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ARTICLE

Genetic variants in the bipolar disorder risk locus *SYNE1* that affect CPG2 expression and protein function

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Abstract

Bipolar disorder (BD) is a common mood disorder characterized by recurrent episodes of mania and depression. Both genetic and environmental factors have been implicated in BD etiology, but the biological underpinnings remain elusive. Recently, genome-wide association studies (GWAS) of neuropsychiatric disorders have identified a risk locus for BD containing the SYNE1 gene, a large gene encoding multiple proteins. The BD association signal spans, almost exclusively, the part of SYNE1 encoding CPG2, a brain-specific protein localized to excitatory postsynaptic sites, where it regulates glutamate receptor internalization. Here we show that CPG2 protein levels are significantly decreased in postmortem brain tissue from BD patients, as compared to control subjects, as well as schizophrenia and depression patients. We identify genetic variants within the postmortem brains that map to the CPG2 promoter region, and show that they negatively affect gene expression. We also identify missense single nucleotide polymorphisms (SNPs) in CPG2 coding regions that affect CPG2 expression, localization, and synaptic function. Our findings link genetic variation in the CPG2 region of SYNE1 with a mechanism for glutamatergic synapse dysfunction that could underlie susceptibility to BD in some individuals. Few GWAS hits in human genetics for neuropsychiatric disorders to date have afforded such mechanistic clues. Further, the potential for genetic distinction of susceptibility to BD from other neuropsychiatric disorders with overlapping clinical traits holds promise for improved diagnostics and treatment of this devastating illness.

Introduction

Bipolar disorder (BD) is a common, chronic mood disorder characterized by recurrent episodes of mania and depression. The lifetime prevalence is estimated at 1–3% of the population [1] and high mortality rates [2], mainly caused by suicide [3], makes BD a major public health problem. Treatment for BD is limited, consisting mainly of pharmaceutical mood stabilizers, antidepressants, and antipsychotic drugs discovered decades ago. Their efficacy in only a subgroup of BD patients highlights the need for development of new drugs based on a molecular understanding of disease etiology [4, 5].

The neuropsychiatric disorders BD, schizophrenia and major depression, as well as other neurodevelopmental disorders such as autism spectrum disorder (ASD) and attention deficit hyperactivity disorder (ADHD), substantially overlap in clinical traits [6]. For example, many BD patients suffer from cognitive deficits and psychotic symptoms qualitatively resembling those of schizophrenia patients [7], and from depressed mood states resembling those of major depression patients. For this reason, precise diagnosing often requires extensive psychiatric evaluation based on clusters of symptoms [6], and in some cases even erroneous pharmacological treatment attempts [8]. Although there is a wide consensus for differential brain structural and connectivity impairments [9–11], there is little specific evidence describing neuronal substrates and

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mechanisms differentiating neuropsychiatric disorders at the cellular level. Consequently, in the absence of more conclusive biological markers, diagnosing is almost exclusively based on psychiatric evaluation [12].

Despite robust evidence for genetic susceptibility to BD [13], with heritability estimated as high as 70–80% based on twin studies [13–16], only a few genetic susceptibility factors have been identified over decades of research [13, 17], with little evidence for BD-specific risk genes [17]. Joint genome-wide association studies (GWAS) show substantial commonalities in risk loci for the major psychiatric disorders, especially between BD and schizophrenia [18–20], suggesting they overlap not only in clinical symptoms but also in their contributing genetic factors [20–22]. A handful of common genetic variants, identified by GWAS as robustly associated with BD and replicated across independent studies, are single-nucleotide polymorphisms (SNPs) in the genes *CACNA1C, ANK3, ODZ4, SYNE1*, and *TRANK1* [23–28]. Some of the identified loci show penetrance for two or more of the neuropsychiatric disorders, underlining a shared genetic etiology [25, 29–31]. The largest GWAS to date for identifying risk loci for neuropsychiatric disorders including BD, schizophrenia, and major depression, identified the region encompassing *SYNE1* as the strongest BD association locus in the genome [32]. Meta-analyses included in the study identified SNPs in *SYNE1* with genome-wide statistically significant association to BD at $P = 4.27 \times 10^{-9}$ [25–27, 32, 33].

