Care and Utilization Committee (Comité de Ética de Experimentación Animal del CBM, CEEA-CBM), and Comunidad de Madrid PROEX 247.1/20.

N	Bank	Sample	Sex	Age	PMI (hrs)	Type	Ethnicity	Treatment	Toxicology (a)	Toxicology (b)	Toxicology (c)	Toxicology (d)	Toxicology (e)	Toxicology (f)	RIN	PCR Amplification product (Yes/No)	CPEB4 RT-PCR (Yes/No)
1	NIH NBB	S11723	M	18	20	CTRL	unk	(-)	(-)	(-)	(-)	(-)	(-)	(-)	7.9	Y	Y
2	NIH NBB	S01509	M	36	21	CTRL	C	(-)	Cotinine	(-)	(-)	(-)	(-)	(-)	2.1	N	N
3	NIH NBB	S12502	M	44	23	CTRL	unk	(-)	Cotinine	Sertraline	(-)	(-)	(-)	(-)	5.4	Y	N
4	NIH NBB	S02296	M	50	21	CTRL	unk	(-)	(-)	(-)	(-)	(-)	(-)	(-)	5.9	Y	N
5	NIH NBB	S14595	M	54	22	CTRL	unk	(-)	Cotinine	(-)	(-)	(-)	(-)	(-)	7.8	Y	N
6	NIH NBB	S00225	M	57	21	CTRL	unk	(-)	(-)	(-)	(-)	(-)	(-)	(-)	7.4	Y	Y
7	NIH NBB	S14640	M	63	20	CTRL	unk	(-)	Chlorpromazine	(-)	(-)	(-)	(-)	(-)	7	Y	N
8	NIH NBB	S05537	M	68	23	CTRL	unk	(-)	(-)	(-)	(-)	(-)	(-)	(-)	7.6	N	N
9	NIH NBB	S06826	M	73	18	CTRL	C	(-)	Duloxetine	(-)	(-)	(-)	(-)	(-)	7.4	Y	N
10	NIH NBB	S03945	F	58	22	CTRL	unk	(-)	Cotinine	(-)	(-)	(-)	(-)	(-)	6	Y	N
11	NIH NBB	S10728	F	62	24	CTRL	unk	(-)	(-)	(-)	(-)	(-)	(-)	(-)	8.1	Y	Y
12	NIH NBB	S19871	F	66	23	CTRL	C	(-)	Duloxetine	(-)	(-)	(-)	(-)	(-)	3.8	N	N
13	NIH NBB	S05759	F	69	21	CTRL	unk	(-)	Lamotrigine	(-)	(-)	(-)	(-)	(-)	6.5	Y	N
14	NIH NBB	S00579	F	80	22	CTRL	unk	(-)	(-)	(-)	(-)	(-)	(-)	(-)	7.6	Y	Y
15	NIH NBB	S10002	F	86	24	CTRL	unk	(-)	(-)	(-)	(-)	(-)	(-)	(-)	6.4	Y	Y
16	BILM	61518	M	23	4	CTRL	C	(-)	(-)	(-)	(-)	(-)	(-)	(-)	8.8	Y	Y
17	BILM	4236	M	25	18	CTRL	C	(-)	(-)	(-)	(-)	(-)	(-)	(-)	6.7	Y	Y
18	BILM	157824	M	26	5	CTRL	C	(-)	(-)	(-)	(-)	(-)	(-)	(-)	8.2	Y	Y
19	BILM	41085	M	28	15	CTRL	C	(-)	(-)	(-)	(-)	(-)	(-)	(-)	7.4	Y	Y
20	BILM	75324	M	29	17	CTRL	C	(-)	(-)	(-)	(-)	(-)	(-)	(-)	7.7	Y	Y
21	BILM	142515	M	31	13	CTRL	C	(-)	(-)	(-)	(-)	(-)	(-)	(-)	7.7	Y	Y
22	BILM	19854	M	32	16	CTRL	C	(-)	(-)	(-)	(-)	(-)	(-)	(-)	6.8	Y	Y
23	BILM	14436	M	32	4	CTRL	C	(-)	(-)	(-)	(-)	(-)	(-)	(-)	8.4	Y	Y
24	BILM	33951	M	32	4	CTRL	C	(-)	Ethanol	Cocaine	(-)	(-)	(-)	(-)	7.2	Y	N
25	BILM	102357	M	33	28	CTRL	C	(-)	Ethanol	(-)	(-)	(-)	(-)	(-)	7	Y	N
26	BILM	104130	M	33	23	CTRL	C	(-)	(-)	(-)	(-)	(-)	(-)	(-)	n.a.	N	N
27	BILM	19554	M	33	17	CTRL	C	(-)	(-)	(-)	(-)	(-)	(-)	(-)	6.6	Y	Y
28	BILM	206082	M	34	18	CTRL	C	(-)	(-)	(-)	(-)	(-)	(-)	(-)	6.1	Y	Y
29	BILM	41136	M	34	16	CTRL	C	(-)	(-)	(-)	(-)	(-)	(-)	(-)	8.6	Y	Y
30	BILM	110397	M	36	33	CTRL	C	(-)	(-)	(-)	(-)	(-)	(-)	(-)	7.1	Y	Y
31	BILM	175533	M	37	11	CTRL	C	(-)	(-)	(-)	(-)	(-)	(-)	(-)	7.2	Y	Y
32	BILM	146730	M	37	3	CTRL	C	(-)	(-)	(-)	(-)	(-)	(-)	(-)	6.2	Y	Y
33	BILM	34809	M	43	10	CTRL	C	(-)	(-)	(-)	(-)	(-)	(-)	(-)	8.1	Y	Y
34	BILM	103206	M	43	9	CTRL	C	(-)	(-)	(-)	(-)	(-)	(-)	(-)	7.9	Y	Y
35	BILM	168654	M	44	29	CTRL	C	(-)	(-)	(-)	(-)	(-)	(-)	(-)	7	Y	Y
36	BILM	95754	M	46	6	CTRL	C	(-)	Ethanol	(-)	(-)	(-)	(-)	(-)	7.7	Y	N
37	BILM	42357	M	50	24	CTRL	C	(-)	THC	(-)	(-)	(-)	(-)	(-)	6.6	Y	N
38	BILM	27654	M	50	2	CTRL	C	(-)	(-)	(-)	(-)	(-)	(-)	(-)	6.6	Y	Y
39	BILM	123330	M	51	18	CTRL	C	(-)	(-)	(-)	(-)	(-)	(-)	(-)	8	Y	Y
40	BILM	20130	M	51	13	CTRL	C	(-)	(-)	(-)	(-)	(-)	(-)	(-)	8.4	Y	Y
41	BILM	144327	M	54	24	CTRL	C	(-)	(-)	(-)	(-)	(-)	(-)	(-)	8	Y	Y
42	BILM	71457	M	56	15	CTRL	C	(-)	(-)	(-)	(-)	(-)	(-)	(-)	7.6	Y	Y
43	BILM	114951	M	57	3	CTRL	C	(-)	(-)	(-)	(-)	(-)	(-)	(-)	6.3	Y	Y
44	BILM	85806	M	58	16	CTRL	C	(-)	(-)	(-)	(-)	(-)	(-)	(-)	7	Y	Y
45	BILM	75606	M	61	23	CTRL	C	(-)	(-)	(-)	(-)	(-)	(-)	(-)	8	Y	Y
46	BILM	71145	M	62	9	CTRL	C	(-)	(-)	(-)	(-)	(-)	(-)	(-)	6.9	Y	Y
47	BILM	80109	M	71	21	CTRL	C	(-)	(-)	(-)	(-)	(-)	(-)	(-)	7.1	Y	Y
48	BILM	17748	F	32	19	CTRL	C	(-)	(-)	(-)	(-)	(-)	(-)	(-)	7.4	Y	Y
49	BILM	200385	F	33	24	CTRL	C	(-)	(-)	(-)	(-)	(-)	(-)	(-)	7.4	Y	Y
50	BILM	102594	F	35	17	CTRL	C	(-)	(-)	(-)	(-)	(-)	(-)	(-)	7.4	Y	Y

Supplementary Table 1: Demographic and clinical data of samples used in this study. BANK: NIH NBB=NIH NeuroBioBank, BLIM= Basque Institute of Legal Medicine; Ethnicity: C=Cucasic, unk= unknown; Toxicology colour legend Red=APDs, Pink=Antidepressant, Light blue=Cotinine, Orange=antiepileptic, Dark green=Cocaine, Light green=Cannabinoids. Samples in grey have been excluded.

5. RNA isolation and cDNA synthesis

Total tissue RNA was extracted from prefrontal cortex (BA9/BA46) of CTRL individuals and matched SCZ using the Maxwell 16 LEV simplyRNA Tissue Kit (Promega, AS1280). Quantification and quality determination of RNA was done on a Nanodrop ND-1000 spectrophotometer and Nanodrop 1000 v.3.7.1 (Thermo Scientific). Only samples with an RNA integrity number (RIN) ≥ 6 and generating an amplification product were included in our experiments (CTRL n=41; FREE-SCZ n=18 and APDs-SCZ n=20) (**Supplementary Table 1**). The same conditions were employed in the case of CTRL and Tg-PN-CPEB4 $\Delta 4$ mice brains. Retrotranscription (RT) reactions were performed using the iScript cDNA Synthesis kit (Bio-Rad, PN170-8891) following the manufacturer's instructions. In brief, 1,000 ng of total RNA from each sample was combined with 10 μ l of master mix (includes all necessary reagents along with a mixture of random primers and oligo-dT for priming). The reaction volume was completed up to 40 μ l with DNase/RNase-free distilled water (Gibco, PN 10977). Thermal conditions consisted of the following steps: 5 min at 25 °C; 20 min at 46 °C and 1 min at 95 °C.

6. Quantification of CPEB4 transcript splicing and differential splicing analysis

Specific primers designed in CPEB4 exon 2 (forward, 5'-GGACGTTTGACATGCACTCAC-3') and exon 5 (reverse, 5'-GAGGTTGATCCCCACGGC-3') able to amplify the four CPEB4 splicing isoforms (full-length, $\Delta 4$, $\Delta 3$ and $\Delta 3\Delta 4$) in human and mouse brain cDNA were used.

PCR amplification protocol was the following: 10 min at 94 °C + 33 cycles (30 s at 94 °C + 30 s at 58 °C + 2 min at 72 °C) + 10 min at 72 °C. PCR products according with the four CPEB4 isoforms were resolved on 2.2% agarose/GelGreen (Biotium, 41004) gels run at 125 V for 1.5 h. Images were scanned with densitometer (Bio-Rad, GS-900) and quantified with Image Laboratory 5.2 (Bio-Rad). Finally, the percentage of each CPEB4 isoform was calculated.

7. Western Blot

Samples from human brain were stored at -80 °C and were ground with a mortar in a frozen environment with liquid nitrogen to prevent thawing of the samples, resulting in tissue powder. For mouse, brains were quickly dissected on an ice-cold plate and the different structures stored at -80 °C. Human and mouse extracts were prepared by homogenizing the brain areas in ice-cold extraction buffer (20 mM HEPES pH 7.4, 100 mM NaCl, 20 mM NaF, 1% Triton X-100, 1 mM sodium orthovanadate, 1 μ M okadaic acid, 5 mM sodium pyrophosphate, 30 mM β -glycerophosphate, 5 mM EDTA, protease inhibitors (Complete, Roche, Cat. No 11697498001)). Homogenates were centrifuged at 15,000g for 15 min at 4 °C. The resulting supernatant was collected, and protein content determined by Quick Start Bradford kit assay (Bio-Rad, 500-0203).

Between 10 and 20 μ g of total protein was electrophoresed on 10% SDS–polyacrylamide gel, transferred to a nitrocellulose blotting membrane (Amersham Protran 0.45 μ m, GE Healthcare Life Sciences, 10600002) and blocked in TBS-T (150 mM NaCl, 20 mM Tris–HCl, pH 7.5, 0.1% Tween 20) supplemented with 5% non-fat dry milk. Membranes were incubated overnight at 4 °C with the primary antibody in TBS-T supplemented with 5% non-fat dry milk. Antibodies used: mouse anti-ATP2A2 (1:1000, Abcam, ab2861), rabbit anti-ATXN7 (1:1000, Invitrogen, PA1-749), rabbit anti-BCL11A (1:250, Sigma-Aldrich, ABE401), rabbit anti-CACNB2 (1:1000, Santa Cruz Biotechnology, sc-81890), rabbit anti-CNTN4 (1:500, Abcam, ab137107), rabbit anti-CTNND1 (1:1000, Sigma-Aldrich, HPA015955), rabbit anti-ELAVL4 (1:500, Abcam, ab96474), rabbit anti-MEF2C (1:1000, Abcam, ab64644), rabbit anti-NEK1 (1:1000, Thermo Scientific, PA5-15336), rabbit anti-OSBPL3 (1:1000, Santacruz Biotechnology, sc-398326), rabbit anti-PDE4B (1:1000, Abcam, ab14611), rabbit anti-STAG1 (1:1000, kindly provided by Spanish National Cancer Research Center-CNIO), rabbit anti-TCF4 (1:1000, Proteintech, 22337-1-AP), rabbit anti-ZEB2 (1:1000, Proteintech, 14026-1-AP), rabbit anti-ZSWIN6 (1:1000, Origene, AP54741PU-N), rabbit anti-VINCULIN (1:10000, Abcam, ab129002) and mouse anti- β -ACTIN (1:25000, Sigma-Aldrich, A2228). Membranes were washed with TBS-T and next incubated with secondary HRP-conjugated anti-mouse IgG (1:2000, DAKO, P0447) or anti-rabbit IgG (1:2000, DAKO, P0448), they were developed using the ECL detection kit (Perkin Elmer, NEL105001EA). Images were scanned with densitometer (Bio-Rad, GS-900) and quantified with Image Laboratory 5.2 (Bio-Rad).

8. Mouse behaviour tests

Prepulse inhibition (PPI) of the acoustic startle response test: Startle response curve as well as PPI test were conducted in 10-week-old mice using a commercially available StartFear (Panlab-Harvard Apparatus). This system allows recording and analysis of the signal generated by the animal movement through a high sensitivity weight transducer system. A standard protocol was adapted⁴⁹. Each mouse has been located in the chamber and during a 5-min acclimation period, while background white noise was continually present. Then, a startle response curve session was performed in order to rule out any impairment in hearing. Startle measures included recordings made every 4 dBs above background (66 dB), up to 118 dB. Each mouse received four times each trial type (40 ms-sound pulses from 70 dB to 118 dB) distributed randomly and separated by 10s-intertrial interval. Response amplitude was considered as the maximum response level recorded during 1 s after the sound pulse. Regarding PPI, trial types, trial type presentation, and background noise levels were performed according to the protocols described previously (Mukai et al., 2004; Stark et al., 2008) with some modifications the following day.

In brief, after a 5 min habituation period (66 dB white noise background), eight sets of four different trials distributed randomly with a variable intertrial time (10, 15 or 20 s) were presented to each mouse: trial 1, 40-ms, 120-dB noise burst alone; trials 2 and 3, 120-dB startle stimulus preceded 100 ms earlier by a 20-ms, 70, 74, 78 or 82-dB noise burst (pre-pulse); trial 4, no stimulus, background noise alone (66 dB). As for startle test, response amplitude was considered as the maximum response level recorded during 1 s after the sound pulse. Percent PPI was calculated as $100 - [(startle\ response\ of\ acoustic\ startle\ from\ acoustic\ prepulse\ and\ startle\ stimulus\ trials / startle\ response\ alone\ trials) \times 100]$.

Grooming time and social interaction tests: Grooming and social interaction was examined in 7-week-old mice. The first day (training), mice were allowed to explore for 5 min a three chamber Plexiglas box. This time of habituation was employed to measure the grooming activity. The next day (test), mice were placed in the same box containing two wire cages, one empty and the other with an unknown (gender paired) mouse in it, located in opposite chambers and separated by the empty chamber. Mice were recorded for 10 min and the time spent interacting with the unknown mouse was measured.

9. Statistical analysis

Statistical analysis was performed with SPSS 26.0 (SPSS Statistic IBM), GraphPad software (La Jolla, CA, USA) or RStudio 2022.02.2 (Boston, MA, USA). The normality of the data was analysed by Shapiro–Wilk test ($n < 50$) or Kolmogorov–Smirnov test ($n > 50$). For comparison of two independent groups two-tail unpaired Student's t-test (data with normal distribution), Mann–Whitney–Wilcoxon or Kolmogorov–Smirnov tests (with non-normal distribution) were performed. For multiple comparisons, data with a normal distribution were analysed by one way-ANOVA or two-way-ANOVA followed by a Tukey's post hoc test. Statistical significance of non-parametric data for multiple comparisons was determined by Kruskal–Wallis one-way ANOVA. Benjamini-Hochberg correction was applied for multiple testing in RNA-seq analysis. Data are represented as mean \pm SEM with 95% confidence intervals. Higher or lower points (outliers) are not plotted. A critical value for significance of $P < 0.05$ was used throughout the study.

VI. RESULTS

1. Enrichment analysis of CPE-harboursing and CPEB4-binder transcripts in SCZ-risk genes

We decided to explore whether CPE-containing and CPEB4-binding transcripts were overrepresented within SCZ-associated genes. We first used MAGMA to perform gene-set analyses based on the summary statistics from the Wave 3 Psychiatric Genomics Consortium (PGC) GWAS of schizophrenia ("core dataset"; 67,390 cases and 94,015 controls) (Trubetskoy et al., 2022). Successively, we examined gene sets comprised of (i) genes containing canonical CPE (cCPE) sequences in their 3'UTR (Pique et al., 2008); (ii) genes identified in genome-wide RNA immunoprecipitation analyses from mouse brain structures as CPEB1-(Parras et al., 2018), CPEB3-(Lu et al., 2021) or CPEB4-(Parras et al., 2018) binders; (iii) gene sets implicated in the pathophysiology of psychiatric disorders: Fragile X Mental Retardation Protein (FMRP) targets (Clifton et al., 2021; Darnell et al., 2011), genes specifically involved in synaptic development and function (Koopmans et al., 2019), and finally (iv) as a comparator, a more general set of all brain-expressed genes (Fagerberg et al., 2014). The results of associations with SCZ-associated genes showed "CPEB4 target" as the top significant gene-set amongst those under study ($P=1.76 \times 10^{-9}$; **Table 1**), together with already known gene-sets implicated in psychiatric disorders such as FMRP targets and brain-expressed genes ($P=4.97 \times 10^{-9}$ and $P=6.13 \times 10^{-19}$, respectively; **Table 1**). MAGMA models were conditioned on a binary indicator to control for overlapping genes amongst gene-sets, given the known substantial overlap between FMRP and CPEB targets (Udagawa et al., 2013), or CPEB target genes and brain expressed genes (Parras et al., 2018). After conditional analysis for each of FMRP targets, synaptic genes, and brain-expressed genes, the "CPEB4 target" gene-set remained the most significant (**Table 1**). As expected, given the overlap among the CPEB1-, CPEB3-, and CPEB4-targets, CPEB1 and CPEB3 targets also showed evidence for enrichment for SCZ associations as did the gene set of "cCPE containing genes" (**Table 1**). These results therefore confirmed enrichment of CPE-containing and CPEB4-binding transcripts in SCZ susceptibility genes.

