Pathogenic mis-splicing of *CPEB4* in schizophrenia

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ABSTRACT

BACKGROUND: Schizophrenia (SCZ) is caused by an interplay of polygenic risk and environmental factors, which might alter regulators of gene expression leading to pathogenic mis-expression of SCZ-risk genes. The CPEB family of RNA-binding proteins (CPEB1-4) regulates translation of target RNAs (approximately 40% of overall genes). We previously identified CPEB4 as a key dysregulated translational regulator in autism spectrum disorder (ASD), as its neuronal-specific microexon (exon4) is mis-spliced in ASD brains, causing underexpression of numerous ASD-risk genes. The genetic and pathogenic mechanisms shared between SCZ and ASD led us to hypothesize CPEB4 mis-splicing in SCZ, leading to underexpression of multiple SCZ-related genes.

METHODS: We performed MAGMA-enrichment analysis in Psychiatric Genomics Consortium GWAS data and analyzed RNA-seq data from the PsychENCODE Consortium. RT-PCR and Western blot were performed on post-mortem brain tissue in which presence/absence of antipsychotics was assessed through toxicological analysis. Finally, mice with mild overexpression of exon4–lacking CPEB4 (CPEB4Δ4) were generated and biochemically and behaviorally analyzed.

RESULTS: We first found enrichment of SCZ-associated genes for CPEB4-binder transcripts. We also found decreased usage of CPEB4 microexon in SCZ probands, correlating with decreased protein levels of CPEB4-target SCZ-associated genes, selectively in antipsychotics-free individuals. Interestingly, differentially expressed genes fit those reported for SCZ, specifically in the SCZ probands with decreased CPEB4-microexon inclusion. Finally, we demonstrate that mice with mild overexpression of CPEB4Δ4 show decreased protein levels of CPEB4-target SCZ genes and SCZ-linked behaviors.

CONCLUSION: We identify 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 (1). Genetic and epidemiological evidence indicate that SCZ is caused by common and rare risk alleles combined with environmental factors (2). 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 (3, 4). 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 (2). 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) (5) accounting for about 40% of the transcriptome (6, 7). 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 (8). CPEBs mediate translational repression or activation of their target transcripts by inducing, respectively, shortening or elongation of the poly(A)-tails (5). 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 (5, 9). More recently, CPEBs have been shown to play important roles in cell division and metabolism (5, 10, 11). 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 (5, 12-16). Consistently, altered CPEBs have been associated with cancer (17, 18) and pathogenic hepatic angiogenesis (19), neurological diseases including epilepsy (20) and Huntington's disease (21) as well as with ASD (7, 22).

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 (7). 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) (7) that encodes an 8-aa sequence with postulated regulatory function (7, 23). 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 (24, 25). 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 (7). Furthermore, we found that transgenic mice overexpressing CPEB4Δ4 showed decreased protein expression of a plethora of ASD risk genes and display ASD-like traits (7).

Schizophrenia and ASD share genetic risk, and by inference pathogenic mechanisms, and have been proposed to lie on a neurodevelopmental continuum (26). This led us to hypothesize that alteration of CPEBs, particularly CPEB4, might also be observed in the brains of individuals with SCZ, and this would lead to pathogenic mis-expression of multiple SCZ-risk-genes.

Methods and Materials

Refer to **supplemental methods and materials** for complete report.

Gene-set enrichment analyses

Analysis of enrichment of CPEBs-related genes among SCZ-risk genes was performed through a gene-level analysis in MAGMA (27). Covariate gene sets used to carry out conditional analyses are reported in supplemental methods and materials. ASD gene-based analysis was performed using the summary statistics from the PGC GWAS of ASD (28).

RNA-seq data analysis

The BrainGVEX RNA-seq study was analyzed (29). See supplemental methods and materials for files download, quality control, and splicing and expression analyses procedures.

Human brain tissue samples

Brain specimens from individuals with SCZ and CTRL used in this study were provided by Basque Institute of Legal Medicine, Bilbao, Spain and the NIH NeuroBioBank (NBB), Maryland, USA. All samples underwent a toxicological screening. See supplemental methods and materials for details.

Mice

Previously generated conditional transgenic mice harbouring human CPEB4 lacking exon 4 (CPEB4Δ4) cDNA under TetO promoter (7) were crossed with a driver mouse line with low expression of the transactivator tTA in forebrain neurons to generate Tg-L-CPEB4Δ4 mice). See details in supplemental methods and materials.

RNA isolation and cDNA synthesis

Total tissue RNA extraction, quantification and quality determination, and retrotranscription reactions were performed following manufacturer's instructions (see supplemental methods and materials).