Increasing evidence, mainly from genetic and pharmacological studies, has implicated abnormal glutamatergic neurotransmission and synaptic plasticity in the etiology of BD [24, 25, 34–36]. Notably, SNPs in *GRIA2*, which encodes the GluA2 subunit of AMPARs, have been associated with time to recurrence of mood episodes in BD patients [37]. Studies have also shown differences in glutamate levels as well as glutamate receptor expression or function between individuals with mood disorders and control subjects [38–42].

SYNE1 has thus far drawn less attention in relation to neuropsychiatric disorders compared to e.g. *CACNA1C*, which encodes voltage-gated calcium channels well-known to play a role in synaptic function [29]. Human *SYNE1* is a large gene comprising 145 exons with multiple transcripts [43]. The most described gene products of *SYNE1*, the nesprins, are mainly related to muscle function [44, 45], and are unlikely to explain how this locus influences susceptibility to BD. Interestingly, the BD association signal in *SYNE1* maps near the transcription start site for candidate plasticity gene 2 (*CPG2*). CPGs are activity-regulated genes identified as potential mediators of synaptic plasticity [46]. *CPG2* is a brain-specific transcript of the *SYNE1* gene. We recently reported the existence of two human *CPG2* transcripts expressed in neocortex, hippocampus, and striatum, encoding proteins that localize to the postsynaptic endocytic zone of excitatory synapses in dendritic spines [43, 47]. Here they facilitate glutamate receptor cycling, consistent with a role in synaptic plasticity. The identification of *CPG2* as a risk locus for BD and CPG2's known function in regulating glutamate receptor internalization, suggest that variation in CPG2 function may affect glutamate receptor cycling in a way that would influence susceptibility to BD.

Here, we combine multiple strategies to identify genetic variations within the *CPG2* locus of *SYNE1* that influence expression or function of the CPG2 protein. Linking BD associated variation with an underlying cellular dysfunction is an opportunity afforded by only a few GWAS hits in human genetics for neuropsychiatric disorders to date. We show that CPG2 protein levels are significantly decreased in postmortem brain tissue of BD patients as compared to schizophrenia and depression patients, as well as control subjects. By deep-sequencing the *CPG2* region of *SYNE1* from the same human subjects, we identified genetic variants within promoter and enhancer regions that negatively affect gene expression. We further show that certain genetic variants in the CPG2 coding region identified by exome sequencing affect CPG2 expression, subcellular localization, and synaptic function.

Materials and methods

Human brain tissue

Fresh frozen human brain tissue samples from BA9/10 and hippocampus were kindly provided by the Harvard Brain Tissue Resource Center, the Stanley Medical Research Institute Neuropathology Consortium collection, Mount Sinai NIH Brain and Tissue Repository (schizophrenia patients), University of Maryland Brain and Tissue Bank (controls), and Massachusetts Alzheimer's Disease Research Center (controls). Information on age, postmortem index (PMI), gender, partial information on medication history, and relative CPG2, Arc and PSD95 protein expression levels is summarized in supplementary table ST1. Informed consent was obtained from all tissue donors or legal signatories.

Western blotting

Protein extraction and Western blotting were performed as described previously [43]. Blots were incubated with guinea pig polyclonal anti-CPG2 (1:1000, A002396; np913, NEO Peptide), mouse anti-Arc (1:1000; Synaptic Systems), mouse anti-PSD95 (1:20,000; UC Davis) or mouse anti-β-actin (1:2000; SIGMA) antibodies and visualized using an Odyssey infrared imaging system (LI-COR). Western blot

signal intensities were quantified using a FIJI (ImageJ) gel lane plot profile tool and groups were compared using one-way ANOVA and Tukey's post-hoc test for multiple comparisons or *t*-tests (two groups). Sample lysates were loaded in a randomized manner and quantified blind to sample identity. For SMRI samples, subject identifying information was revealed to the experimenter only after submission of the final dataset to the SMRI consortium. One sample was below detection level and excluded from the protein level analyses. The Western blots for CPG2 showed two bands that were quantified in combination due to insufficient signal segregation and apparent co-regulation.

Targeted region deep-sequencing and variant calling

Genomic DNA was extracted from human brain tissue using a ChargeSwitch gDNA Mini Tissue Kit (Invitrogen) and purified by ethanol precipitation. High-quality gDNA samples were deep-sequenced in the extended *CPG2* region of *SYNE1* (NM_182961: exon 8–70 incl. introns) by CD Genomics (sequencing depth: 30–100×).