Gene-set name	N genes	P-value	Conditional Analysis (P-value)		
			Brain-expressed	Synaptic genes	FMRP targets
cCPE-containing genes	7,712	2.92E-04	0.036	5.93E-04	0.003
CPEB1 targets	2,207	1.39E-07	2.08E-04	7.06E-07	3.71E-06
CPEB3 targets	3,508	6.95E-04	0.065	0.002	0.003
CPEB4 targets	2,749	1.76E-09	5.77E-05	2.19E-08	7.98E-08
FMRP targets	813	4.97E-09	7.19E-06	4.76E-07	-
Synaptic genes	1,046	4.36E-07	5.55E-05	-	4.43E-05
Brain-expressed genes	9,695	6.13E-19	-	6.68E-17	7.55E-16

Table 1: GWAS summary statistics from the PGC3 of SCZ was used for a gene-level analysis via MAGMA. For each gene-set is reported the number of genes (N genes) and P-value of enrichment with SCZ-related genes after gene-based analysis.

2. Assessment of CPEB4 splicing in RNA-seq data from individuals with SCZ

To explore if the splicing alteration of CPEB4 seen in brains of ASD cases also happens in SCZ, we performed vast-tools (Irimia et al., 2014; Tapial et al., 2017) analysis on the (BA46) cortex RNA-seq data from people with SCZ (n=95) and matched controls (n=75) of the PsychENCODE Consortium BrainGVEX RNA-seq study (Psych et al., 2015). After quality control (QC) procedures (see materials and methods), 54 control and 66 SCZ samples met the thresholds for subsequent splicing analyses. Regarding the four CPEB genes, the only skipped exon (SE) event that differed significantly between controls and SCZ was exon 4 of CPEB4 (i.e. the 24 nucleotide microexon) (Table 2).

GENE	COMPLEX	LENGTH	COORDINATES	Δ PSI	P-value
CPEB4	C2	24	chr5:173370029-173370052	-3,63	0,023*
CPEB3	C3	141	chr10:93904702-93904842	-0,91	0,086
CPEB1	S	199	chr15:83218210-83218408	-0,83	0,094
CPEB3	S	57	chr10:93940720-93940776	-0,54	0,124
CPEB3	S	115	chr10:93851587-93851701	-0,48	0,151
CPEB3	S	90	chr10:93902786-93902875	-0,29	0,325
CPEB2	S	24	chr4:15042088-15042111	0,63	0,355
CPEB1	C2	193	chr15:83297115-83297307	4,16	0,407
CPEB2	S	138	chr4:15054686-15054823	-0,20	0,431
CPEB3	C1	24	chr10:93917806-93917829	-0,86	0,475
CPEB1	C3	91	chr15:83297413-83297503	2,52	0,481
CPEB1	C3	90	chr15:83222217-83222306	-0,34	0,482
CPEB2	S	90	chr4:15009962-15010051	-1,18	0,509
CPEB1	C3	50	chr15:83301923-83301972	4,15	0,562
CPEB1	C3	70	chr15:83307316-83307385	-1,93	0,574
CPEB2	S	91	chr4:15029066-15029156	-0,17	0,594
CPEB3	S	227	chr10:94002926-94003152	0,93	0,641
CPEB3	S	160	chr10:93952234-93952393	0,03	0,695
CPEB1	C3	114	chr15:83222628-83222741	-0,13	0,698
CPEB1	S	92	chr15:83252120-83252211	-0,22	0,704
CPEB1	C3	137	chr15:83221230-83221366	-0,12	0,707
CPEB2	S	115	chr4:15060811-15060925	0,01	0,708
CPEB1	C3	209	chr15:83309408-83309616	-2,75	0,708
CPEB1	C2	248	chr15:83224620-83224867	0,07	0,868
CPEB4	C1	51	chr5:173359453-173359503	0,14	0,907
CPEB1	S	107	chr15:83262993-83263099	0,08	0,927
CPEB1	C2	227	chr15:83226510-83226736	0,02	0,975

Table 2: GENE: official gene symbol; COMPLEX: S, C1, C2, C3 are SE events with increasing degrees of complexity; LENGTH: length of the alternative sequence expressed in nucleotides; COORDINATES: genomic coordinate of the alternative sequence; Δ PSI: difference in PSI between the two groups. P-value: significance applying a student's *t*-test. CPEB4 microexon is the only event significantly different (*) between the two groups and is highlighted in light blue.

Akin to ASD, inclusion of this microexon was significantly reduced in those with SCZ compared with controls. More precisely, in average, 64.1% of the CPEB4 transcripts contained the microexon in the control samples versus 60.4% in the SCZ samples, resulting in a significant percent spliced in index difference (ΔPSI) = -3.63 ($P=0.023$) (Figure 9).

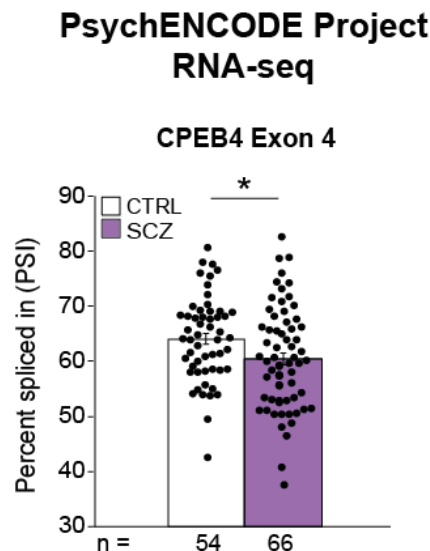


Figure 9: PsychENCODE BrainGVEX analysis by vast-tools: representation of CPEB4 exon 4 PSI in SCZ and CTRL. Student's *t*-test; * $P<0.05$. Data are mean with \pm SEM.

3. Exploration of transcriptomic signatures correlating with CPEB4 microexon inclusion levels

When we analysed the relative frequencies of the percentages of inclusion of CPEB4 exon 4, we noticed that the distributions significantly differed between control and SCZ samples ($P=0.048$) (Figure 10, lower panel). When restricting the analysis to only male samples, two peaks could be clearly observed in the SCZ distribution, the major peak corresponded to a value of percent spliced in index (PSI) ≈ 55 , while the minor peak corresponded to PSI ≈ 70 , the latter matching with the mode value in the distribution of control samples (Figure 10, upper panel). Therefore, in terms of inclusion of exon 4 of CPEB4, the observed distribution in SCZ seem to represent two subpopulations, one that resembles controls, and one whose peak value differs from that in controls with $\Delta\text{PSI} \approx -15$. To further explore whether the two parts of the distribution may represent two different subpopulations, we interrogated global differences in transcript levels with respect to controls. In view of the inflections of SCZ distributions at PSI=65 (Figure 10, lower panel), we stratified the 66 SCZ samples into two pools based on this cut-off. Interestingly, gene expression at the PSI>65-SCZ subpopulation ($n=21$) was similar that of control samples. In contrast, the PSI<65-SCZ subpopulation ($n=45$) showed 771 differentially expressed genes (DEG), 492 upregulated and 279 downregulated. Remarkably, the DEG signature in the PSI<65-SCZ subpopulation, particularly the downregulated genes, is highly concordant with

DEG signatures previously reported for dorsolateral prefrontal cortex (DLPFC) of SCZ subjects (Hashimoto et al., 2008; Yang et al., 2020). More precisely, the representation factor (see materials and methods) was 9.3 ($P < 1.17 \times 10^{-7}$) regarding RNA-seq based signatures (Yang et al., 2020) and 12.7 ($P < 1.94 \times 10^{-6}$) regarding gene-chip analysis (Hashimoto et al., 2008), with marked expression deficits in GABA neurotransmission-related transcript such as GAD1 (GAD67) or the neuropeptides somatostatin (SST) and neuropeptide Y (NPY) (**Table 3**). These results indicate that the SCZ individuals seem to segregate into two subpopulations, one that matches controls in terms of both CPEB4 exon 4 inclusion (PSI \approx 70) and global gene expression, and the other with lower CPEB4 exon 4 inclusion (PSI \approx 55) and a paradigmatic SCZ DEG signature. This suggests interrelated alterations of transcription and of CPEB4-dependent translational regulation in SCZ.

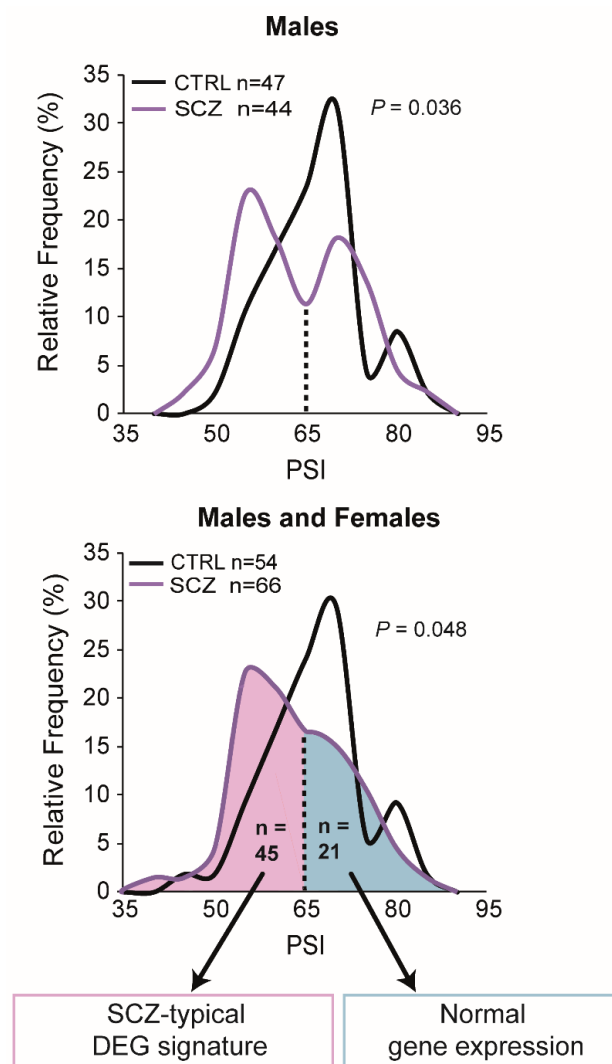


Figure 10: CPEB4 exon 4 PSI relative frequency (%) distributions in CTRL and SCZ: in males (upper panel) and males and females (lower panel). In the lower panel, SCZ subjects with PSI<65% are highlighted in pink, those with PSI>65% in light blue. DEGs analysis was performed comparing each group with CTRL. Two-sample Kolmogorov-Smirnov test for the distributions comparison; student's *t*-test followed by Benjamini-Hochberg correction for multiple comparison for DEGs. * $P < 0.05$.

Gene Name	FC	Gene Name	FC
TNFSF10	0,57	BEX5	0,76
RERGL	0,60	NPY	0,77
PENK	0,62	DUSP2	0,77
DUSP4	0,63	NXPH2	0,77
TAC1	0,65	GIMAP7	0,77
CD52	0,65	IL34	0,77
RPS4Y1	0,65	C19orf26	0,77
CRH	0,65	MMD2	0,77
PVALB	0,67	A2M	0,77
SEMA3G	0,68	RASL11A	0,77
LBH	0,68	FGL2	0,78
SST	0,68	NNAT	0,78
CXCR4	0,68	CRYM	0,78
OSTN	0,68	ARL4D	0,78
ABCG2	0,68	HLA-DPB1	0,79
HLA-DMB	0,69	USMG5	0,79
TMSB4Y	0,69	KLF10	0,79
KDM5D	0,69	NUDT14	0,79
CX3CR1	0,69	ITGAX	0,79
P2RY12	0,69	NPTX2	0,79
NEUROD6	0,69	ATP5G1	0,79
ZFY	0,71	RLBP1	0,79
OLR1	0,71	KCNS3	0,79
SLC38A5	0,71	FABP3	0,79
LYVE1	0,72	C17orf96	0,79
PNOC	0,72	LGI2	0,79
C1orf133	0,73	KCNIP3	0,79
COX7A2	0,73	COX7A1	0,80
DUSP1	0,73	PROM1	0,80
FRZB	0,73	BDNF	0,80
OLFML3	0,73	ADAP2	0,80
MCHR1	0,73	UQCRH	0,80
SMPX	0,73	NME5	0,80
RGS8	0,74	HSPA12B	0,80
GS1-211B7.1	0,74	BOLA3	0,80
PLA2G5	0,74	VGF	0,80
SPATA2L	0,74	RASL10A	0,80
PLD4	0,74	FBLN7	0,80
CRHBP	0,74	MGST3	0,80
NDUFA4	0,74	GAD1	0,80
COL5A3	0,74	SLC13A5	0,80
HSD11B1	0,75	TUBA1B	0,81
VIP	0,76	UBA7	0,81
ETV5	0,76	TRPC3	0,81
DUSP6	0,76	PPEF1	0,81
SPEF1	0,76	DLK2	0,81
IGFBP6	0,76	LPAR6	0,81
AGPAT9	0,76	ACAT2	0,81
GIMAP6	0,76	CNTN6	0,81
LMO2	0,76	SCG2	0,81

Table 3: Top 100 significantly downregulated genes comparing SCZ with CPEB4 Ex4 PSI<65 (n=45) and CTRL group (n=54). Minimum counts= 1.5 cRPKM; FDR=5%. FC= fold-change.

4. Analysis of CPEB4 splicing in human SCZ post-mortem brains by RT-PCR

There is evidence of antipsychotic medication correlating with diminished alteration of protein expression in post-mortem brains from individuals with schizophrenia (Chan et al., 2011). This led us to speculate that the two SCZ subpopulations arbitrarily delimited with the PSI=65 cut-off value might correlate with a different degree of exposure to antipsychotic drugs (APDs). Interestingly, the PsychENCODE database metadata provide “Lifetime Antipsychotics” index value for 45 of the 66 SCZ analyzed samples and, when we analyzed the mean Lifetime APDs index values for each group, we found that it was significantly higher in the PSI>65-SCZ (n=15) subpopulation compared to PSI<65-SCZ (n=30) subpopulation ($95,237 \pm 20,179$ vs. $42,770 \pm 7,786$; $P=0.048$) (**Figure 11**). This observation suggests that marked alteration in CPEB4 exon 4 inclusion might be specific to SCZ individuals with lower exposure to antipsychotic medication.

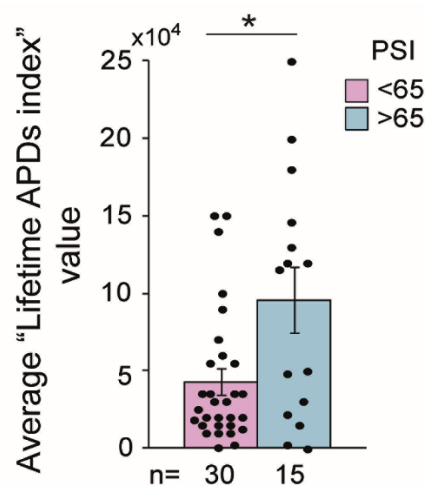


Figure 11: The graph shows the values of the lifetime APDs index in SCZ individuals with CPEB4 exon 4 PSI<65 (pink) and in those with CPEB4 exon 4 PSI>65 (light blue). Mann-Whitney test * $P<0.05$. Data are mean with \pm SEM.

To confirm in an independent cohort of samples the decreased usage of exon 4 in SCZ observed through RNA-seq analysis, and to settle or discard the correlation with antipsychotic medication, we decided to perform RT-PCR analysis in SCZ post-mortem DLPFC samples from the Basque Institute of Legal Medicine and the NIH NeuroBioBank (CTRL n=57 and SCZ n=42), in which a complete toxicological examination was performed by mass spectrometry to detect the presence of antipsychotics, as well as mood stabilizers, cotinine, antidepressants and benzodiazepines, at the time of death. Controls positive for any substance, as well as samples with low RNA quality, were excluded from the analysis (see materials and methods).

Since exon 3 (57 nucleotides) of CPEB4 is also alternatively spliced, there are four CPEB4 isoforms that can be detected with PCR primers hybridizing to exons 2 and 5, two transcripts that include exon 4 (CPEB4FL and CPEB4 Δ 3) and two lacking it (CPEB4 Δ 4 and CPEB4 Δ 3 Δ 4) (**Figure 12 A**). In control samples, the CPEB4FL transcript predominates and, as expected from the RNA-seq data, CPEB4FL decreases in samples from SCZ individuals that were free of antipsychotics at the time of death (FREE-SCZ) (**Figure 12 B-C**). As expected, patients of the FREE-SCZ sample show an increase of CPEB4 Δ 4 compared to controls, with some of the FREE-SCZ samples showing a completely opposite pattern of isoforms respect to controls, as CPEB4 Δ 4 and CPEB4 Δ 3 Δ 4 clearly predominate (**Figure 12 B-C**). As suggested by both the RNA-seq and Lifetime APDs index analyses, SCZ individuals under APDs medication at the time of death (APDs-SCZ) significantly differed from FREE-SCZ samples, showing CPEB4FL and CPEB4 Δ 4 isoform levels to be indistinguishable from those in controls (**Figure 12 B-C**). Consequently, the Δ Ex4/Ex4+ isoform ratio is markedly increased in FREE-SCZ samples, while unaltered in APDs-SCZ samples (**Figure 12 D**). Together, these results demonstrate that individuals with SCZ show a shift in the ratio of exon 4-dependent CPEB4 transcripts, selectively in the absence of antipsychotic medication.