Quantification of CPEB4 transcript splicing and differential splicing analysis

Previously reported (7) CPEB4 splicing isoforms amplifying primers and the PCR amplification protocol were used (see supplemental methods and materials).

Tissue Homogenization and Western blot

Tissue homogenization and western blot procedures and the list of antibodies used in this study are fully detailed in supplemental methods and materials.

Mouse Behaviour tests

Prepulse inhibition (PPI) of the acoustic startle response test was performed following standard protocols (30), as also the grooming time and social interaction tests (7, 31).

Statistical Analysis

All statistical analyses are described fully in supplemental methods and materials.

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) (32). Successively, we examined gene sets comprised of (i) genes containing canonical CPE (cCPE) sequences in their 3'UTR (6); (ii) genes identified in genome-wide RNA immunoprecipitation analyses from mouse brain structures as CPEB1- (7), CPEB3- (33) or CPEB4- (7) binders; (iii) gene sets implicated in the pathophysiology of psychiatric disorders: Fragile X Mental Retardation Protein (FMRP) targets (34, 35), genes specifically involved in synaptic development and function (36), and finally (iv) as a comparator, a more general set of all brain-expressed genes (37) (Table S1). The results of associations with SCZ-associated genes showed "CPEB4 targets" as the top significant gene-set amongst those under study (P=1,76x10^{-9}; Table 1), together with already known gene-sets implicated in psychiatric disorders such as "FMRP targets" and "brain-expressed genes" (P=4.97x10^{-9} and P=6.13x10^{-19}, respectively; Table 1). Then, the 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 (22), or CPEB target genes and brain expressed genes (7). After conditional analysis for each of "FMRP targets", "synaptic genes", and "brain-expressed genes" sets, the "CPEB4 targets" 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. Additionally, we verified that the signal from CPEB4-targets is not driven by the overlapping of genes from sets already

known to be implicated in psychiatric phenotypes ("FMRP targets", "brain-expressed genes" and "synaptic genes") (Table S2 and Figure S1).

Given the previous evidence of CPEB4 targets in ASD, we assessed the overlap of gene-based associations for CPEB4 targets between ASD and SCZ using GWAS data. The result showed a significant overlap (n=156 genes) between CPEB4 target genes found nominally associated with ASD (n=319) and those associated with SCZ (1,089) (*P*=2.2E-04) (Figure S2).

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 (38) 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 (39). 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) (Table S3). 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*=0.0415) (Figure 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 SZC samples (*P*=0.048) (Figure 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)≈55, while the minor peak corresponded to PSI≈70, the latter matching with the mode value in the distribution of control samples (Figure 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 ΔPSI=-15. To discard potential confounders, we fit a multiple linear regression model to control for the effects of covariates like brain bank, sex, ethnicity, cause of death, age of death, post-mortem interval (PMI), RNA integrity, RNA-seq platform or total number of reads (Table S4) and none of them influenced the distribution of PSI values.

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 1B), we stratified the 66 SCZ samples analyzed in Figure 1A into two pools based on this cut-off. Interestingly, gene expression at the PSI>65-SCZ subpopulation (n=21) was similar to that of control samples, while the PSI<65-SCZ subpopulation (n=45) showed 771 differentially expressed genes (DEG), 492 upregulated and 279 downregulated (Figure 1B and Table S5). Remarkably, the DEG signature in the PSI<65-SCZ subpopulation, particularly the downregulated genes (Table S6), is highly concordant with DEG signatures previously reported for dorsolateral prefrontal cortex (DLPFC) of SCZ subjects in two different studies, with representation factors (see methods) 9.3 (P<1.17x10^{-7}) (40) and 12.7 (P<1.94x10^{-6}) (41), respectively, and marked expression deficits in GABA neurotransmission-related transcript such as GAD1 (GAD67) or the neuropeptides somatostatin (SST) and neuropeptide Y (NPY). 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≈70) and global gene expression, and the other with lower CPEB4 exon 4 inclusion (PSI≈55) and a paradigmatic SCZ DEG signature (Figure 1B). 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 (42). 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 (95,2±20,2 vs. 42,8±7,8; *P*=0.048) (Figure 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 (Table S7). 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 (Figure 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-CPEB4) and CPEB4Δ3) and two lacking it (CPEB4Δ4 and CPEB4Δ3Δ4) (Figure 2B). In control samples, the FL-CPEB4 transcript predominates (Figure 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) (Figure 2B-C). As expected, patients of the FREE-