The raw sequencing data was mapped to human genome assembly GRCh37 using BWA aligner [48]. The BAM-files were indexed and the mapped reads within the *SYNE1* gene locus (chr6:152740704–152831506) were selected using the SAMtools view module [49]. The generated *SYNE1* BAM-files were piled up using SAMtools mpileup module for union genotype calling using BCFtools with –mv and –Oz options [50]. Variant annotation was performed using ANNOVAR tool [51]. Human brain region-specific chromatin states were collected [52] and visualized using the web-based NeuVar tool http://bioinfo5pilm46.mit.edu:318/neuvar. Statistical correlation of genetic variants with CPG2 protein expression was tested using Mann–Whitney binary tests.

Molecular cloning

Putative promoter DNA regions from purified control or patient gDNA samples were amplified using LongAmp Taq polymerase and proofreading Q5 polymerase (NEB). PCR products from promoter regions were separated on agarose gels, excised, purified and cloned into the pGL3-Basic vector (Promega) in front of the Firefly Luciferase (Luc^+) gene using KpnI and XhoI restriction sites.

For rescue experiments in the absence or presence of CPG2 coding variants, full-length human *CPG2* (*hCPG2*) was cloned into a lentiviral transfer vector for rat *Cpg2* KD (pFUGW-*Cpg2*-shRNA), in which the stop codon after GFP was removed and *hCPG2* inserted using EcoRI and XhoI restriction sites yielding a GFP-hCPG2 fusion protein (pFUGW-*GFP-hCPG2*) [43]. Human CPG2 coding variants were introduced into pFUGW-*GFP-hCPG2* by site-directed mutagenesis. All constructs were validated by Sanger sequencing.

Neuronal cultures

Rat cortical or hippocampal cultures were prepared as described previously [43, 53].

Luciferase assay

At 8 DIV, cortical neurons were transfected by calcium phosphate precipitation for 1 h with 1 µg of various Firefly Luciferase plasmids and 1.2 µg of the normalization control pRL-TK Renilla Luciferase (RLuc) vector plasmid (Promega) [54]. At 14 DIV, neurons were lysed and assessed using a Dual-Luciferase® Reporter Assay System (Promega) according to the manufacturer's protocol. Plates were read on an EnSpire® Multimode Plate Reader (PerkinElmer). Where indicated, neurons were activated with KCl stimulation solution (170 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES (pH 8.3)) diluted in culture media 1:6 for final concentrations of 28 mM KCl and 50 µM picrotoxin for 6 h before cells were lysed and assayed. Relative Luciferase activity was statistically compared using one-way or two-way (KCl + PcTx) ANOVAs and Tukey's post-hoc tests for multiple comparisons.

Spine localization assay

At 8 DIV, neurons were infected with lentivirus for KD of rat *Cpg2* (pFUGW-*Cpg2*-shRNA) or at 15 DIV with lentivirus for replacement of the endogenous rat CPG2 with the GFP-hCPG2 fusion reference protein (pFUGW-*GFP-hCPG2*) or mutated variants. At 21 DIV cells were fixed in 4% formaldehyde for 15 min and then permeabilized for 25 min with 0.2% saponin in PBS with 10% goat serum. Cells were then incubated for 3 h with primary antibodies; mouse anti-CPG2 (1:500, 200A6; Nedivi lab) and rabbit anti-GFP (1:3000; Abcam), followed by 1 h incubation with secondary antibodies; goat anti-mouse IgG-Alexa Fluor 555 (1:500) and goat anti-rabbit IgG-Alexa Fluor 488 (1:500; Molecular Probes). Coverslips were mounted using Fluoromount-G (Southern Biotech) and imaged using a Nikon Eclipse E600 upright microscope with a 40×/1.40 Plan Apo oil immersion objective (Nikon).