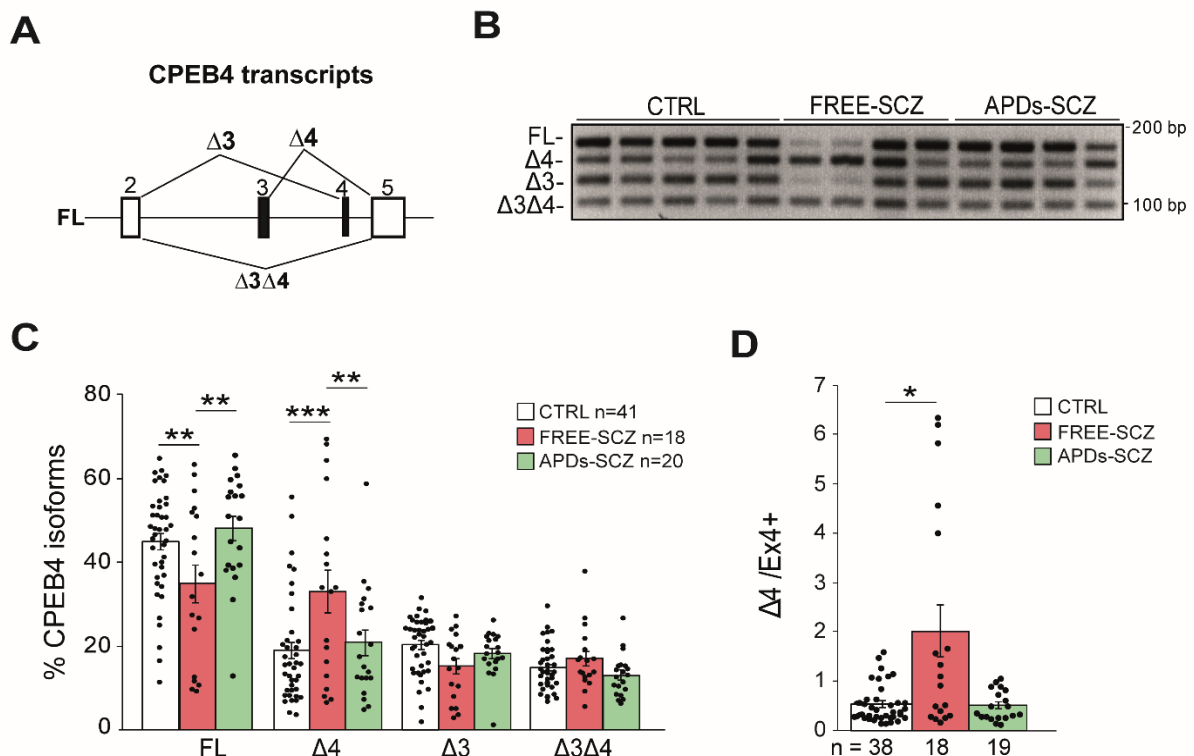


Figure 12: **A)** Diagram of the four splicing variants of CPEB4; **B)** Representative RT-PCR analysis of brain tissue from controls and SCZ subjects negative (FREE-SCZ) or positive to antipsychotic drugs (APDs-SCZ) at the moment of death; **C)** CPEB4 isoforms (%) quantification and **D)** Δ Ex4/Ex4+ ratio. C) Two-Way ANOVA with Bonferroni Correction; D) Kruskal-Wallis test with Dunn's multiple correction. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Data are mean with \pm SEM.

5. Analysis of CPEB4 microexon inclusion in RNA-seq data from cells and model animals treated with antipsychotics

The following aim was the examination of published RNA-seq studies in order to establish whether APDs affect CPEB4 splicing, as suggested by our observations regarding the PsychEncode Consortium BrainGVEX RNA-seq study (Psych et al., 2015) analysis and by the RT-PCR experiment just described. The first to be analysed was a RNA-seq performed in SH-SY5Y human neuroblastoma cells treated either with volinanserin (n=4) or risperidone (n=4) or amisulpride (n=4), with DMSO as a control (n=4) (Malekizadeh et al., 2020). Replicates were pooled together by vast-tools in order to achieve a deeper read coverage, although such step deprives the analysis of statistical significance (see materials and methods). Cells treated with DMSO showed a CPEB4 exon 4 PSI =0 and volinanserin did not affect this condition. However, in cells treated both with risperidone and amisulpride, CPEB4 exon 4 inclusion level increased (Δ PSI=12.73 and Δ PSI=21.12, respectively) (**Figure 13**). Thus, risperidone and amisulpride could alter CPEB4 splicing.

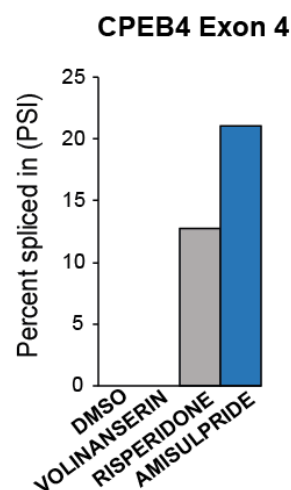


Figure 13: CPEB4 microexon PSI in SH-SY5Y cells treated with volinanserin or risperidone (gray) or amisulpride (blue).

Then, a second published RNA-seq data obtained from PFC and Nucleus Accumbens tissues of *Mus musculus* treated either with haloperidol (n=4), risperidone (n=4) or saline (n=8) as a control (Zygmunt et al., 2018) was explored by vast-tools (**Figure 14 A-B**). In PFC, neither haloperidol nor risperidone changed CPEB4 exon 4 PSI, as we observed non-significant $\Delta\text{PSI} = -2.55$ and $\Delta\text{PSI} = 5.04$, respectively (**Figure 14 A**). Similarly, in the Nucleus Accumbens only tendencies could be detected, with $\Delta\text{PSI} = -2.38$ in the case of haloperidol and $\Delta\text{PSI} = 10.05$ when mice were injected with risperidone (**Figure 14 B**). Thus, both haloperidol and risperidone were unable to change CPEB4 exon 4 inclusion level in two *Mus musculus* brain structures.

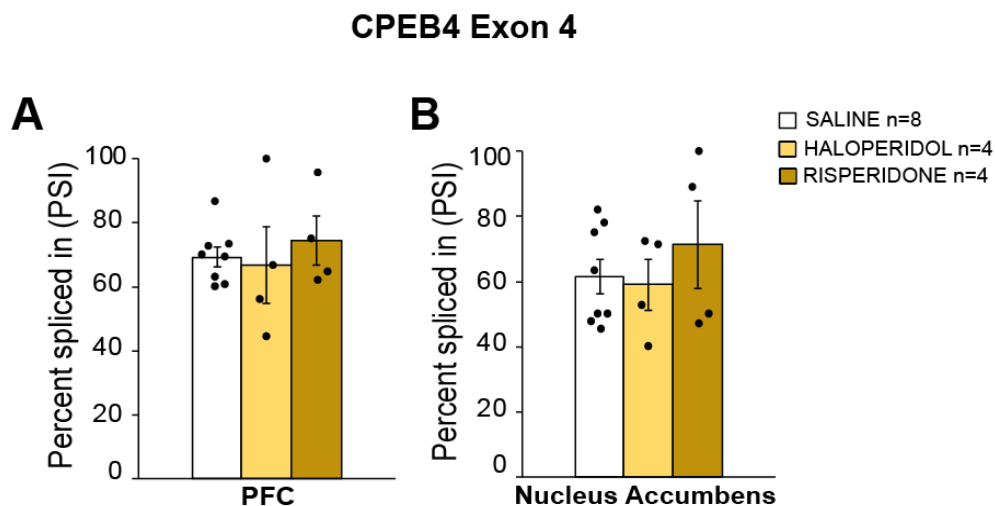


Figure 14: Effect on CPEB4 exon 4 PSI in mice treated with haloperidol or risperidone with respect to saline as control. Two brain structure were analysed, PFC and Nucleus Accumbens, by vast-tools.

Finally, the analysis of CPEB4 microexon inclusion in the “CMC_Macaque” RNA-seq study was carried out. Such study is performed on DLPFC (BA46) tissue from *Macaca mulatta* animals chronically treated with haloperidol. The following experimental groups have been considered: animals treated with high doses of haloperidol (n=7), animals treated with low doses of haloperidol (n=10) and animals treated with placebo as controls (n=8) (Hoffman et al., 2019). The CPEB4 splicing analysis was carried out through rMATS. The examination revealed that CPEB4 exon 4 inclusion levels did not significantly change upon haloperidol administration in the brain of this primate, independently from the dosage (**Figure 15 A-B**). More in details, when CPEB4 exon 4 was preceded by exon 3, it showed not statistically different ΔPSI values upon both low and high dosage of haloperidol treatment ($\Delta\text{PSI} = -1.7$ and a $\Delta\text{PSI} = -3.7$, respectively) (**Figure 15 A**). When CPEB4 exon 4 was preceded by exon 2, it presented a similar behaviour, with a $\Delta\text{PSI} = -6.5$ following the treatment with low doses of haloperidol and a $\Delta\text{PSI} = 1.8$ following haloperidol at high doses (**Figure 15 B**). Thus, haloperidol does not affect CPEB4 exon 4 inclusion level in *Macaca mulatta*.

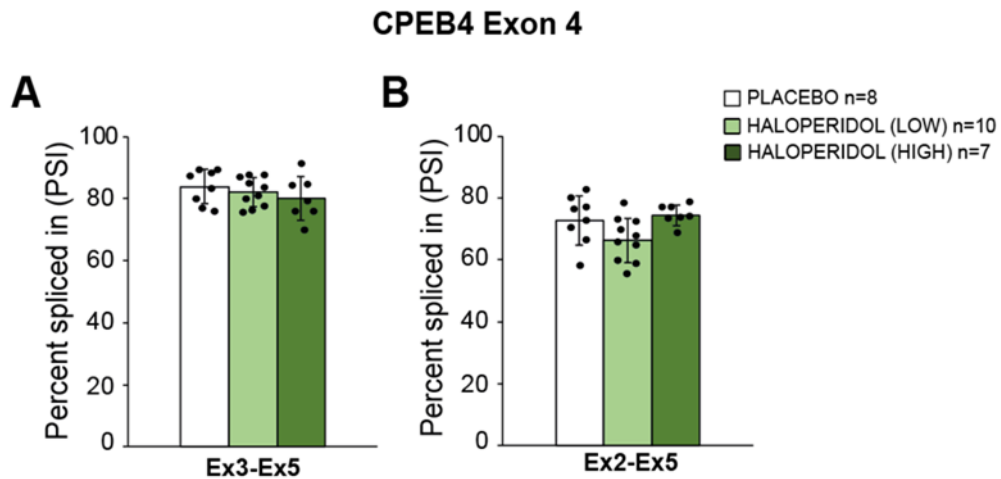


Figure 15: Effects of haloperidol in low and high doses on CPEB4 microexon inclusion in macaque DLPFC when the microexon belongs to different CPEB4 transcripts: **A)** when upstream exon is exon 3 and **B)** when upstream exon is exon 2. The tool rMATS was used.

Altogether, these studies suggest that APDs are not capable of affecting CPEB4 splicing neither *in vitro* not *in vivo* when using experimental models with normal levels of CPEB4 microexon inclusion.

6. Evaluation of protein levels of CPEB4-related SCZ-risk genes in SCZ-post-mortem brains

As mentioned, the increase of CPEB4Δ4 transcript in brains of idiopathic ASD patients correlates with concerted decreased protein expression of multiple ASD risk genes that are targets of CPEB4 (Parras et al., 2018). Since we have found an enrichment of CPE-containing and CPEB4-binding transcripts among SCZ susceptibility genes (**Table 1**), we hypothesized that the observed increase of CPEB4Δ4 in DLPFC of antipsychotic-free SCZ cases might result in decreased protein levels of multiple CPEB4-target SCZ risk genes. To test this by Western blot, we identified, among the top 5% SCZ risk genes with the most significant *P*-values in the MAGMA analysis, those i) that are brain expressed, ii) that are binders of CPEB4 (but not of CPEB1, to maximize chances of detecting CPEB4Δ4-associated changes), iii) that show evolutionary conserved presence in the 3'UTR of canonical CPE sequences in human and mice, and iv) whose transcript levels do not vary between control and SCZ samples in our analysis of PsychEncode RNA-seq data (in order to be able to show effects on translation independent of mRNA). This resulted in a short list of 43 top candidate genes (**Table 4**). We assayed antibodies for 14 of them in Western blot analyses. Seven of these antibodies yielded protein signal at the predicted molecular weight: BCL11A, CACNB2, CNTN4, CTNND1, OSBPL3, STAG1 and TCF4 (**Table 4**).

N	Gene symbol	Top 5% <i>P</i> -value in MAGMA PGC3 Gene-based	n. cCPE (human)	n. cCPE (mouse)	n. cCPE (h+m)	Tested Antibodies	WB detection (Yes/No)
1	RBFOX1	1,14E-12	10	10	20		
2	CNOT7	7,74E-06	8	11	19		
3	ATXN1	2,70E-05	10	6	16		
4	STAG1	5,90E-11	8	7	15	(CNIO)	Y
5	BCL11A	3,46E-07	10	2	12	ABE401	Y
6	PTBP2	7,48E-07	10	2	12		
7	GTF2A1	9,27E-06	8	4	12		
8	PCDH7	9,71E-09	7	5	12		
9	ZEB2	1,88E-10	7	5	12	14026-1-AP	N
10	ATXN7	8,00E-07	4	8	12	PA1-749	N
11	FRMD4B	1,46E-05	10	1	11		
12	OSBPL3	2,84E-05	8	2	10	sc-398326	Y
13	SLF2	3,16E-05	8	2	10		
14	KLHL2	8,19E-06	4	6	10		
15	AKAP6	1,70E-06	8	1	9		
16	SOCS4	2,20E-05	7	2	9		
17	CD47	2,17E-06	6	3	9		
18	NEGR1	1,69E-10	6	2	8		
19	ATP2A2	2,43E-08	5	3	8	ab2861	N
20	FXR1	6,09E-15	6	1	7		
21	PDE4B	1,78E-06	4	3	7	ab14611	N
22	TBC1D15	4,67E-07	4	3	7		
23	TP53BP1	7,05E-08	3	4	7		
24	PAG1	1,92E-05	4	2	6		
25	PTBP3	2,75E-05	4	2	6		
26	UBE2D3	3,16E-07	3	3	6		
27	THOC7	8,05E-07	4	1	5		
28	ZSWIM6	5,20E-09	3	2	5	AP54741PU-N	N
29	TCF4	1,43E-12	2	3	5	22337-1-AP	Y
30	ZDHHC5	3,56E-10	2	3	5		
31	MEF2C	2,42E-05	3	1	4	ab64644	N
32	ELAVL4	4,73E-06	2	2	4	ab200342	N
33	NPAS3	3,19E-10	2	2	4		
34	RILPL2	1,28E-11	2	2	4		
35	SHISA9	8,13E-06	2	2	4		
36	CTNND1	1,08E-10	2	1	3	HPA015955	Y
37	CACNB2	3,85E-14	1	1	2	sc-81890	Y
38	CDK5R1	4,26E-05	1	1	2		
39	CNTN4	6,44E-09	1	1	2	ab137107	Y
40	GALNT2	2,49E-08	1	1	2		
41	GPM6A	1,25E-11	1	1	2		
42	TENM3	8,21E-07	1	1	2		
43	YPEL4	1,24E-09	1	1	2		

Table 4: List of the 43 top candidate CPEB4 binders SCZ-risk genes obtained by considering the most significant *P*-values in the MAGMA analysis and that passed the filters above described. Both mouse and human cCPE are reported, as well as the reference of the antibody when used. Whether the Western blot analysis provided a proper band (Y, in green) or not (N, in red) is also indicated. Of these, only genes whose protein levels change in SCZ are indicated in bold.

Interestingly, the protein levels of BCL11A, CTNND1, OSBPL3, STAG1 and TCF4 were reduced in the FREE-SCZ samples, but not in the APDs-SCZ samples (**Figure 16**). Furthermore, through a manual curation we identified, among the 5% SCZ risk genes with the most significant *P*-values in the MAGMA analysis, four genes (GABBR2, HCN1, NEK1 and SOX5) that could not have been detected as CPEB4 binders in the CPEB4-RIP experiment performed on striatal tissue (Parras et al., 2018) because they show minimal expression in striatum (according to our published RNA-seq datasets (Elorza et al., 2021) but that are potentially interesting to this study because they are expressed in prefrontal cortex (according to our analysis of PsychEncode RNA-seq data) and they contain numerous CPEs (at least 4 in both human and mouse). Interestingly, among them, NEK1 has been reported to be translationally regulated by CPEBs in a CPEB4-related paper (Pascual et al., 2020) and we found that NEK1 protein levels are also decreased in the FREE-SCZ samples, but not in the APDs-SCZ samples (**Figure 16**). Together, these results indicate that the increase of transcripts excluding exon 4 observed in post-mortem DLPFC samples of SCZ cases free of antipsychotic medication correlates with decreased protein levels -despite unaltered transcript levels-, of multiple SCZ-associated genes that are targets of CPEB4.