SCZ sample show an increase of CPEB4Δ4 compared to controls (Figure 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 (Figure 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 (Figure 2C), showing FL-CPEB4 and CPEB4Δ4 isoform levels to be indistinguishable from those in controls (Figure 2C). Consequently, the ΔEx4/Ex4+ isoform ratio is markedly increased in FREE-SCZ samples, while unaltered in APDs-SCZ samples (Figure 2D). Similar results were obtained through quantitative PCR with primer pairs specific for each splicing isoform (Figure 2E). This pathological mis-splicing of CPEB4 occurring selectively in FREE-SCZ brains strengthen the notion of an etiological parallelism with ASD. Interestingly, the non-pathological decrease in CPEB4 protein levels reported in ASD brains (7) is also observed in FREE-SCZ cases, without alteration of other CPEBs (Figure S3). In conclusion, these results demonstrate that individuals with SCZ show the pathogenic CPEB4 splicing alteration previously seen in ASD (altered 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 CPEB4Δ4 transcript in brains of idiopathic ASD patients correlates with concerted decreased protein expression of multiple ASD risk genes that are targets of CPEB4 (7). 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 levels). This resulted in a short list of 43 top candidate genes (Table S8) and we assayed antibodies for 15 of them in Western blot analyses. Eight of these antibodies yielded protein signal at the predicted molecular weight: BCL11A, CACNB2, CNTN4, CTNND1, OSBPL3, RBFOX1, STAG1 and TCF4 (Table S8). 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 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 (7) because they show minimal expression in striatum (according to our published RNA-seq datasets (43)) 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 (44) and we found that NEK1 protein levels are also decreased in the FREE-SCZ samples, but not in the APDs-SCZ samples (Figure 3). As a control of the specificity of the role of CPEB4 mis-splicing in altered protein expression of target genes, we analyzed SRRM3, whose transcript does not contain CPE sequences nor binds CPEB4 (7) and found its protein levels unaltered (Figure S4). This protein was interesting to analyze because it is one of the few splicing factors reported to modulate inclusion of microexons (45). 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) (7). Since strong CPEB4Δ4 overexpression starting at embryonic stages leads to consistent ASD like phenotypes already evident in pups (7), 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 (Figure 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 (Figure 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 Figure 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 (Figure 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 (5) 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 (7). Using data from the largest GWAS meta-analysis in SCZ (32), 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 (46). 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 (47) 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 (48). 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 (49). 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 (50, 51), 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 (52).

This study further supports that, within the 'neurodevelopmental model' of psychiatric diseases (53), 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 (7).

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 (26, 53). 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 stating 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 (7). 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.

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Disclosures

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. and C.R.-L. performed 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. R.M. revised the manuscript and made contribution to the discussion. C.T. performed bioinformatics analysis 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.

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Legends for tables and figures

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-level analysis performed via MAGMA of relevant gene-sets: i) categories under this study: genes containing canonical CPE, genes bound by CPEB1, CPEB3, and CPEB3; 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, the number of genes (N genes) and the *P*-values of enrichment with SCZ-associated genes after gene-based analysis are shown.

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) Wilcoxon signed-rank test. B) Two-sample Kolmogorov-Smirnov test for distribution comparison and Student's t-test followed by Benjamini-Hochberg correction for multiple comparison for DEGs (bottom panel). **P*<0.05. Data are mean with ±SEM.

Figure 2: CPEB4 mis-splicing selectively occurs in antipsychotic-free SCZ brains. A) PsychENCODE BrainGVEX project (39) 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) ΔEx4/Ex4+ ratio. E) quantification of the percentage of CPEB4 isoforms respect to controls from Q-PCR experiment. A) Mann-Whitney test; C) Two-Way ANOVA test with Bonferroni Correction; D-E) Kruskal-Wallis test with Dunn's multiple correction. *P<0.05, **P<0.01, ***P<0.001. Data are mean with ±SEM.

Figure 3: Decreased protein levels of the SCZ risk genes in FREE-SCZ brain samples. BCL11A, CTNND1, OSBPL3, STAG1, TCF4 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 ±SEM.

Figure 4: Transgenic overexpression of CPEB4Δ4 isoform in mouse brains suffices to induce decreased BCL11A, OSBPL3, TCF4 and NEK1 protein levels. A) Scheme of the transgenic mouse lines combined to generate 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) BCL11A, OSBPL3, TCF4 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 ±SEM.

Figure 5: Tg-PN-CPEB4Δ4 mice show schizophrenia-linked rodent behaviors. A) 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 ±SEM.