Quantification of immunocytochemistry was conducted using ImageJ software to obtain pixel intensity values in a linear range within regions of interest (ROI). ROIs were positioned over spine heads and dendritic regions based on GFP-stained neuronal morphology and the corresponding staining intensity was measured. Spine localization of GFP-hCPG2 or mutated variants was quantified as the ratio

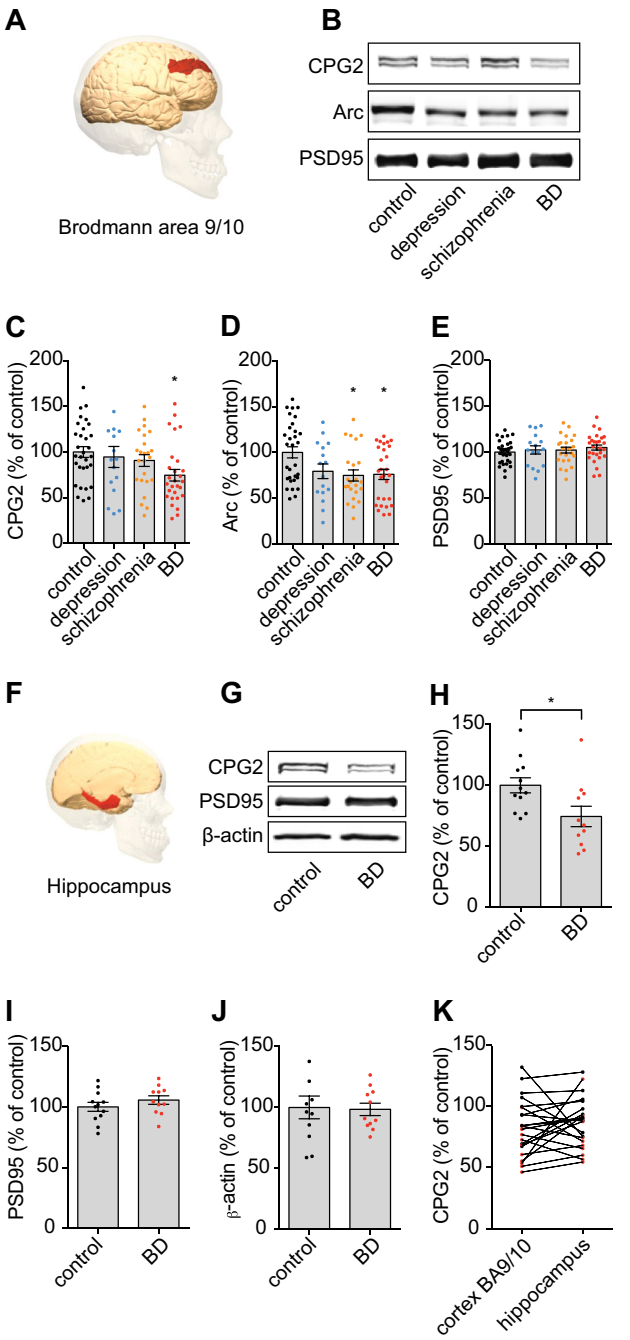
between fluorescence intensity in spine heads and adjacent dendritic shafts. For quantification of spine size, a threshold was applied to make the fluorescence signal binary, and signal areas were quantified and calibrated to the 40× objective specific pixel size (μm^2). All quantifications were done blind to sample identity. Statistical difference from controls was tested using one-way ANOVA and Dunnett's post-hoc test.

Surface receptor internalization assay

The receptor internalization assay was performed as described previously [47] with the following modifications: Cortical cultures were infected at 8 DIV with lentivirus for CPG2 KD (pFUGW-*Cpg2*-shRNA) and at 10 DIV with lentivirus for GFP-hCPG2 (pFUGW-*GFP-hCPG2*) reference protein or mutated variant molecular replacement. Western blots were probed with rabbit anti-GluA2 (1:1000; Abcam), mouse anti-GluN1 (1:2000; Temicula) or mouse anti-TfR (1:1000; Invitrogen) primary antibodies and developed using the Odyssey infrared system (LI-COR). Receptor internalization was quantified as described [47] and groups were compared using one-way ANOVA and Tukey's *post hoc* test for multiple comparisons.

General statistics and data analysis

All statistical analyses were performed with GraphPad Prism software (version 7). The data is graphed as the mean ± standard error of the mean (SEM). Appropriate statistical tests were chosen based on the experimental conditions (see assay specific methods). Gaussian distribution of non-normalized data was assumed in all but the genetic variant analyses. Here, non-parametric tests were used as described. Brown-Forsythe and Bartlett's tests were included in the ANOVAs assuring similar variance between statistically compared groups. Statistical significance was defined as $*p \le 0.05$. For cell culture experiments, poorly transfected cultures were excluded from data analysis. Test conditions were randomized across culture wells/plates.