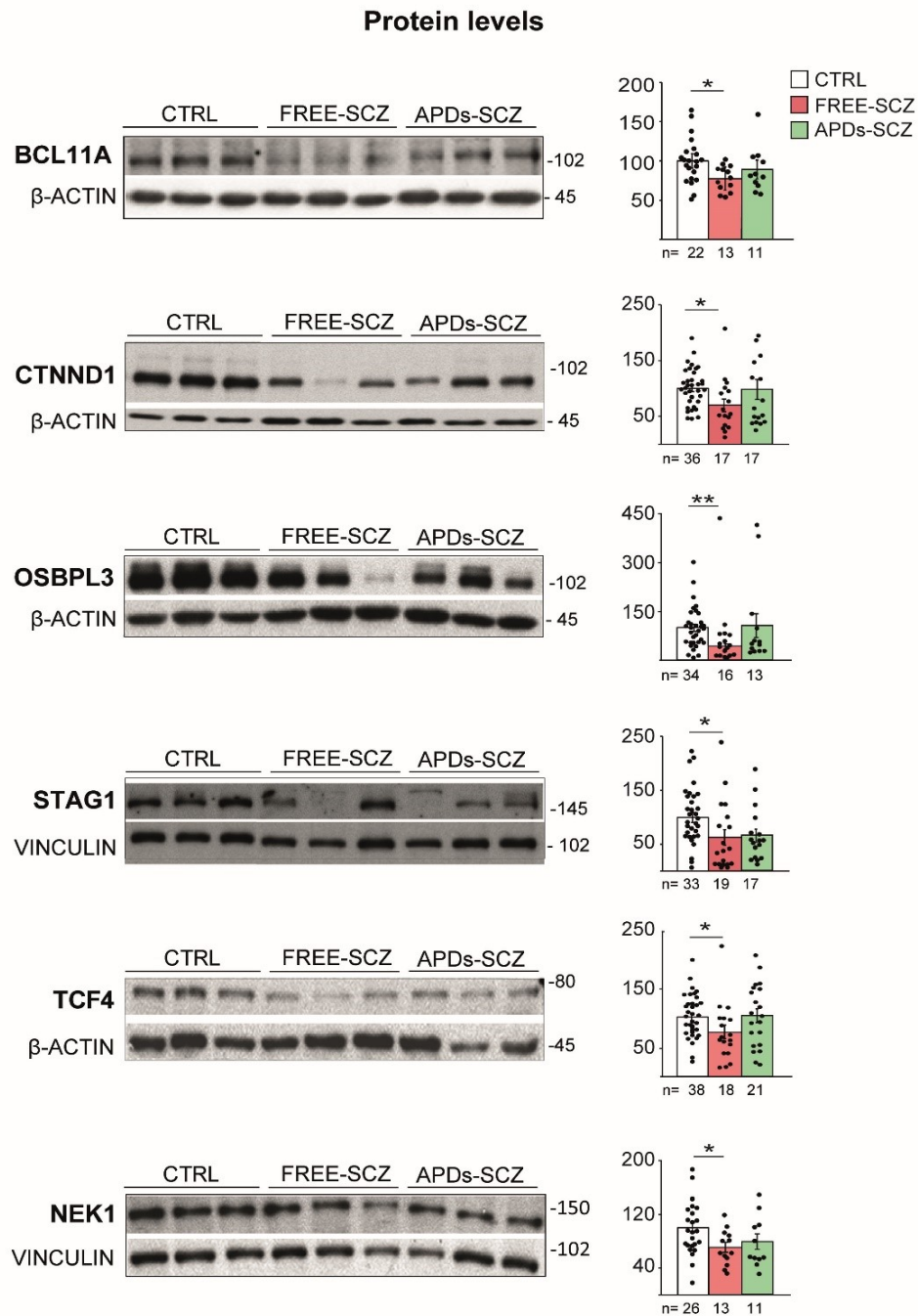


Figure 16: BCL11A, CTNND1, OSBPL3, STAG1, TCF4 and NEK1 protein levels in CTRL, FREE-SCZ and APDs-SCZ samples. Kruskal-Wallis test with Dunn's multiple comparison test or One-Way ANOVA test. * $P<0.05$, ** $P<0.01$. Data are mean with \pm SEM.

7. Analysis of protein levels of SCZ-risk genes in a mouse model overexpressing the microexon-lacking isoform of CPEB4 (Tg-PN-CPEB4Δ4)

Thus far, we have provided correlative evidence of the role of CPEB4 mis-splicing on expression of SCZ risk genes. To test *in vivo* whether an increase of CPEB4Δ4 transcript suffices to induce concerted decreased protein levels of multiple SCZ risk genes, we leveraged of a previously generated transgenic mouse line that allows conditional overexpression of CPEB4Δ4 in forebrain neurons at different time points and levels (through a tetracycline-system controlled transactivator) (Parras et al., 2018). Since strong CPEB4Δ4 overexpression starting at embryonic stage (Tg-E- CPEB4Δ4) leads to consistent ASD like phenotypes already evident in pups (Parras et al., 2018), in the context of this SCZ related study, we decided to use a transactivator mouse line with milder expression starting postnatally (PN), to generate Tg-PN-CPEB4Δ4 mice (**Figure 17 A**). Young (6 weeks) Tg-PN-CPEB4Δ4 mice show detectable overexpression of CPEB4Δ4 transcript (**Figure 17 B**).

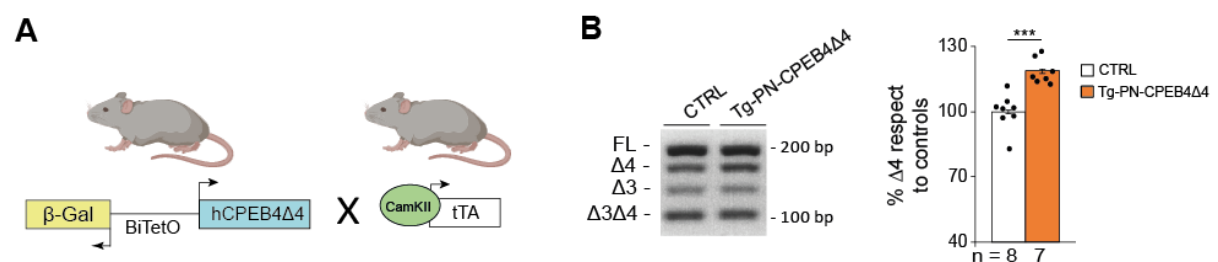


Figure 17: A) Scheme of the transgenes in the Tg-PN-CPEB4Δ4 mouse model and **B)** Representative RT-PCR of CPEB4Δ4 isoform in control and Tg-PN-CPEB4Δ4 mouse brain tissue with corresponding quantification. Student's *t*-test; ****P*<0.001. Data are mean ±SEM.

When we performed Western blot analysis of the CPE-containing SCZ genes that we found decreased in the human FREE-SCZ samples, we also observed decreased protein levels of BCL11A, OSBPL3, TCF4 and NEK1 in Tg-PN-CPEB4Δ4 mice (**Figure 18**). This therefore demonstrates that modest overexpression of CPEB4Δ4 suffices to cause concerted underexpression of multiple proteins encoded by genes associated to SCZ.

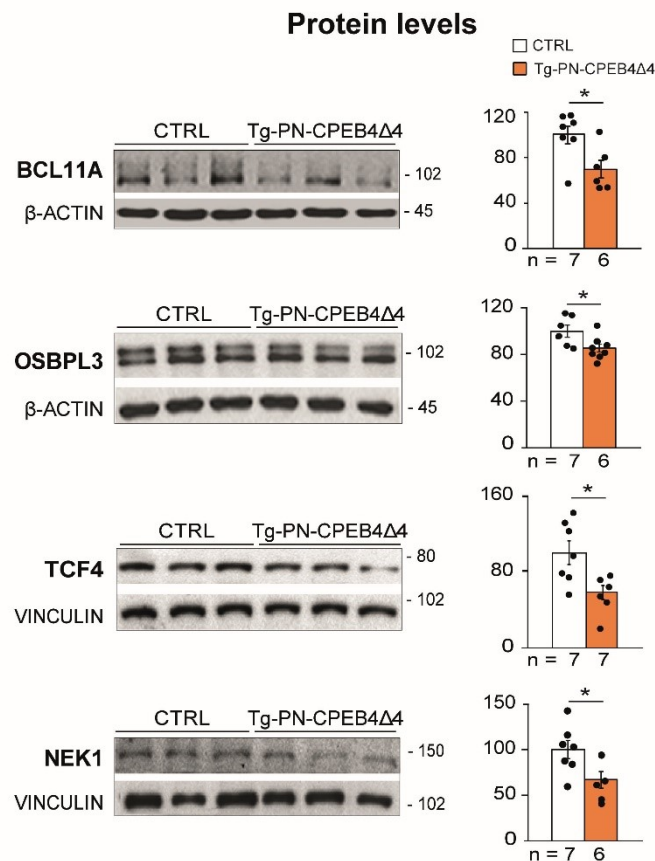


Figure 18: BCL11A, OSBPL3, TCF4 and NEK1 protein levels in control and Tg-PN-CPEB4Δ4 mouse brains. Student's *t*-test; * $P < 0.05$, Data are mean \pm SEM.

8. Behavioural characterization of Tg-PN-CPEB4Δ4 mice

To gain insight into whether the overexpression of CPEB4Δ4 with subsequent misexpression of SCZ susceptibility genes lead to SCZ-like behaviours in mice, we analysed the pre-pulse inhibition (PPI) of the startle response (SR) in Tg-PN-CPEB4Δ4 mice. As shown in **Figure 19 A**, SR response is normal in Tg-PN-CPEB4Δ4 mice at all frequencies, thus ruling out any hearing impairment and, importantly, Tg-PN-CPEB4Δ4 mice showed impaired PPI of the SR (**Figure 19 B**), therefore confirming a SCZ-like rodent phenotype.

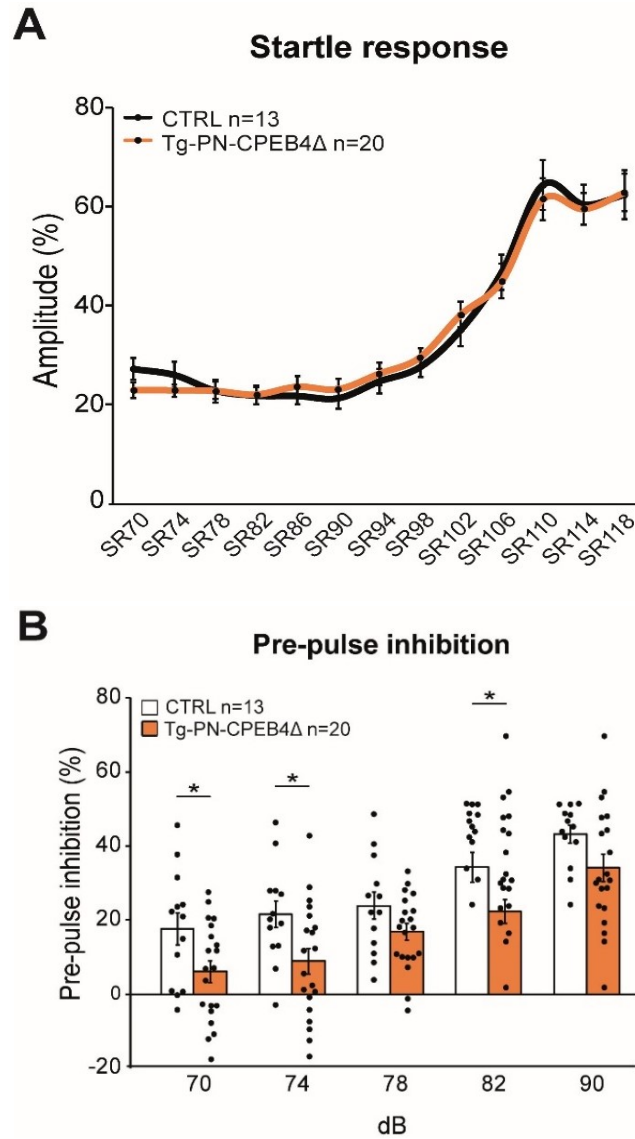


Figure 19: A) Representation of the amplitude of acoustic startle response (%) corresponding to acoustic stimuli of increasing decibels (from 70 to 118 dB) in control and Tg-PN-CPEB4Δ mice and **B)** quantification of the PPI of the acoustic startle response at 70, 74, 78, 82 and 90 Db (right). Student's *t*-test; **P*<0.05; Data are mean with ±SEM.

Interestingly, we performed this analysis also in a similar mouse model in which transgenic expression of CPEB4Δ4 begins during the embryonic life, also under the control of the CAMKII promoter (the Tg-E-CPEB4Δ4 mice). These mice have been previously characterized as an animal model of ASD (Parras et al., 2018). As shown in **Figure 20 A**, SR response is substantially normal in Tg-E-CPEB4Δ4 mice (only one difference can be observed between the two groups at 78Db), thus excluding any relevant hearing impairment. Also, Tg-E-CPEB4Δ4 mice showed no altered PPI of the SR, therefore suggesting that the timing of CPEB4 isoform imbalance is important for this SCZ-like phenotype (**Figure 20 B**).

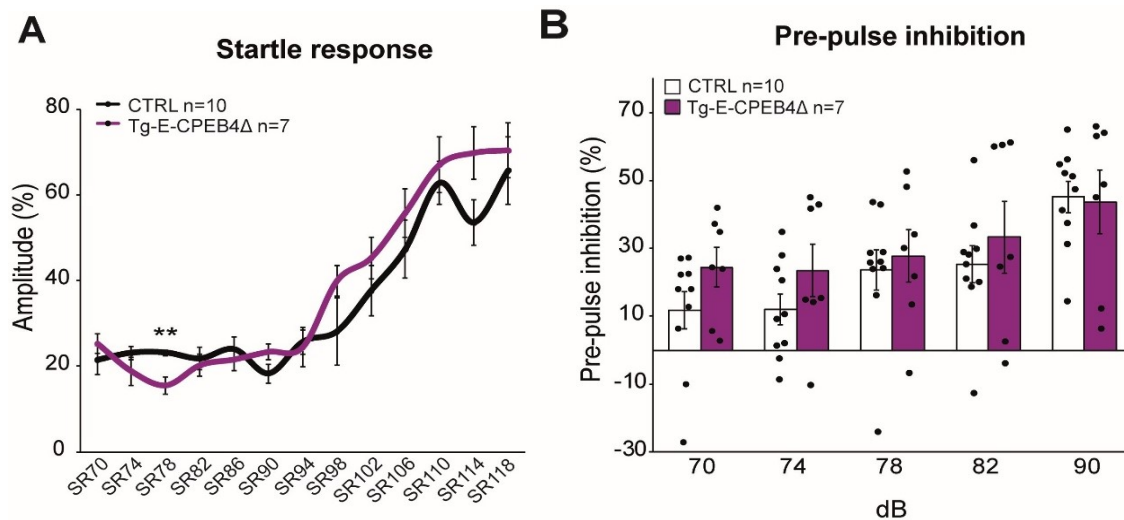


Figure 20: A) Representation of the amplitude of acoustic startle response (%) corresponding to acoustic stimuli of increasing decibels (from 70 to 118 dB) in control and Tg-E-CPEB4Δ mice and **B)** quantification of the PPI of the acoustic startle response at 70, 74, 78, 82 and 90 Db (right). Student's *t*-test; **P*<0.05; Data are mean with ±SEM.

Tg-PN-CPEB4Δ mice also showed other altered behaviours frequent in animal models of SCZ, such as increased grooming (**Figure 21 A**) and decreased social interaction (**Figure 21 B**).

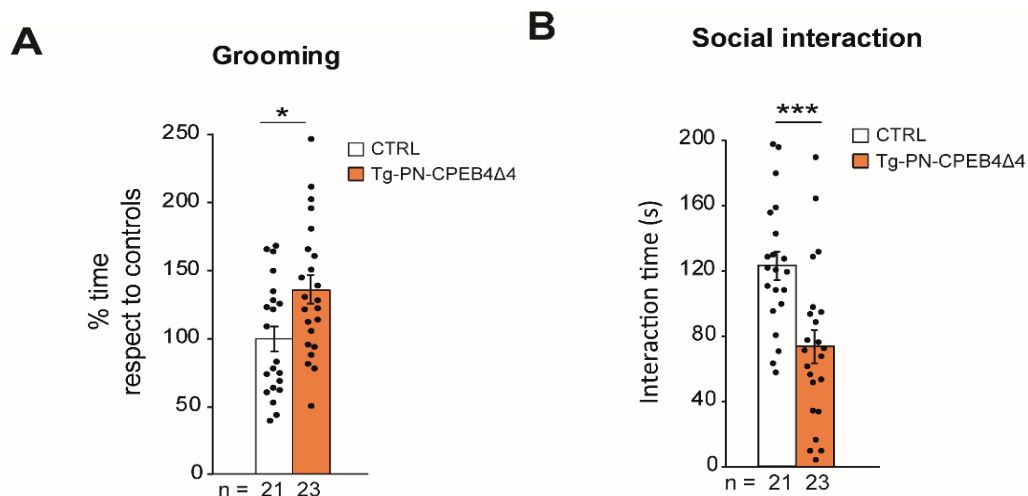


Figure 21: A) Grooming time during a 5 min trial. **B)** Interaction time with an unfamiliar mouse in social interaction test. Student's *t*-test; **P*<0.05, ****P*<0.001. Data are mean with ±SEM.

These data therefore demonstrate that in vivo overexpression of CPEB4Δ suffices to induce SCZ-related behavioural alterations of mouse models.

VII. DISCUSSION

In this study we show altered splicing of CPEB4 in DLPFC of SCZ patients. CPEB4 belongs to the family of CPEBs, which are RNA-binding proteins that regulate the translation of specific mRNAs containing CPE sequences in their 3' untranslated regions (UTRs), targeting about 40% of transcripts. CPEBs play important roles in development and neuronal plasticity (Ivshina et al., 2014) and it has been previously demonstrated that CPEB4 mis-splicing in ASD brains leads to concerted mis-expression of a plethora of high confidence ASD risk genes (Parras et al., 2018). Since ASD and SCZ are part of a neurodevelopmental continuum (Owen & O'Donovan, 2017), because of their genetic overlap and environmental factors conferring risk for both diseases (van Os et al., 2010), the aim of this work has been to explore whether the CPEB4-related alterations observed in ASD also occur in SCZ.

Using data from the largest GWAS meta-analysis in SCZ (Trubetskoy et al., 2022), we find that both CPE-harboring and CPEB4-binder gene subsets are significantly enriched in SCZ associated genes. Through RT-PCR and Western blot analyses on post-mortem DLPFC tissue, we further characterize the CPEB4 transcript isoform switch in SCZ, which occurs specifically in antipsychotic medication free individuals. Furthermore, the imbalance in CPEB4 isoforms correlates with diminished protein levels of top SCZ associated genes being either CPE-harboring and/or CPEB4-binder. Finally, we demonstrate that mild overexpression of CPEB4Δ4 in transgenic mice (to mimic the CPEB4 transcript isoform imbalance observed in SCZ brains) suffices to cause decreased protein levels of target SCZ-risk genes and to induce SCZ-associated behaviours found in mouse models of the disease.