Results

Low CPG2 protein expression in brains of postmortem BD patients

To investigate if the *CPG2* region of *SYNE1* could play a role in susceptibility for BD, we first asked whether CPG2 protein expression was compromised in BD patients. To this purpose, we isolated synaptic protein fractions from postmortem human brain tissue of control subjects, as well as BD, schizophrenia, and major depression patients (see supplementary table ST1 for source information). Since fMRI and postmortem studies have implicated the prefrontal cortex in BD [55–58], we ran Western blots on tissue from Brodmann Area 9/10 (Fig. 1a), and compared CPG2 protein levels in the three patient groups with that of control subjects (Fig. 1b). We found CPG2 protein levels to be significantly lower in the BD group as compared to control subjects (One-way ANOVA: $F(3,95) = 2.74$, $*p = 0.047$; Tukey's post-hoc: control vs. BD $*p = 0.032$, $n = 102$ subjects) but not in other patient groups tested (Tukey's post-hoc: control vs. depression $p = 0.96$, control vs.

◀ **Fig. 1** CPG2 protein levels are reduced in postmortem brain tissue from BD patients. **a** Synaptic protein fractions were extracted from a total of 102 postmortem human brain tissue samples from Brodmann Area 9/10 (image adapted from commons.wikimedia.org). For SDS-PAGE, 20 µg of total protein was loaded. **b** Representative Western blots comparing CPG2, Arc, and PSD95 protein expression in controls, depression, schizophrenia, and BD patients. Arc is another glutamatergic synaptic protein that like CPG2 is the product of an activity-regulated gene, and the synaptic protein PSD95 serves as a positive marker of glutamatergic synapse presence. **c–e** Quantification of CPG2, Arc, and PSD95 protein levels, respectively, shows that CPG2 levels are significantly lower in the BD patient population as compared to control subjects, whereas Arc is decreased in both schizophrenia and BD groups. Comparable PSD95 levels in all groups indicates that reduced CPG2 or Arc expression does not reflect synaptic loss. (*$p < 0.05$, One-way ANOVAs, Tukey's post-hoc tests). **f** Synaptic protein fractions were extracted from a total of 22 postmortem human brain tissue samples from hippocampus (image adapted from commons.wikimedia.org). **g** Representative Western blots showing CPG2 protein expression in controls and BD patients. The synaptic marker protein PSD95 and β-actin serve as controls. **h** Quantification of CPG2 protein expression. **i** Quantification of PSD95 protein expression. **j** Quantification of β-actin protein expression. **k** Direct comparison of CPG2 protein expression in cortical and hippocampal tissue samples from individual subjects (black dots represent controls and red dots represent BD patients). As in neocortex, CPG2 protein expression is significantly decreased in hippocampal tissue from BD patients. (*$p < 0.05$, Student's *t*-tests)

schizophrenia $p = 0.76$) (Fig. 1c). The Western blots for CPG2 show two bands, which we speculate could represent protein products from different *CPG2* transcripts, or could be a result of, as yet, unidentified posttranslational modifications. Both bands are expressed in the synaptic protein fraction, and their relative signal intensity is constant independent of patient group or other variables, suggesting that expression of the two protein products is regulated by the same mechanism either at the transcriptional, post-transcriptional, or posttranslational level.

Neuropsychiatric disorders are generally associated with lower activity in the prefrontal cortex [10, 11, 59]. To confirm that low levels of CPG2 were not secondary to reduced neural activity and a resulting reduction in levels of this activity-regulated transcript [46], we tested for levels of another activity-regulated gene product, Arc, a protein that like CPG2 is localized to glutamatergic synapses [60]. We observed a positive correlation of CPG2 and Arc levels (Fig. S1A; linear reg.: $F(1,95) = 52.4$, $p < 0.0001$ and Fig. S1B). However, unlike CPG2, which was reduced exclusively in the BD population, Arc was significantly decreased also in other patient groups (One-way ANOVA: $F(3,94) = 4.13$, **$p = 0.0085$; Tukey's post-hoc: control vs. BD *$p = 0.020$, control vs. schizophrenia *$p = 0.022$, control vs. depression $p = 0.14$, $n = 102$ subjects) (Fig. 1d). Levels of the postsynaptic density protein PSD95, a marker of glutamatergic synapses, was similar among the patient groups and as compared to control subjects (One-way ANOVA: $F(3,95) = 0.55$, $p = 0.65$, $n = 102$ subjects). The correlation between CPG2 and PSD95 levels was slight enough (Fig. S1C; linear reg.: $F(1,96) = 16.2$, $p = 0.0001$) to indicate that reduced CPG2 or Arc expression is not merely caused by synaptic loss in BD and/or Schizophrenia patients (Fig. 1e and Fig. S1D; One-way ANOVA: $F(3,95) = 4.51$, **$p = 0.0083$; Tukey's post-hoc: control vs. BD **$p = 0.0027$, $n = 102$ subjects). We did not observe significant correlation of Arc and PSD95 levels (Fig. S1E; linear reg.: $F(1,95) = 1.92$, $p = 0.17$ and Fig. S1F; One-way ANOVA: $F(3,95) = 4.83$, **$p = 0.0036$; Tukey's post-hoc: control vs. BD *$p = 0.012$, control vs. schizophrenia **$p = 0.0017$, $n = 102$ subjects). Further, we found no statistically significant correlation between CPG2, Arc, or PSD95 protein expression and variation in patient age (linear reg.: $F(1,97) = 0.64$, $p = 0.43$; $F(1,97) = 0.16$, $p = 0.69$; $F(1,97) = 2.0$, $p = 0.16$, respectively), postmortem index (PMI) (linear reg.: $F(1,97) = 0.023$, $p = 0.88$; $F(1,97) = 1.2$, $p = 0.28$; $F(1,97) = 3.2$, $p = 0.079$, respectively) or gender (*t*-tests: $t(97) = 1.7$, $p = 0.094$; $t(97) = 1.8$, $p = 0.073$ and $t(97) = 0.52$, $p = 0.61$, respectively) (Fig. S2). Thus, our findings show a specific correlation between low CPG2 levels and incidence of BD that is not shared with schizophrenia or major depression patients.

To test if low CPG2 protein expression in BD patients was specific to prefrontal cortex, we also isolated synaptic protein fractions from postmortem hippocampal tissue (Fig. 1f). Western blotting showed significantly lower CPG2 protein levels in hippocampal tissue from BD patients compared to controls (*t*-test: $t(21) = 2.5$, *$p = 0.021$, $n = 22$ subjects) (Fig. 1g, h), with no change in level of either of the two control proteins, PSD95 (*t*-test: $t(21) = 1.1$, $p = 0.29$), or the loading control β-actin (*t*-test: $t(20) = 0.15$, $p = 0.88$) (Fig. 1i, j). Cortical and hippocampal CPG2 protein levels were well correlated between tissue samples from the same individuals (Fig. 1k). This suggests that lower CPG2 protein levels is a feature common across different brain regions of BD patients.

Low CPG2 protein expression correlates with the presence of common genetic variants in *CPG2* regulatory regions

Low CPG2 protein levels in the BD patient group could derive from several factors, including reduced *CPG2* gene expression. We hypothesized that genetic variation associated with BD might affect regulatory elements of the gene that are important for transcription, such as promoter/enhancer regions, transcription factor binding sites, or in the *CPG2* untranslated regions (UTRs). To test this hypothesis, we purified gDNA from all samples and performed targeted deep-sequencing in the *SYNE1* region (NM_182961: exon 8–70 incl. introns) encompassing the *CPG2* locus. Our

sequencing data yielded hundreds of genetic variants within the *CPG2* locus (see supplementary information, ST5), most of which were common variants also represented in population databases (1000 genomes, HapMap). Of all the identified variants, five SNPs were previously shown to be BD associated (rs4523096 [61], rs7747960 [62], rs9371601 [26], rs214972 [33], and rs215006 [33] supplementary table ST2).

Active promoter and enhancer elements within the *CPG2* gene region were mapped based on histone methylation states seen in ChIP-seq data from human cultured neurons and from human prefrontal cortex [43, 52] (Fig. 2a). Interestingly, three of the BD associated SNPs (rs4523096, rs7747960, and rs9371601) map in or near the proposed *CPG2* promoter region. The two other SNPs (rs214972 and rs215006) map near the 3′UTR of a short *CPG2* isoform and near the 3′UTR end of a long *CPG2* isoform, respectively [43].