Since our mouse genetics data demonstrate a causal role of CPEB4 mis-splicing on SCZ-associated gene expression and behaviours, our study pinpoints CPEB4 mis-splicing as a potential novel target for therapeutic intervention in SCZ. Interestingly, the splicing alteration is not observed in patients that were on antipsychotics medication at the time of death, suggesting that the current medication that improves symptoms in SCZ patients, also results in normalization of CPEB4 splicing. However, the analysis of multiple RNA-seq data exploring the study of antipsychotics effects using *in vitro* and *in vivo* models, revealed no direct effect on CPEB4 splicing. This might indicate that APDs have the potential to normalize the splicing of CPEB4 microexon when its inclusion is altered, while not having effect when inclusion levels are normal. In other words, it is possible that a schizophrenic-like pathological context is required for antipsychotic drugs to affect CPEB4 splicing. Interestingly, splicing modifying therapies, particularly antisense oligonucleotides (ASOs) have recently reached the clinic for chronic neural and neuromuscular conditions such as spinal muscular atrophy and Duchenne muscular

dystrophy (Rinaldi & Wood, 2018). It is therefore plausible that CPEB4 splicing-modifying ASOs, administered in combination with antipsychotics, might improve the efficacy of the treatment for SCZ.

Broad splicing alteration has been suggested to play a role in SCZ (Zhang et al., 2022) and it would be interesting to disentangle the molecular mechanism underneath that global aberrant RNA splicing in SCZ, and behind CPEB4 mis-splicing in particular. This might be useful to design interventions with small molecule drugs, as it has been proposed for cancer (Dong & Chen, 2020). Since the CPEB4 splicing alteration is not seen in patients taking antipsychotics and the SCZ associated transcriptomic signature is observed only in the RNA-seq-datasets of patients that show aberrant inclusion of CPEB4 exon 4 (PSI<65) -which in turn correlate with lower Lifetime antipsychotics index-, screening the RNA-seq data for splicing factors mis-expressed in SCZ PSI<65 but not in SCZ PSI>65 might be informative to identify potential splicing factors responsible for both broad and CPEB4-specific mis-splicing in SCZ.

Regarding the mechanism leading to altered splicing machinery selectively in individuals not receiving antipsychotic therapy, markers of GABAergic neurons are abundant among top downregulated genes in SCZ PSI<65 samples (while SCZ PSI>65 samples are indistinguishable from controls in terms of gene expression). The latter fits the excitation/inhibition (E/I) imbalance model of SCZ and ASD aetiology (Gao & Penzes, 2015). It is therefore conceivable that E/I imbalance in SCZ results in altered gene expression -including splicing factors governing the inclusion of CPEB4 microexon-, with antipsychotics attenuating the E/I imbalance (Llado-Pelfort et al., 2016; Smucny et al., 2022), thus counteracting abnormal expression of the relevant splicing factors and, hence, CPEB4 mis-splicing. In good agreement, it has been reported that inclusion of the program of ASD-associated microexons is neuronal activity-dependent (Quesnel-Vallieres et al., 2016).

This study further supports that, within the 'neurodevelopmental model' of psychiatric diseases (Birnbaum & Weinberger, 2017), ASD and SCZ share common molecular mechanisms as defective inclusion of CPEB4 microexon has previously been reported in ASD, with subsequent diminished protein levels of ASD-risk genes (Parras et al., 2018). A common assumption in the neurodevelopmental model is that the etiological process is relatively subtle in SCZ, such that it could be compensated for early in life, while it is stronger in ASD, leading to greater developmental pathology or disruption of neural functions that is not compensable early in life (Birnbaum & Weinberger, 2017; Owen & O'Donovan, 2017).

In line with the neurodevelopmental model of psychiatric disease, moderate overexpression of the CPEB4 Δ 4 transcript isoform starting postnatally in Tg-PN-CPEB4 Δ 4 mice results in SCZ-relevant phenotypes, like decreased PPI and social interaction, in the absence of overt visible phenotypes. On the contrary, robust overexpression of CPEB4 Δ 4 transcript starting at late embryonic stages (the Tg-E- CPEB4 Δ 4 mice) results in evident phenotypes such as stereotypic running in the periphery of the mouse home cage and hydrocephalous, apart from subtler ASD-associated behaviours (Parras et al., 2018). Interestingly, although decreased social interaction is common in mice with moderate and strong expression of CPEB4 Δ 4, diminished PPI is only found in Tg-PN-CPEB4 Δ 4 mice, as Tg-E- CPEB4 Δ 4 mice show no altered PPI, and even a tendency to increased PPI at low intensity prepulses.

In summary, this work unveils CPEB4 mis-splicing -consisting in reduced inclusion of a neuronal microexon- and concomitant decreased protein levels of CPEB4-target SCZ genes as a new molecular mechanism in SCZ and further support the etiological parallelism between SCZ and ASD.

VIII. CONCLUSIONS

1. SCZ susceptibility loci show enrichment for CPE-harboured and CPEB4-binder transcripts.
2. The neuronal specific microexon of CPEB4 displays a reduced inclusion in DLPF of SCZ cases.
3. CPEB4 exon 4 skipping correlates with typical SCZ-transcriptomic signature.
4. CPEB4 mis-splicing is not observed in antipsychotic-treated patients.
5. Cultured neuroblastoma cells and brains of *Mus musculus* or *Macaca mulata* with normal levels of CPEB4 microexon inclusion do not modify the latter after exposure to APDs.
6. CPEB4 target SCZ genes are decreased at protein level in DLPFC of antipsychotic-free SCZ cases.
7. Protein levels of CPE-containing and CPEB4-target SCZ genes are diminished in Tg-PN-CPEB4 Δ 4 mice.
8. CPEB4 Δ 4 overexpressing mice show SCZ-linked behaviours.

VIII. CONCLUSIONES

1. Los loci de susceptibilidad a SCZ muestran un enriquecimiento de transcritos que albergan secuencias CPE y que se unen a CPEB4.
2. El microexón específico neuronal de CPEB4 muestra una inclusión reducida en DLPFC de casos de SCZ.
3. La omisión del exón 4 de CPEB4 se correlaciona con la firma transcriptómica típica de cerebros de casos de SCZ.
4. No se observa splicing incorrecto de CPEB4 en casos de SCZ tratados con antipsicóticos.
5. Células de neuroblastoma cultivadas y cerebros de *Mus musculus* o *Macaca mulata* con niveles normales de inclusión de microexón de CPEB4 no modifican este último tras exposición a APDs.
6. Los genes de susceptibilidad a SCZ que son diana de CPEB4 muestran niveles disminuidos de proteína en DLPFC de casos de SCZ libres de antipsicóticos.
7. Los niveles de proteína de los genes de susceptibilidad a SCZ que contienen secuencias CPE y se unen a CPEB4 están disminuidos en ratones que sobreexpresan CPEB4 Δ 4.
8. Ratones que sobreexpresan CPEB4 Δ 4 muestran comportamientos vinculados a SCZ.

IX. REFERENCES

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X. ANNEX I: SUPPLEMENTARY FILES

Extra files related to this study, such as the complete MAGMA analysis output and the list of 771 DEGs described in paragraphs 1 and 3 of the “Results” section of this thesis, respectively, are public available for download at <https://doi.org/10.1101/2022.09.22.508890>.

XI. ANNEX II: Ph.D. PUBBLICATIONS

1. Parras, A., de Diego-Garcia, L., Alves, M., Beamer, E., Conte, G., Jimenez-Mateos, E. M., **Ollà I.**, Hernandez-Santana Y.,...Engel, T. (2020). Polyadenylation of mRNA as a novel regulatory mechanism of gene expression in temporal lobe epilepsy. *Brain*, 143(7), 2139-2153. <https://doi.org/10.1093/brain/awaa168>
2. Conte, G., Parras, A., Alves, M., **Ollà, I.**, De Diego-Garcia, L., Beamer, E., . . . Engel, T. (2020). High concordance between hippocampal transcriptome of the mouse intra-amygdala kainic acid model and human temporal lobe epilepsy. *Epilepsia*, 61(12), 2795-2810. <https://doi.org/10.1111/epi.16714>
3. **Olla, I.**, Santos-Galindo, M., Elorza, A., & Lucas, J. J. (2020). P2X7 Receptor Upregulation in Huntington's Disease Brains. *Front Mol Neurosci*, 13, 567430. <https://doi.org/10.3389/fnmol.2020.567430>
4. **Ollà, I.**, Pardiñas, A. F., Parras, A., Hernández, I. H., Santos-Galindo, M., Picó, S., . . . Lucas, J. J. (2022). Pathogenic mis-splicing of CPEB4 in schizophrenia. *bioRxiv*, 2022.2009.2022.508890. <https://doi.org/10.1101/2022.09.22.508890>

Pathogenic mis-splicing of *CPEB4* in schizophrenia

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Running title: *CPEB4* mis-splicing in schizophrenia

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ABSTRACT

Schizophrenia (SCZ) is caused by a complex interplay of polygenic risk and environmental factors, which might alter regulators of gene expression leading to pathogenic mis-expression of SCZ risk genes. The RNA binding protein family CPEB (CPEB1, CPEB2, CPEB3, CPEB4) regulates the translation of target RNAs bearing CPE sequences in their 3'UTR (approximately 40% of overall genes). We previously identified CPEB4 as a key dysregulated translational regulator in autism spectrum disorder (ASD), proving that its neuronal-specific microexon (exon 4) is mis-spliced in brains of ASD probands, leading to concerted underexpression of a plethora of high confidence ASD-risk genes. The genetic and pathogenic mechanisms shared between SCZ and ASD make it plausible that mis-splicing of CPEB4 might occur also in SCZ patients, leading to downstream altered brain expression of multiple SCZ-related genes. In this study, we first analyzed Psychiatric Genomics Consortium GWAS data and found significant enrichment of SCZ-associated genes for CPEB4-binder transcripts. We also found decreased inclusion of CPEB4 microexon in postmortem prefrontal cortex of SCZ probands. This mis-splicing is associated with decreased protein levels of SCZ-associated genes that are targets of CPEB4. Interestingly, this happens specifically in individuals with low exposure to antipsychotic medication. Finally, we show that mild overexpression of a CPEB4 transcript lacking exon 4 (CPEB4Δ4) in mice suffices to induce decreased protein levels of SCZ genes targeted by CPEB4; these mice are also characterized by SCZ-linked behaviors. In summary, this study identifies aberrant CPEB4 splicing and downstream mis-expression of SCZ-risk genes as a novel etiological mechanism in SCZ.

Introduction

Schizophrenia (SCZ) is a severe psychiatric disorder characterized by abnormalities in thought and cognition which affects nearly 1% of the adult population (Owen et al., 2016). Genetic and epidemiological evidence indicate that SCZ is caused by common and rare risk alleles combined with environmental factors (Henriksen et al., 2017). In the last decade, advances in genomics and collaborative efforts from international consortia have identified around 300 risk alleles although biological and pathophysiological mechanisms are still largely unknown (Hall et al., 2015; PGC, 2015). The identified genetic risk variants range from rare copy number variants, multiple rare single nucleotide variants and loci containing common genetic variants, the latter exerting individually small effect sizes (Henriksen et al., 2017). Since the precise molecular determinants that integrate polygenic risk and environmental risk factors in SCZ are not fully elucidated, it is important to investigate altered regulators of gene expression in the brains of individuals with SCZ that could explain orchestrated pathogenic mis-expression of numerous risk genes during both neurodevelopment and adult life.

Cytoplasmic polyadenylation element binding proteins (CPEBs) are a family of RNA-binding proteins that regulate the stability and translation of specific mRNAs containing CPE sequences in their 3' untranslated regions (UTRs) (Ivshina et al., 2014) accounting for about 40% of the transcriptome (Parras et al., 2018; Pique et al., 2008). In vertebrates, the family consist of four paralogs (CPEB1, CPEB2, CPEB3 and CPEB4) where CPEB2-4 are closely related and CPEB1 is the most distant member of the family (Wang and Cooper, 2010). CPEBs mediate translational repression or activation of their target transcripts by inducing, respectively, shortening or elongation of the poly(A)-tails (Ivshina et al., 2014). CPEBs were first discovered through their role in development, as they regulate the expression of multiple mRNAs in response to embryonic environmental clues, such as hormones (Ivshina et al., 2014; Sarkissian et al., 2004). More recently, CPEBs have been shown to play important roles in cell division and metabolism

(Alexandrov et al., 2012; Burns and Richter, 2008; Ivshina et al., 2014). In the adult brain, CPEBs are known to regulate many genes involved in synaptic plasticity, thus contributing to complex processes such as learning and memory consolidation (Drisaldi et al., 2015; Fioriti et al., 2015; Ivshina et al., 2014; Pavlopoulos et al., 2011; Si et al., 2003; Wu et al., 1998). Consistently, altered CPEBs have been associated with cancer (Ortiz-Zapater et al., 2011; Perez-Guijarro et al., 2016) and pathogenic hepatic angiogenesis (Calderone et al., 2016), neurological diseases including epilepsy (Parras et al., 2020) and Huntington's disease (Picó et al., 2021) as well as with ASD (Parras et al., 2018; Udagawa et al., 2013).

In ASD, CPEB4 has emerged as a key pathogenic effector that is altered in brain tissues of cases, resulting in the simultaneous mis-expression of most high confidence ASD-risk genes (Parras et al., 2018). More precisely, we reported that individuals with ASD show an imbalance of CPEB4 transcript isoforms resulting from a decreased inclusion of a neuronal-specific microexon (exon 4) (Parras et al., 2018). Microexons, which are exons of 27 or fewer nucleotides, show a pattern of neural specific alternative splicing (AS) that has been shown to be dysregulated globally in ASD (Irimia et al., 2014; Li et al., 2015). We have also shown that the resulting increase of CPEB4Δ4 transcript isoforms in ASD brain tissues correlates with decreased protein levels of multiple ASD-risk genes whose transcripts harbour CPE sequences in their 3'UTR (Parras et al., 2018). Furthermore, we found that transgenic mice overexpressing CPEB4Δ4 showed decreased protein expression of a plethora of ASD risk genes and display ASD-like traits (Parras et al., 2018).

Schizophrenia and ASD share genetic risk, and by inference pathogenic mechanisms, and have been proposed to lie on a neurodevelopmental continuum (Owen and O'Donovan, 2017). This led us to hypothesize that alteration of CPEBs, particularly CPEB4, might also be observed in

the brains of individuals with SCZ, and that this would lead to pathogenic mis-expression of multiple SCZ-risk-genes.

Results

SCZ susceptibility loci show enrichment for CPE-harboring and CPEB4-binder transcripts

We decided to explore whether CPE-containing and CPEB4-binding transcripts were over-represented within SCZ-associated genes. We first used MAGMA to perform gene-set analyses based on the summary statistics from the Wave 3 Psychiatric Genomics Consortium (PGC) GWAS of schizophrenia ("core dataset"; 67,390 cases and 94,015 controls) (Trubetskoy et al., 2022). Successively, we examined gene sets comprised of (i) genes containing canonical CPE (cCPE) sequences in their 3'UTR (Pique et al., 2008); (ii) genes identified in genome-wide RNA immunoprecipitation analyses from mouse brain structures as CPEB1- (Parras et al., 2018), CPEB3- (Lu et al., 2021) or CPEB4- (Parras et al., 2018) binders; (iii) gene sets implicated in the pathophysiology of psychiatric disorders: Fragile X Mental Retardation Protein (FMRP) targets (Clifton et al., 2021; Darnell et al., 2011), genes specifically involved in synaptic development and function (Koopmans et al., 2019), and finally (iv) as a comparator, a more general set of all brain-expressed genes (Fagerberg et al., 2014). The results of associations with SCZ-associated genes showed "CPEB4 target" as the top significant gene-set amongst those under study ($P=1.76 \times 10^{-9}$; Table 1), together with already known gene-sets implicated in psychiatric disorders such as FMRP targets and brain-expressed genes ($P=4.97 \times 10^{-9}$ and $P=6.13 \times 10^{-19}$, respectively; Table 1). MAGMA models were conditioned on a binary indicator to control for overlapping genes amongst gene-sets, given the known substantial overlap between FMRP and CPEB targets (Udagawa et al., 2013), or CPEB target genes and brain expressed genes (Parras et al., 2018). After conditional analysis for each of FMRP targets, synaptic genes, and brain-expressed genes, the "CPEB4 target" gene-set remained the most significant (Table 1). As expected, given the overlap among the CPEB1-, CPEB3-, and CPEB4-targets, CPEB1 and CPEB3 targets also showed evidence for enrichment for SCZ associations as did the gene set of "cCPE-

containing genes” (Table 1). These results therefore confirmed enrichment of CPE-containing and CPEB4-binding transcripts in SCZ susceptibility genes.

Decreased inclusion of the neuronal specific microexon of CPEB4 in SCZ

To explore if the splicing alteration of CPEB4 seen in brains of ASD cases also happens in SCZ, we performed vast-tools (Tapial et al., 2017) analysis on the (BA46) cortex RNA-seq data from people with SCZ (n=95) and matched controls (n=75) of the PsychENCODE Consortium BrainGVEX RNA-seq study (PsychEncode-Consortium et al., 2015). After quality control (QC) procedures (see methods), 54 control and 66 SCZ samples met the thresholds for subsequent splicing analyses. Regarding the four CPEB genes, the only skipped exon (SE) event that differed significantly between controls and SCZ was exon 4 of CPEB4 (i.e. the 24 nucleotide microexon) (Supplementary Table 2). Akin to ASD, inclusion of this microexon was significantly reduced in those with SCZ compared with controls. More precisely, in average, 64.1% of the CPEB4 transcripts contained the microexon in the control samples versus 60.4% in the SCZ samples, resulting in a significant percent spliced in index difference (ΔPSI) = -3.63 ($P=0.023$) (Fig. 1A).

Marked exon 4 skipping correlates with typical SCZ-transcriptomic signature

When we analyzed the relative frequencies of the percentages of inclusion of CPEB4 exon 4, we noticed that the distributions significantly differed between control and SCZ samples ($P=0.048$) (Fig. 1B, lower panel). When restricting the analysis to only male samples, two peaks could be clearly observed in the SCZ distribution, the major peak corresponded to a value of percent spliced in index (PSI) \approx 55, while the minor peak corresponded to $\text{PSI}\approx$ 70, the latter matching with the mode value in the distribution of control samples (Fig. 1B, upper panel). Therefore, in terms of inclusion of exon 4 of CPEB4, the observed distribution in SCZ seem to represent two subpopulations, one that resembles controls, and one whose peak value differs

from that in controls with $\Delta\text{PSI}=-15$. To further explore whether the two parts of the distribution may represent two different subpopulations, we interrogated global differences in transcript levels with respect to controls. In view of the inflections of SCZ distributions at $\text{PSI}=65$ (Fig. 1B), we stratified the 66 SCZ samples analyzed in Fig. 1a into two pools based on this cut-off. Interestingly, gene expression at the $\text{PSI}>65$ -SCZ subpopulation ($n=21$) was similar that of control samples (Supplementary Table 3). In contrast, the $\text{PSI}<65$ -SCZ subpopulation ($n=45$) showed 771 differentially expressed genes (DEG), 492 upregulated and 279 downregulated (Supplementary Table 3). Remarkably, the DEG signature in the $\text{PSI}<65$ -SCZ subpopulation, particularly the downregulated genes, is highly concordant with DEG signatures previously reported for dorsolateral prefrontal cortex (DLPFC) of SCZ subjects³²⁻³³. More precisely, the representation factor (see methods) was 9.3 ($P<1.17\times 10^{-7}$) regarding RNA-seq based signatures (Yang et al., 2020) and 12.7 ($P<1.94\times 10^{-6}$) regarding gene-chip analysis (Hashimoto et al., 2008), with marked expression deficits in GABA neurotransmission-related transcript such as GAD1 (GAD67) or the neuropeptides somatostatin (SST), neuropeptide Y (NPY) and cholecystinin (CCK) (Supplementary Table 4). These results indicate that the SCZ individuals seem to segregate into two subpopulations, one that matches controls in terms of both CPEB4 exon 4 inclusion ($\text{PSI}\approx 70$) and global gene expression, and the other with lower CPEB4 exon 4 inclusion ($\text{PSI}\approx 55$) and a paradigmatic SCZ DEG signature. This suggests interrelated alterations of transcription and of CPEB4-dependent translational regulation in SCZ.

CPEB4 mis-splicing is not observed in antipsychotic-treated patients

There is evidence of antipsychotic medication correlating with diminished alteration of protein expression in post-mortem brains from individuals with schizophrenia (Chan et al., 2011). This led us to speculate that the two SCZ subpopulations arbitrarily delimited with the $\text{PSI}=65$ cut-off value might correlate with a different degree of exposure to antipsychotic drugs (APDs).

Interestingly, the PsychENCODE database metadata provide “Lifetime Antipsychotics” index value for 45 of the 66 SCZ analyzed samples and, when we analyzed the mean Lifetime APDs index values for each group, we found that it was significantly higher in the PSI>65-SCZ (n=15) subpopulation compared to PSI<65-SCZ (n=30) subpopulation ($95,237 \pm 20,179$ vs. $42,770 \pm 7,786$; $P=0.048$) (Fig. 2A). This observation suggests that marked alteration in CPEB4 exon 4 inclusion might be specific to SCZ individuals with lower exposure to antipsychotic medication.

To confirm in an independent cohort of samples the decreased usage of exon 4 in SCZ observed through RNA-seq analysis and to settle or discard the correlation with antipsychotic medication, we decided to perform RT-PCR analysis in SCZ postmortem DLPFC samples from the Basque Institute of Legal Medicine and the NIH NeuroBioBank (CTRL n=57 and SCZ n=42) in which a complete toxicological examination was performed by mass spectrometry to detect the presence of antipsychotics, as well as mood stabilizers, cotinine, antidepressants and benzodiazepines, at the time of death. Controls positive for any substance, as well as samples with low RNA quality, were excluded from the analysis (see methods). Since exon 3 (57 nucleotides) of CPEB4 is also alternatively spliced (Fig. 2B), there are four CPEB4 isoforms that can be detected with PCR primers hybridizing to exons 2 and 5, two transcripts that include exon 4 (full-length (FL) and CPEB4Δ3) and two lacking it (CPEB4Δ4 and CPEB4Δ3Δ4) (Fig. 2B). In control samples, the FL-CPEB4 transcript predominates (Fig. 2B-C) and, as expected from the RNA-seq data, FL-CPEB4 decreases in samples from SCZ individuals that were free of antipsychotics at the time of death (FREE-SCZ) (Fig. 2B-C). As expected, patients of the FREE-SCZ sample show an increase of CPEB4Δ4 compared to controls (Fig. 2B-C), with some of the FREE-SCZ samples showing a completely opposite pattern of isoforms respect to controls, as CPEB4Δ4 and CPEB4Δ3Δ4 clearly predominate (Fig. 2B). As suggested by both the RNA-seq and Lifetime APDs index analyses, SCZ individuals under APDs medication at the time of death

(APDs-SCZ) significantly differed from FREE-SCZ samples (Fig. 2C), showing FL-CPEB4 and CPEB4 Δ 4 isoform levels to be indistinguishable from those in controls (Fig. 2C). Consequently, the Δ Ex4/Ex4+ isoform ratio is markedly increased in FREE-SCZ samples, while unaltered in APDs-SCZ samples (Fig. 2D). Together, these results demonstrate that individuals with SCZ show a shift in the ratio of exon 4-dependent CPEB4 transcripts, selectively in the absence of antipsychotic medication.

Decreased protein levels of CPEB4 target SCZ genes in antipsychotic-free SCZ brains

As mentioned, the increase of CPEB4 Δ 4 transcript in brains of idiopathic ASD patients correlates with concerted decreased protein expression of multiple ASD risk genes that are targets of CPEB4 (Parras et al., 2018). Since we have found an enrichment of CPE-containing and CPEB4-binding transcripts among SCZ susceptibility genes (Table 1), we hypothesized that the observed increase of CPEB4 Δ 4 in DLPFC of antipsychotic-free SCZ cases might result in decreased protein levels of multiple CPEB4-target SCZ risk genes. To test this by Western blot, we identified, among the top 5% SCZ risk genes with the most significant *P*-values in the MAGMA analysis, those i) that are brain expressed, ii) that are binders of CPEB4 (but not of CPEB1, to maximize chances of detecting CPEB4 Δ 4-associated changes), iii) that show evolutionary conserved presence in the 3'UTR of canonical CPE sequences in human and mice, and iv) whose transcript levels do not vary between control and SCZ samples in our analysis of PsychEncode RNA-seq data (in order to be able to show effects on translation independent of mRNA). This resulted in a short list of 43 top candidate genes (Supplementary Table 5) and we assayed antibodies for 14 of them in Western blot analyses. Seven of these antibodies yielded protein signal at the predicted molecular weight: *BCL11A*, *CACNB2*, *CNTN4*, *CTNND1*, *OSBPL3*, *STAG1* and *TCF4* (Supplementary Table 5). Interestingly, the protein levels of *BCL11A*, *CTNND1*, *OSBPL3*, *STAG1* and *TCF4* were reduced in the FREE-SCZ samples, but not in the APDs-SCZ samples (Fig. 3). Furthermore, through a manual curation we identified, among the 5% SCZ risk

genes with the most significant *P*-values in the MAGMA analysis, four genes (*GABBR2*, *HCN1*, *NEK1* and *SOX5*) that could not have been detected as CPEB4 binders in the CPEB4-RIP experiment performed on striatal tissue (Parras et al., 2018) because they show minimal expression in striatum (according to our published RNA-seq datasets (Elorza et al., 2021)) but that are potentially interesting to this study because they are expressed in prefrontal cortex (according to our analysis of PsychEncode RNA-seq data) and they contain numerous CPEs (at least 4 in both human and mouse). Interestingly, among them, NEK1 has been reported to be translationally regulated by CPEBs in a CPEB4-related paper (Pascual et al., 2020) and we found that NEK1 protein levels are also decreased in the FREE-SCZ samples, but not in the APDs-SCZ samples (Fig. 3). Together, these results indicate that the increase of transcripts excluding exon 4 observed in postmortem DLPFC samples of SCZ cases free of antipsychotic medication correlates with decreased protein levels -despite unaltered transcript levels-, of multiple SCZ-associated genes that are targets of CPEB4.

Decreased protein levels of CPE-containing and CPEB4-target SCZ genes in CPEB4Δ4 overexpressing mice

Thus far, we have provided correlative evidence of the role of CPEB4 mis-splicing on expression of SCZ risk genes. To test *in vivo* whether an increase of CPEB4Δ4 transcript suffices to induce concerted decreased protein levels of multiple SCZ risk genes, we leveraged of a previously generated transgenic mouse line that allows conditional overexpression of CPEB4Δ4 in forebrain neurons at different time points and levels (through a tetracycline-system controlled transactivator) (Parras et al., 2018). Since strong CPEB4Δ4 overexpression starting at embryonic stages leads to consistent ASD like phenotypes already evident in pups (Parras et al., 2018), in the context of this SCZ related study, we decided to use a transactivator mouse line with milder expression starting postnatally (PN), to generate Tg-PN-CPEB4Δ4 mice. Young (6 weeks) Tg-PN-CPEB4Δ4 mice show detectable overexpression of CPEB4Δ4 transcript (Fig.

4A) and when we performed Western blot analysis of the CPE-containing SCZ genes that we found decreased in the human FREE-SCZ samples, we also observed decreased protein levels of BCL11A, OSBPL3, TCF4 and NEK1 in Tg-PN-CPEB4Δ4 mice (Fig. 4B). This therefore demonstrates that modest overexpression of CPEB4Δ4 suffices to cause concerted under-expression of multiple proteins encoded by genes associated to SCZ.

CPEB4Δ4 overexpressing mice show SCZ-linked behaviors

To gain insight into whether the overexpression of CPEB4Δ4 with subsequent mis-expression of SCZ susceptibility genes lead to SCZ-like behaviors in mice, we analyzed the pre-pulse inhibition (PPI) of the startle response (SR) in Tg-PN-CPEB4Δ4 mice. As shown in Fig. 5A, SR response is normal in Tg-PN-CPEB4Δ4 mice at all frequencies, thus ruling out any hearing impairment and, importantly, Tg-PN-CPEB4Δ4 mice showed impaired PPI of the SR, therefore confirming a SCZ-like rodent phenotype. Tg-PN-CPEB4Δ4 mice also showed other altered behaviors frequent in animal models of SCZ, such as increased grooming and decreased social interaction (Fig. 5B-C). These data therefore demonstrate that *in vivo* overexpression of CPEB4Δ4 suffices to induce SCZ-related behavioral alterations of mouse models.

Discussion

In this study we show altered splicing of *CPEB4* in DLPFC of SCZ patients. CPEB4 belongs to a family of RNA-binding proteins that regulate the translation of specific mRNAs containing CPE sequences in their 3' untranslated regions (UTRs), targeting about 40% of transcripts. CPEBs play important roles in development and neuronal plasticity (Ivshina et al., 2014) and we have previously demonstrated CPEB4 mis-splicing in ASD brains leading to concerted mis-expression of a plethora of high confidence ASD risk genes (Parras et al., 2018). Using data from the largest GWAS meta-analysis in SCZ (Trubetskoy et al., 2022), we find that both CPE-harboring and CPEB4-binder gene subsets are significantly enriched in SCZ associated genes. Through RT-PCR and Western blot analyses on postmortem DLPFC tissue, we further characterize the CPEB4 transcript isoform switch in SCZ, which occurs specifically in antipsychotic medication-free individuals. Furthermore, the imbalance in CPEB4 isoforms correlates with diminished protein levels of top SCZ associated genes being either CPE-harboring and/or CPEB4-binder. Finally, we demonstrate that mild overexpression of CPEB4Δ4 in transgenic mice (to mimic the CPEB4 transcript isoform imbalance observed in SCZ brains) suffices to cause decreased protein levels of target SCZ-risk genes and to induce SCZ-associated behaviors found in mouse models of the disease.

Since our mouse genetics data demonstrate a causal role of CPEB4 mis-splicing on SCZ-associated gene expression and behaviors, our study pinpoints CPEB4 mis-splicing as a potential novel target for therapeutic intervention in SCZ. Interestingly, the splicing alteration is not observed in patients that were on antipsychotics medication at the time of death, suggesting that the current medication that improves symptoms in SCZ patients, also results in normalization of CPEB4 splicing. Recently, splicing modifying therapies, particularly antisense oligonucleotides (ASOs) have reached the clinic for chronic neural and neuromuscular conditions such as spinal muscular atrophy and Duchenne muscular dystrophy (Rinaldi and

Wood, 2018). It is therefore conceivable that CPEB4 splicing-modifying ASOs, if administered in combination with antipsychotics, might improve the efficacy of the treatment.

Broad splicing alteration has been suggested to play a role in SCZ (Zhang et al., 2022) and it would be interesting to disentangle the molecular mechanism underneath that global aberrant RNA splicing in SCZ, and behind CPEB4 mis-splicing in particular. This might be useful to design interventions with small molecule drugs, as has been proposed for cancer (Dong and Chen, 2020). Since the CPEB4 splicing alteration is not seen in patients taking antipsychotics and the SCZ associated transcriptomic signature is observed only in the RNAseq-datasets of patients that show aberrant inclusion of CPEB4 exon 4 (PSI<65) -which in turn correlate with lower Lifetime antipsychotics index-, screening the RNA-seq data for splicing factors mis-expressed in SCZ PSI<65 but not in SCZ PSI>65 might be informative to identify potential splicing factors responsible for both broad and CPEB4-specific mis-splicing in SCZ .

Regarding the mechanism leading to altered splicing machinery selectively in individuals not receiving antipsychotic therapy, markers of GABAergic neurons are abundant among top downregulated genes in SCZ PSI<65 samples (while SCZ PSI>65 samples are indistinguishable from controls in terms of gene expression). The latter fits the excitation/inhibition (E/I) imbalance model of SCZ and ASD etiology (Gao and Penzes, 2015). It is therefore conceivable that E/I imbalance in SCZ results in altered gene expression -including splicing factors governing the inclusion of CPEB4 microexon-, with antipsychotics attenuating the E/I imbalance (Llado-Pelfort et al., 2016; Smucny et al., 2022), thus counteracting abnormal expression of the relevant splicing factors and, hence, CPEB4 mis-splicing. In good agreement, it has been reported that inclusion of the program of ASD-associated microexons is neuronal activity-dependent (Quesnel-Vallieres et al., 2016).

This study further supports that, within the ‘neurodevelopmental model’ of psychiatric diseases (Birnbaum and Weinberger, 2017), ASD and SCZ share common molecular mechanisms as defective inclusion of CPEB4 microexon has previously been reported in ASD, with subsequent diminished protein levels of ASD-risk genes (Parras et al., 2018). A common assumption in the neurodevelopmental model is that the etiological process is relatively subtle in SCZ, such that it could be compensated for early in life, while it is stronger in ASD, leading to greater developmental pathology or disruption of neural functions that is not compensable early in life (Birnbaum and Weinberger, 2017; Owen and O'Donovan, 2017). In line with this model, moderate overexpression of the CPEB4Δ4 transcript isoform starting postnatally in Tg-PN-CPEB4Δ4 mice results in SCZ-relevant phenotypes, like decreased PPI and social interaction, in the absence of overt visible phenotypes. On the contrary, robust overexpression of CPEB4Δ4 transcript starting at embryonic stages results in evident phenotypes such as stereotypic running in the periphery of the mouse home cage and hydrocephalous, apart from more subtle ASD-associated behaviors (Parras et al., 2018). Interestingly, although decreased social interaction is common in mice with moderate and strong expression of CPEB4Δ4, diminished PPI is only found in Tg-PN-CPEB4Δ4 mice, as mice with prenatally-starting strong CPEB4Δ4 overexpression show a different behavior in the PPI test consisting on no effect of high intensity prepulses and even a tendency to increased PPI at low intensity prepulses (data not shown).

In summary, our study unveils CPEB4 mis-splicing -consisting in reduced inclusion of a neuronal microexon- and concomitant decreased protein levels of CPEB4-target SCZ genes as a new molecular mechanism in SCZ and further support the etiological parallelism between SCZ and ASD.

Materials / Subjects and Methods

CPEBs-related genes enrichment analysis among SCZ risk genes

To establish a relationship between SCZ genes and CPEBs, we carried out gene-set enrichment analyses through MAGMA, a bioinformatics tool for gene-level analyses of GWAS data sets (de Leeuw et al., 2015). European and East Asian SCZ GWAS summary statistics were obtained from the latest public multi-stage study of the Psychiatric Genomics Consortium (Trubetskoy et al., 2022), and processed through a gene-level analysis in MAGMA as described therein. Briefly, this involved computing gene-wide mean *P*-values with each ancestry-specific subsample, which were then meta-analysed leading to combined gene-level data from 67,390 SCZ cases and 94,015 controls. For these analyses, gene boundaries were retrieved from Refseq (GRCh38.p13; last updated on 23-11-2020), lifted over to GRCh37 coordinates and expanded by 35kb/10kb upstream/downstream flanking regions to encompass potential regulatory elements (Maston et al., 2006). Regarding CPE-containing genes in humans, we considered a previously published database (Pique et al., 2008). CPEB1 and CPEB4 gene lists were derived from an RNA immunoprecipitation (RIP) study performed in mouse striatum (Parras et al., 2018). A list of CPEB3 binders has been recently obtained through a RIP experiment performed in mouse cortical tissue (Lu et al., 2021). Covariate gene sets used to carry out conditional analyses included (i) targets of the FMRP regulon (Darnell et al., 2011), (ii) genes with significant expression in the brain (Fagerberg et al., 2014); as processed by (Genovese et al., 2016) and (iii) genes specifically involved in synaptic development and function (Koopmans et al., 2019).

RNA-seq data analysis

To explore the alternatively spliced events of CPEBs in SCZ patients, we examined the data in the BrainGVEX RNA-seq study³¹. Such study comprises well-characterized human post-mortem prefrontal (BA46) cortex samples from SCZ subjects (n=95) and matched controls (n=75),

originating from the Stanley Medical Research Institute (SMRI). Fastq files were downloaded from the PsychENCODE Consortium (PEC) Knowledge Portal repository (<https://psychencode.synapse.org/>). For quality control analysis, we run FastQC (v0.11.9). Then, the four parameters i) *per base GC content*, ii) *per sequence GC content*, iii) *sequence duplication level* and iv) *overrepresented sequences* were monitored for each strand (forward and reverse), thus resulting in a total of 8 tests. Finally, only samples that overall pass at least four of the latter were considered. Following the quality filter, a total of n=54 controls and n=66 SCZ samples got access to the final step of the analysis pipeline. Reads were aligned to human hg19 reference genome and counts and per cent spliced in (PSI) values were computed through complete Vertebrate Alternative Splicing and Transcription Tools (vast-tools) v.2.5.1 program (Irimia et al., 2014; Tapial et al., 2017). Regarding CPEB4 Ex4 PSI values relative frequency distribution, a bin width of 10 has been considered. Differentially expressed genes between CTRL and SCZ were obtained calculating the mean of the cRPKM values of each gene and computing the fold change (FC) value. *P*-values obtained with a T-test were corrected by false discovery rate (FDR) method for multiple comparison. Downregulated genes in SCZ with a CPEB4 exon 4 PSI<65 compared to control were obtained with a FDR<1%. In the case of the 100 top genes, a FDR<5% has been chosen. Enrichment analysis of already published SCZ-related DEG signature among our DEGs were performed using http://nemates.org/MA/progs/overlap_stats.html. The representation factor (RF) is the number of overlapping genes divided by the expected number of overlapping genes drawn from two independent groups. A RF>1 indicates more overlap than expected of two independent groups, while a RF<1 indicates less overlap than expected.