To quantify whether the identified BD associated SNPs correlate with low CPG2 expression, we defined mean CPG2 expression in the BD group as the threshold between high and low expression subjects. Within our limited sample set, we did not find statistically significant over-representation of any single SNP allele in the low CPG2 expression subjects when compared to the high CPG2 expression subjects (Fig. 2b). However, the BD associated alleles of the two SNPs (rs4523096[T] and rs7747960[T]) closest to the *CPG2* transcription start site (TSS) trended towards higher frequency in the low, as compared to high, CPG2 expression subjects. This prompted us to focus on the locus encompassing the *CPG2* TSS region. From our sequence data, we identified ~25 genetic variants within this region (supplementary table S3). Based on allele frequencies from the 1000 Genomes database, we found that six SNPs (rs9478332, rs12055686, rs4343926, rs4318888, rs7771568, and rs6908747) within the *CPG2* TSS flanking region were in linkage disequilibrium (LD) with BD associated SNPs ($D' = \sim 1$, $r^2 = > 0.8$) (Fig. 2c, and supplementary table ST2). One of the six SNPs, rs9478332 was also found in the UCSC database of annotated transcription factor binding sites based on ENCODE ChIP-seq data [52], and mapped within a HFH-1 consensus motif upstream of the annotated *CPG2* TSS.

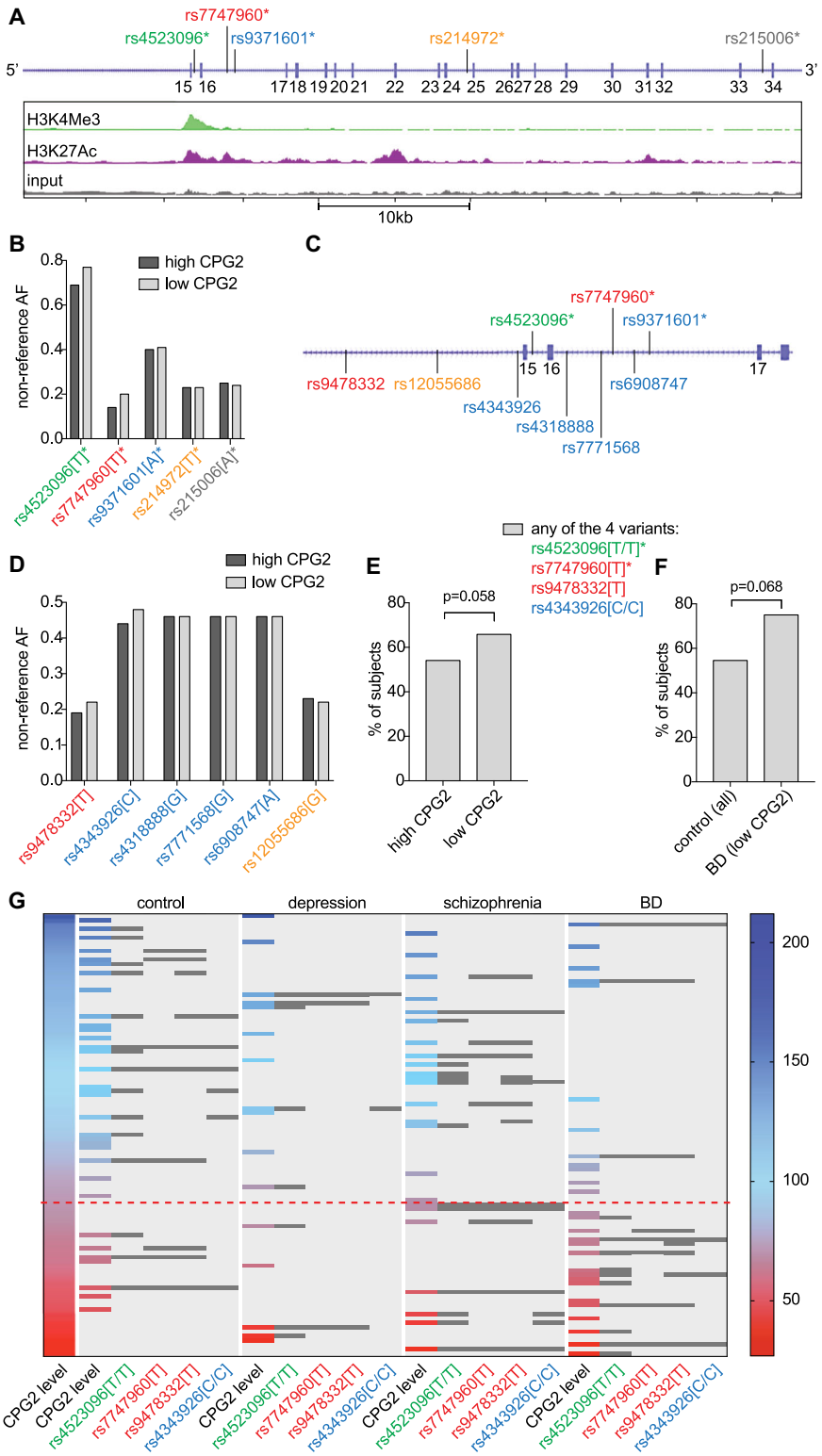
We then tested whether any of the six *CPG2* TSS flanking region SNPs in LD with the BD associated SNPs had alleles overrepresented in our low CPG2 expression subjects (Fig. 2d). Two of the non-reference alleles, rs9478332[T] and rs4343926[C]), trended towards higher frequency within our limited pool of low CPG2 expression subjects, when compared to high CPG2 expression subjects. When considering all four non-reference allele SNPs with higher frequency in low versus high CPG2 expression subjects, (rs4523096[T], rs7747960[T], rs9478332[T], and rs4343926[C]), we found a trend for low CPG2 expression subjects to have at least one, or more, of the four alleles (Mann–Whitney test: $U = 942$, $p = 0.058$, $n = 60$ (any of the four alleles) of 102 subjects) (Fig. 2e). Furthermore, ~75% of the low CPG2 expression BD subjects had at least one of the identified alleles, as compared to ~50% of subjects in the control group (Mann–Whitney test: $U = 195$, $p = 0.068$, $n = 36$ (any of the four alleles) of 50 subjects)) (Fig. 2f).