Human brain tissue samples

Brain specimens from the frontal cortex (BA9/BA46) of individuals with SCZ and matched controls used in qRT-PCR and immunoblotting were obtained at autopsies performed in the

Basque Institute of Legal Medicine, Bilbao, Spain and by the NIH NeuroBioBank (NBB), Maryland, USA (CTRL n=57 and SCZ n=42). Demographic and clinical feature of individuals are reported in Supplementary Table 6. A toxicological screening for a panel of drugs, including antipsychotics, antidepressant, cotinine and ethanol, was performed on both cohorts by the Central Analysis Service at the University of the Basque Country, Spain. A variety of standard procedures including radioimmunoassay, enzymatic immunoassay, high-performance liquid chromatography and gas chromatography–mass spectrometry have been performed. Following toxicological screening, only controls who resulted negative to any substance were included in the study (n=45), while schizophrenic individuals were divided in a treatment-free group (FREE-SCZ n=21) and a group of antipsychotic treated patients (APDs-SCZ n=21).

Mice

Conditional transgenic mice carrying a cDNA of human CPEB4 lacking exon 4 (CPEB4Δ4) under control of the inducible TetO promoter were previously generated (Parras et al., 2018) and maintained in a C57BL/6J background. CPEB4Δ4 mice were crossed with a driver mouse line with low expression of the transactivator tTA (Tet-Off) under the CamkII promoter (Low-CamkII-tTA mice) to obtain conditional double transgenic mice with low forebrain neuronal expression of CPEB4Δ4 (Tg-PN-CPEB4Δ4 mice). All mice were housed in the CBMSO animal facility. Mice were housed four per cage with food and water available *ad libitum* and maintained in a temperature-controlled environment on a 12 h–12 h light–dark cycle with light onset at 08:00. Animal housing and maintenance protocols followed local authority guidelines. Animal experiments were performed under protocols approved by the Centro de Biología Molecular Severo Ochoa Institutional Animal Care and Utilization Committee (Comité de Ética de Experimentación Animal del CBM, CEEA-CBM), and Comunidad de Madrid PROEX 247.1/20.

RNA isolation and cDNA synthesis

Total tissue RNA was extracted from prefrontal cortex (BA9/BA46) of control individuals and matched SCZ using the Maxwell 16 LEV simplyRNA Tissue Kit (Promega, AS1280). Quantification and quality determination of RNA was done on a Nanodrop ND-1000 spectrophotometer and Nanodrop 1000 v.3.7.1 (Thermo Scientific). Only samples with an RNA integrity number (RIN) ≥ 6 and generating an amplification product were included in our experiments (CTRL n=41; FREE-SCZ n=18 and APDs-SCZ n=20) (Supplementary Table 6). The same conditions were employed in the case of control and Tg-PN-CPEB4 Δ 4 mice brains. Retrotranscription (RT) reactions were performed using the iScript cDNA Synthesis kit (Bio-Rad, PN170-8891) following the manufacturer's instructions. In brief, 1,000 ng of total RNA from each sample was combined with 10 μ l of master mix (includes all necessary reagents along with a mixture of random primers and oligo-dT for priming). The reaction volume was completed up to 40 μ l with DNase/RNase-free distilled water (Gibco, PN 10977). Thermal conditions consisted of the following steps: 5 min at 25°C; 20 min at 46°C and 1 min at 95°C.

Quantification of CPEB4 transcript splicing and differential splicing analysis

Specific primers designed in CPEB4 exon 2 (forward, 5'-GGACGTTTGACATGCACTCAC-3') and exon 5 (reverse, 5'-GAGGTTGATCCCCACGGC-3') able to amplify the four CPEB4 splicing isoforms (full-length, Δ 4, Δ 3 and Δ 3 Δ 4) in human and mouse brain cDNA were used. PCR amplification protocol was the following: 10 min at 94°C + 33 cycles (30 s at 94°C + 30 s at 58°C + 2 min at 72°C) + 10 min at 72°C. PCR products according with the four CPEB4 isoforms were resolved on 2.2% agarose/GelGreen (Biotium, 41004) gels run at 125 V for 1.5 h. Images were scanned with densitometer (Bio-Rad, GS-900) and quantified with Image Laboratory 5.2 (Bio-Rad). Finally, the percentage of each CPEB4 isoform was calculated.

Western Blot

Samples from human brain were stored at -80°C and were ground with a mortar in a frozen environment with liquid nitrogen to prevent thawing of the samples, resulting in tissue powder. For mouse, brains were quickly dissected on an ice-cold plate and the different structures stored at -80°C . Human and mouse extracts were prepared by homogenizing the brain areas in ice-cold extraction buffer (20 mM HEPES pH 7.4, 100 mM NaCl, 20 mM NaF, 1% Triton X-100, 1 mM sodium orthovanadate, $1\mu\text{M}$ okadaic acid, 5 mM sodium pyrophosphate, 30 mM β -glycerophosphate, 5 mM EDTA, protease inhibitors (Complete, Roche, Cat. No 11697498001)). Homogenates were centrifuged at 15,000g for 15 min at 4°C . The resulting supernatant was collected, and protein content determined by Quick Start Bradford kit assay (Bio-Rad, 500-0203). Between 10 and $20\mu\text{g}$ of total protein was electrophoresed on 10% SDS–polyacrylamide gel, transferred to a nitrocellulose blotting membrane (Amersham Protran 0.45 μm , GE Healthcare Life Sciences, 10600002) and blocked in TBS-T (150 mM NaCl, 20 mM Tris–HCl, pH 7.5, 0.1% Tween 20) supplemented with 5% non-fat dry milk. Membranes were incubated overnight at 4°C with the primary antibody in TBS-T supplemented with 5% non-fat dry milk. Antibodies used: mouse anti-ATP2A2 (1:1000, Abcam, ab2861), rabbit anti-ATXN7 (1:1000, Invitrogen, PA1-749), rabbit anti-BCL11A (1:250, Sigma-Aldrich, ABE401), rabbit anti-CACNB2 (1:1000, Santa Cruz Biotechnology, sc-81890), rabbit anti-CNTN4 (1:500, Abcam, ab137107), rabbit anti-CTNND1 (1:1000, Sigma-Aldrich, HPA015955), rabbit anti-ELAVL4 (1:500, Abcam, ab96474), rabbit anti-MEF2C (1:1000, Abcam, ab64644), rabbit anti-NEK1 (1:1000, Thermo Scientific, PA5-15336), rabbit anti-OSBPL3 (1:1000, Santacruz Biotechnology, sc-398326), rabbit anti-PDE4B (1:1000, Abcam, ab14611), rabbit anti-STAG1 (1:1000, kindly provided by Spanish National Cancer Research Center-CNIO)(Remeseiro et al., 2012), rabbit anti-TCF4 (1:1000, Proteintech, 22337-1-AP), rabbit anti-ZEB2 (1:1000, Proteintech, 14026-1-AP), rabbit anti-ZSWIN6 (1:1000, Origene, AP54741PU-N), rabbit anti-VINCULIN (1:10000, Abcam, ab129002) and mouse anti- β -ACTIN (1:25000, Sigma-Aldrich, A2228). Membranes were washed with TBS-T and next incubated with secondary HRP-conjugated anti-mouse IgG

(1:2000, DAKO, P0447) or anti-rabbit IgG (1:2000, DAKO, P0448), they were developed using the ECL detection kit (Perkin Elmer, NEL105001EA). Images were scanned with densitometer (Bio-Rad, GS-900) and quantified with Image Laboratory 5.2 (Bio-Rad).

Mouse Behaviour tests

Prepulse inhibition (PPI) of the acoustic startle response test: Startle response curve as well as PPI test were conducted in 10-week-old mice using a commercially available StartFear (Panlab-Harvard Apparatus). This system allows recording and analysis of the signal generated by the animal movement through a high sensitivity weight transducer system. A standard protocol was adapted (Stark et al., 2008). Each mouse has been located in the chamber and during a 5-min acclimation period, while background white noise was continually present. Then, a startle response curve session was performed in order to rule out any impairment in hearing. Startle measures included recordings made every 4 dBs above background (66 dB), up to 118 dB. Each mouse received four times each trial type (40 ms-sound pulses from 70 dB to 118 dB) distributed randomly and separated by 10s-intertrial interval. Response amplitude was considered as the maximum response level recorded during 1 s after the sound pulse. Regarding PPI, trial types, trial type presentation, and background noise levels were performed according to the protocols described previously (Mukai et al., 2004; Stark et al., 2008) with some modifications the following day. In brief, after a 5 min habituation period (66 dB white noise background), eight sets of four different trials distributed randomly with a variable intertrial time (10, 15 or 20 s) were presented to each mouse: trial 1, 40-ms, 120-dB noise burst alone; trials 2 and 3, 120-dB startle stimulus preceded 100 ms earlier by a 20-ms, 70, 74, 78 or 82-dB noise burst (pre-pulse); trial 4, no stimulus, background noise alone (66 dB). As for startle test, response amplitude was considered as the maximum response level recorded during 1 s after the sound pulse. Percent PPI was calculated as $100 - [(startle\ response\ of$

acoustic startle from acoustic prepulse and startle stimulus trials/startle response alone trials) $\times 100$].

Grooming time and social interaction tests: Grooming and social interaction was examined in 7-week-old mice. The first day (training), mice were allowed to explore for 5 min a three chamber Plexiglas box. This time of habituation was employed to measure the grooming activity. The next day (test), mice were placed in the same box containing two wire cages, one empty and the other with an unknown (gender paired) mouse in it, located in opposite chambers and separated by the empty chamber. Mice were recorded for 10 min and the time spent interacting with the unknown mouse was measured.

Statistical Analysis

Statistical analysis was performed with SPSS 26.0 (SPSS Statistic IBM), GraphPad software (La Jolla, CA, USA) or RStudio 2022.02.2 (Boston, MA, USA). The normality of the data was analysed by Shapiro–Wilk test ($n < 50$) or Kolmogorov–Smirnov test ($n > 50$). For comparison of two independent groups two-tail unpaired Student’s t-test (data with normal distribution), Mann–Whitney–Wilcoxon or Kolmogorov–Smirnov tests (with non-normal distribution) were performed. For multiple comparisons, data with a normal distribution were analysed by one way-ANOVA or two-way-ANOVA followed by a Tukey’s post hoc test. Statistical significance of non-parametric data for multiple comparisons was determined by Kruskal–Wallis one-way ANOVA. Benjamini-Hochberg correction was applied for multiple testing in RNA-seq analysis. Data are represented as mean \pm SEM with 95% confidence intervals. Higher or lower points (outliers) are not plotted. A critical value for significance of $P < 0.05$ was used throughout the study.

ACKNOWLEDGEMENTS

RNA-seq data for this publication were obtained from NIMH Repository & Genomics Resource, a centralized national biorepository for genetic studies of psychiatric disorders, specifically, data were generated as part of the PsychENCODE Consortium. For providing brain samples, the authors thank the Basque Institute of Legal Medicine and the Universidad del Pais Vasco (UPV/EHU) as well as Harvard Brain Tissue Resource Center, funded through NIH-NeuroBiobank HHSN-271-2013-00030C, the National Institute of Mental Health (NIMH), National Institute of Neurological Diseases and Stroke (NINDS), National Institute on Aging (NIA), Eunice Kennedy Shriver National Institute of Child Health and Human Development (NICHD), and brain donors and their families for the tissue samples used in these studies.

The authors would like to thank the staff members of the Basque Institute of Legal Medicine for their cooperation in the study. We also thank excellent technical assistance by Miriam Lucas and by the following core facilities: CBMSO-Genomics & Massive Sequencing, CBMSO-Animal Facility and the CETA-CIEMAT computing center.

We thank Dr. M. Irimia (CRG, Barcelona) for advice on AS analysis tools and for critical reading of the manuscript and Dr. Ana Losada (CNIO, Madrid) for providing the anti-STAG1 antibody.

This work was supported by CIBERNED-ISCIII collaborative grant PI2018/06-1 to JLL; grants from Spanish Ministry of Economy and Competitiveness/Ministry of Science, Innovation and Universities to JLL: SAF2015-65371-R (MINECO/AEI/FEDER, UE), RTI2018-096322-B-I00 (MCIU/AEI/FEDER,UE) and PID2021-123141OB-I00 (MCIU/AEI/FEDER,UE); to RM: PID2020-119533GB-I00 (MINECO) and to CT: PID2020-114996RB-I00 (MCIU/AEI/FEDER,UE) and RyC2018-024106-I; and grants from Basque Government (IT-1211-19 and 1512-22) to JJM and LFC and from World Cancer Research Fund International (2020_021) to RM. Cardiff University's work was supported by Medical Research Council Centre (MR/L010305/1), Program (MR/P005748/1), and Project (MR/L011794/1, MC_PC_17212) grants to JTRW, MCOB and MJO. AFP was supported by an Academy of Medical Sciences "Springboard" award

(SBF005\1083). IO was hired through European Union's Horizon 2020 research and innovation program under the Marie Skłodowska-Curie grant agreement (766124).

AUTHOR CONTRIBUTIONS

I.O. was involved in all assays and data collection, data interpretation and statistical analysis. A.F.P performed the MAGMA analyses. A.P. contributed to experimental design and data interpretation. I.H.H. performed the bioinformatics analyses and contributed to data interpretation. M.S.G. performed bioinformatics analyses and western blotting. S.P. and A.E. contributed to data interpretation, experimental design and discussion. L.F.C. provided patient samples with toxicological data. G.F.M. and E.B. made intellectual contributions, provided reagents and optimized protocols. J.T.R.W and M.C.O'D. made intellectual contributions to and edited the manuscript. C.T. performed bioinformatics analysis and made contribution to the discussion R.M. revised the manuscript and made contribution to the discussion. J.J.M. provided patient samples with toxicological data and made contribution to the discussion. M.J.O. revised the manuscript and made contributions to the discussion. J.J.L. directed the study and designed experiments, drafted and revised the manuscript with input from all authors.

CONFLICT OF INTEREST

JTRW, MCO and MJO are investigators on a grant from Takeda Pharmaceuticals Ltd. to Cardiff University, for a project unrelated to the work presented here. The remaining authors report no competing interests.

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FIGURE LEGENDS

Figure 1: Decreased inclusion of CPEB4 exon 4 in SCZ. Vast-tools splicing analysis of PsychENCODE BrainGVEX data. A) Representation of CPEB4 exon 4 PSI and B) its relative frequency (%) distributions in controls and SCZ patients. Males (upper panel), males and females (lower panel). In the lower panel, SCZ individuals with CPEB4 exon 4 PSI<65% are highlighted in pink and those with CPEB4 exon 4 PSI>65% in light blue. Analysis of differentially expressed genes (DEGs) was performed comparing each group with controls. A) Student's T-test. B) Two-sample Kolmogorov-Smirnov test (upper panel) and Student's t-test followed by Benjamini-Hochberg correction for multiple comparison (bottom panel). * $P<0.05$. Data are mean with \pm SEM.

Figure 2: CPEB4 mis-splicing selectively occurs in antipsychotic-free SCZ brains. A) PsychENCODE BrainGVEX project (PsychEncode-Consortium et al., 2015) provides the value of "Lifetime antipsychotic drugs consumption index" for 45 of the SCZ samples used in our RNA-seq analysis. The graph shows the values of this parameter in the SCZ individuals with CPEB4 exon 4 PSI<65 and in those with CPEB4 exon 4 PSI>65. B) At the top, diagram of the four splicing variants of CPEB4. At the bottom, representative RT-PCR analysis of brain tissue from controls and SCZ subjects negative (FREE-SCZ) or positive to antipsychotic drugs (APDs-SCZ) at the moment of death, with C) the corresponding quantification of the percentage of CPEB4 isoforms. D) Δ Ex4/Ex4+ ratio. A) Mann-Whitney test C) Two-Way ANOVA test with Bonferroni Correction D) Kruskal-Wallis test with Dunn's multiple correction. * $P<0.05$, ** $P<0.01$, *** $P<0.001$. Data are mean with \pm SEM.

Figure 3: Decreased protein levels of the SCZ risk genes in FREE-SCZ brain samples. TCF4, STAG1, CTNND1, OSBPL3 and NEK1 protein levels in control, FREE-SCZ and APDs-SCZ samples.

Kruskal-Wallis test with Dunn's multiple comparison test or One-Way ANOVA test. * $P < 0.05$, ** $P < 0.01$. Data are mean with \pm SEM.

Figure 4: Transgenic overexpression of CPEB4Δ4 isoform in mouse brains suffices to induce decreased TCF4, OSBPL3 and NEK1 protein levels. A) Scheme of the transgenes in the Tg-PN-CPEB4Δ4 mouse model (top). Representative RT-PCR of CPEB4Δ4 isoform in control and Tg-PN-CPEB4Δ4 mouse brain tissue with corresponding quantification (bottom). B) TCF4, OSBPL3 and NEK1 protein levels in control and Tg-PN-CPEB4Δ4 mouse brains. A) and B) Student's t-test; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Data are mean \pm SEM.

Figure 5: Tg-PN-CPEB4Δ4 mice show schizophrenia-linked rodent behaviors. A) Representation of the amplitude of acoustic startle response corresponding to acoustic stimuli of increasing decibels (from 70 to 118 dB) in control and Tg-CPEB4Δ4 mice (left); quantification of the pre-pulse inhibition (PPI) of the acoustic startle response at 70, 74, 78, 82 and 90 Db (right). B) Grooming time during a 5 min trial. C) Interaction time with an unfamiliar mouse in social interaction test. A)-B)-C) Student's t-test; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Data are mean with \pm SEM.

Table 1: Gene-level analysis performed with CPE-containing, CPEB-targets and psychiatric-related gene sets using the latest GWAS summary statistics from the PGC of schizophrenia.

Gene-set name	N genes	P-value	Conditional Analysis (P-value)		
			Brain-expressed	Synaptic genes	FMRP targets
cCPE-containing genes	7,712	2.92E-04	0.036	5.93E-04	0.003
CPEB1 targets	2,207	1.39E-07	2.08E-04	7.06E-07	3.71E-06
CPEB3 targets	3,508	6.95E-04	0.065	0.002	0.003
CPEB4 targets	2,749	1.76E-09	5.77E-05	2.19E-08	7.98E-08
FMRP targets	813	4.97E-09	7.19E-06	4.76E-07	-
Synaptic genes	1,046	4.36E-07	5.55E-05	-	4.43E-05
Brain-expressed genes	9,695	6.13E-19	-	6.68E-17	7.55E-16

Gene-level analysis performed via MAGMA of relevant gene-sets: i) categories under this study: genes containing canonical CPE, genes bound by CPEB1, CPEB3, and CPEB4; ii) genes belonging to categories known to play a substantial role in psychiatry: FMRP interactor targets, specific genes at synapses, and brain-expressed genes. The latter psychiatric-related gene sets were used also to perform a conditional analysis. For each gene-set is shown the number of genes (N genes) and *P-value* of enrichment with SCZ-associated genes after gene-based analysis.

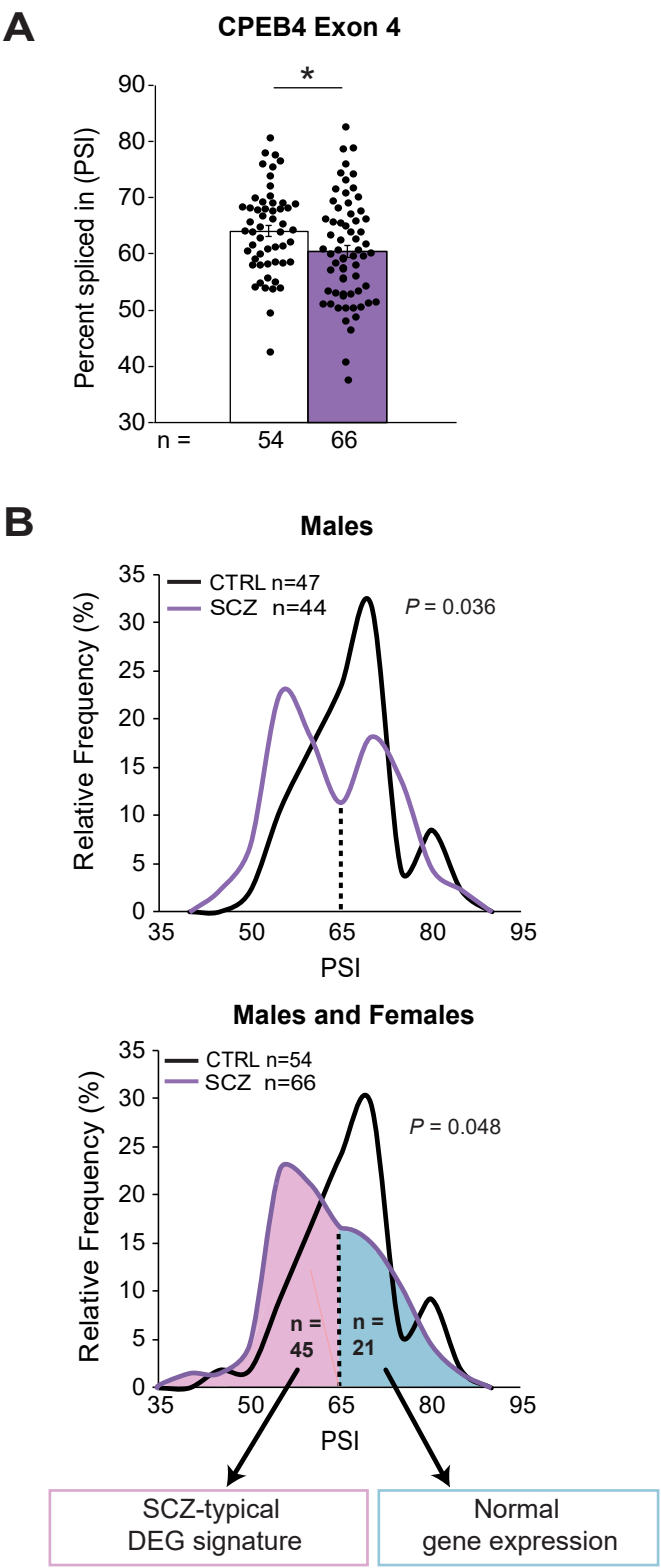


Figure 1

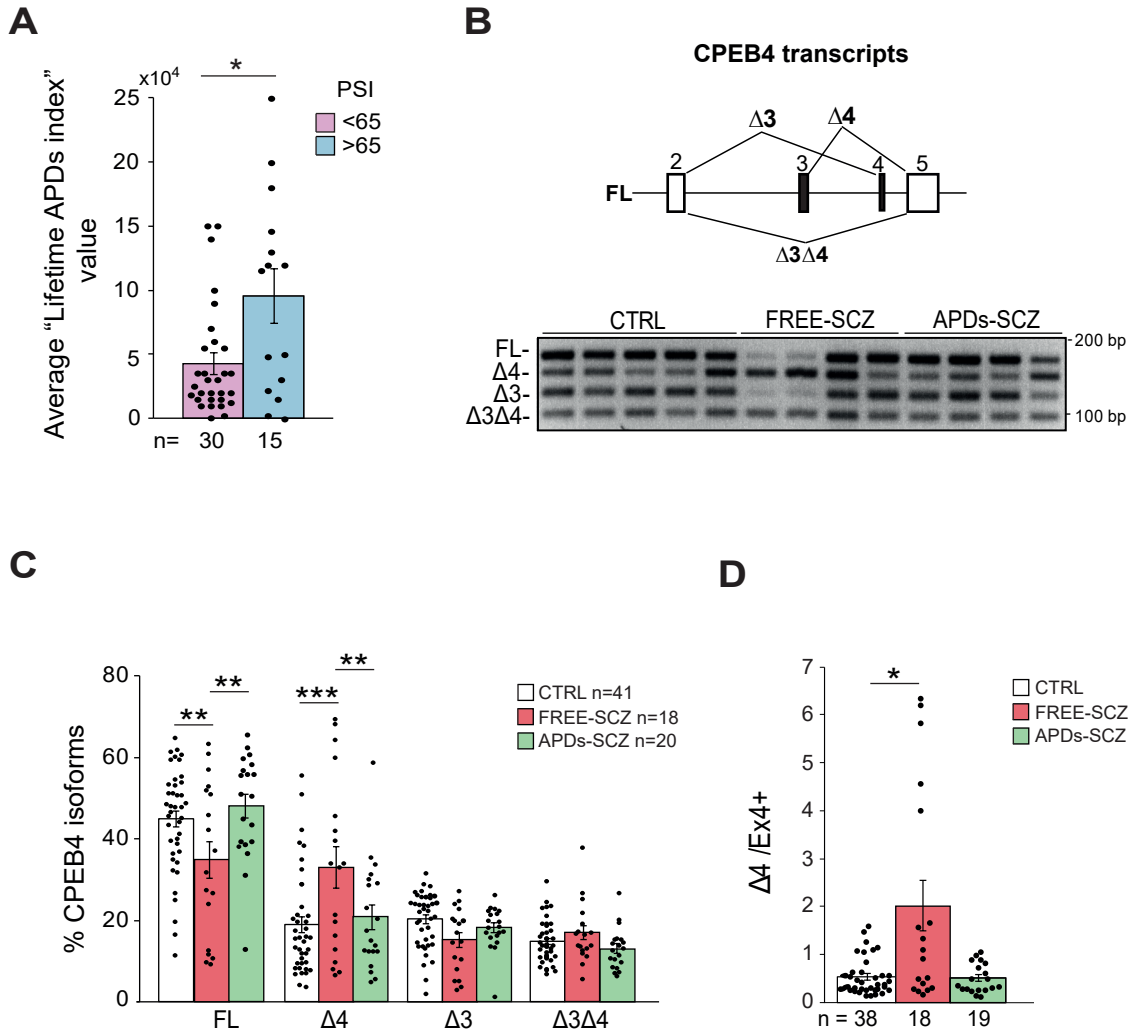


Figure 2

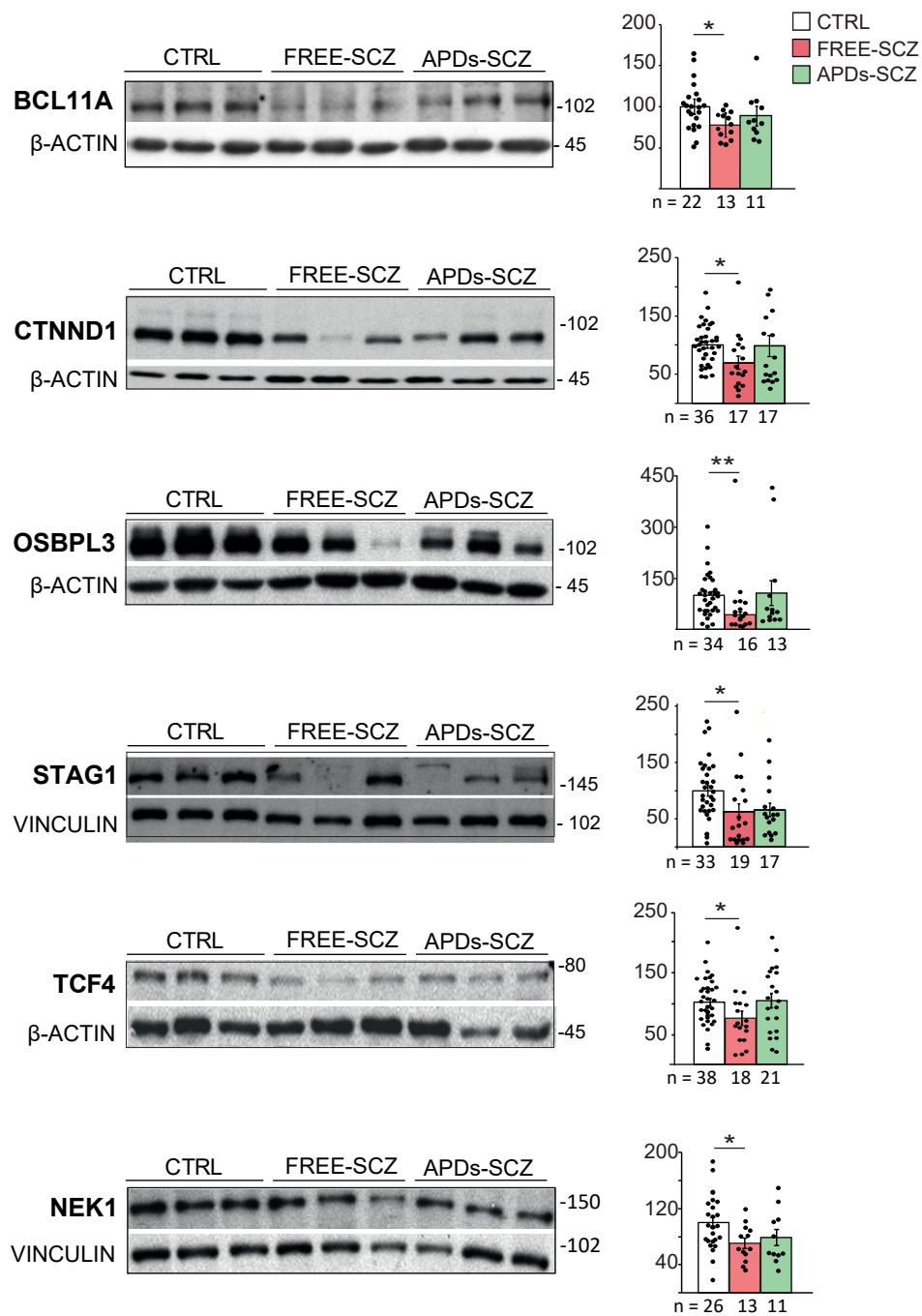


Figure 3

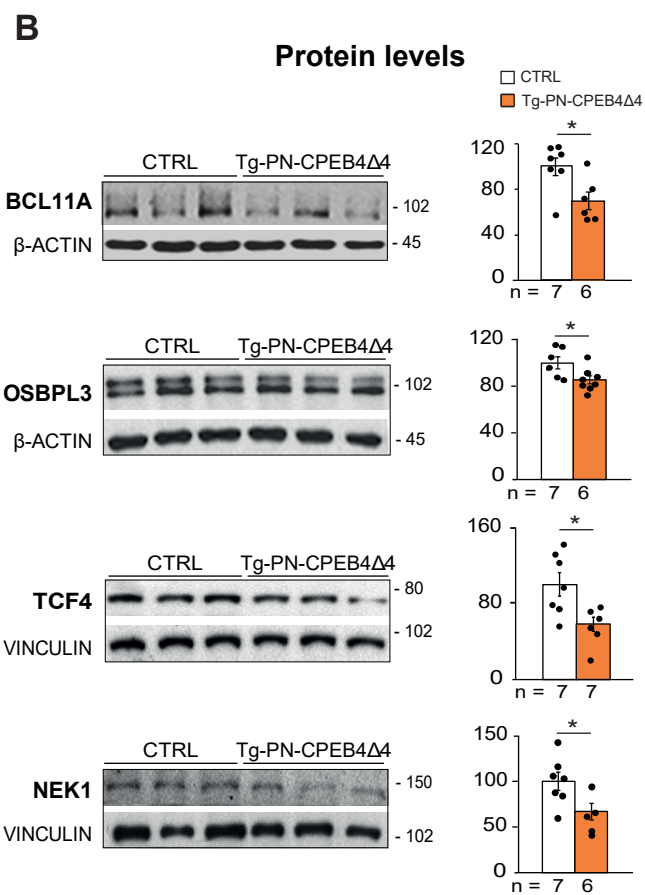
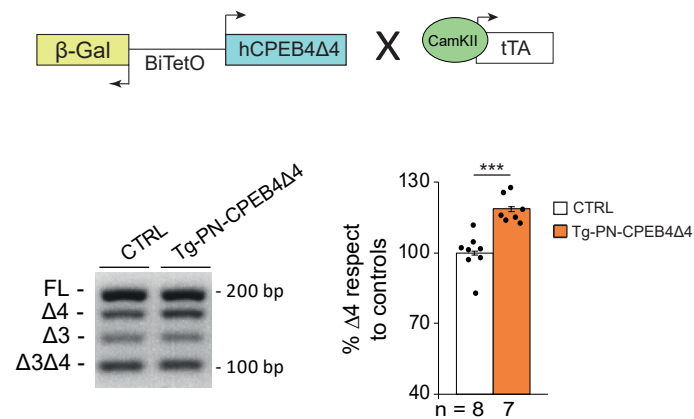


Figure 4

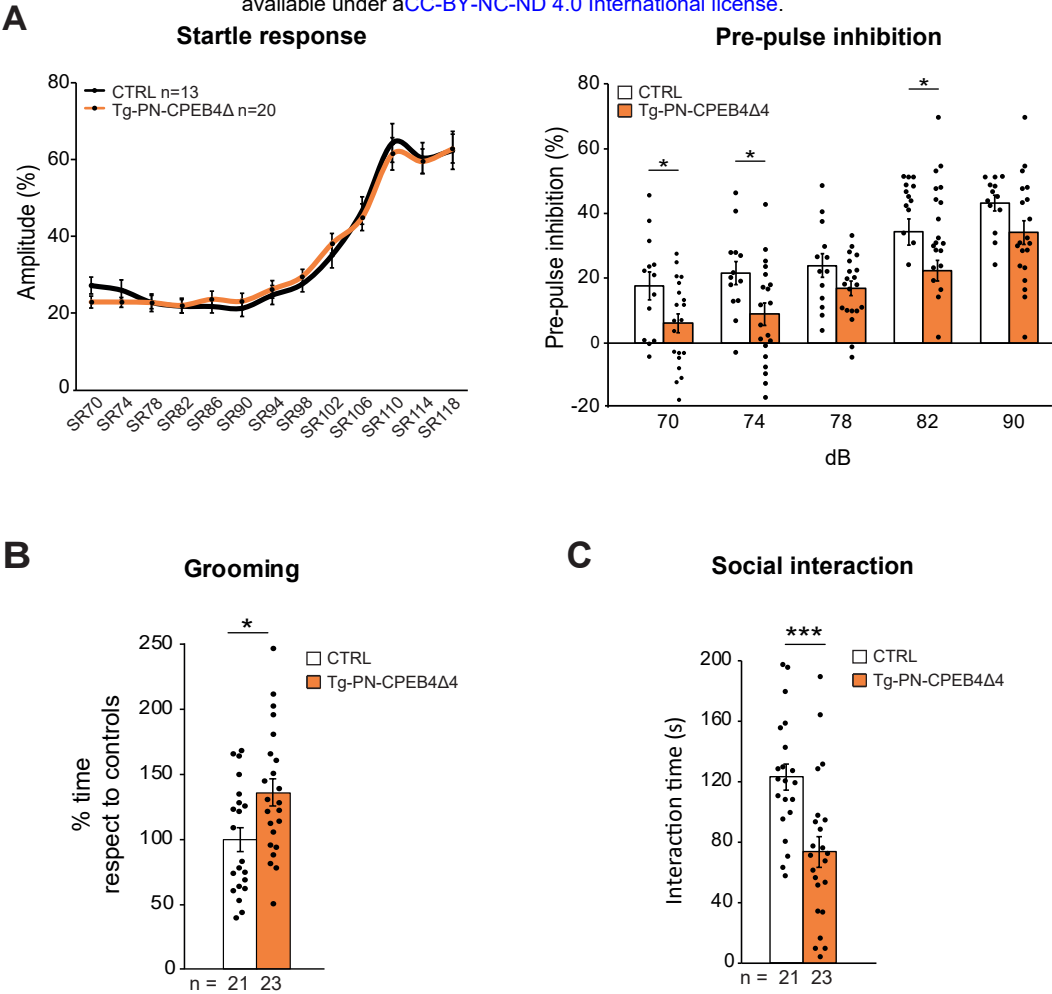


Figure 5