The representation of the non-reference alleles with higher frequency in low CPG2 expression subjects is illustrated in Fig. 2g for all patient groups. Here, CPG2 protein expression for individual subjects in the different patient groups is displayed on a continuum ranging from low to high expression, where each colored bar represents CPG2 levels in a single subject. Gray bars illustrate the presence of each of the four selected alleles in individual subjects. The dashed line demarcates the threshold between low and high CPG2 expression (as defined above, mean CPG2 expression level for the BD group). Our data suggests that no single allele is associated with the low CPG2 protein expression found in the BD patient subjects. Nonetheless, the presence of a handful of common alleles in or near the proposed *CPG2* promoter region might correlate with low CPG2 protein expression in a larger sample set and could potentially mark BD susceptibility loci within the gene.

Non-reference SNP alleles within the *CPG2* promoter region and their effects on gene expression

To examine whether genetic variants in *CPG2* regulatory loci can influence transcription, we first functionally mapped *CPG2* regulatory domains. We cloned putative promoter and enhancer regions from human brain gDNA samples based on genomic mapping of the *CPG2* locus in *SYNE1* (Fig. 3a), and tested their ability to drive expression of a Luciferase reporter gene in cultured cortical neurons. When plasmids, containing potential *CPG2* promoter regions cloned in front of the Luciferase gene, were transfected into primary neurons, we found a ~2 kb region encompassing the *CPG2 TSS* with robust promoter activity (Fig. 3b and S3). Subsequent deletion analysis on this fragment resolved two regions with promoter activity: One within the *CPG2* 5′UTR, and one within the *SYNE1* intron before exon 16 (i16), both downstream of the predicted TSS (One-way ANOVA: $F(12,188) = 167$, $^{****}p < 0.0001$, Tukey's post-hoc tests, $^{****}p < 0.0001$, $n = 6$–15 transfections, 3–6 cultures) (Fig. 3b and S3).

Of the SNP alleles with higher prevalence in the low CPG2 population, SNP allele rs4523096[T] mapped within the i16 promoter region. To test whether its presence could influence promoter function, we introduced rs4523096[T] into the i16 promoter luciferase construct, and performed



the luciferase reporter assay on transfected primary neurons (Fig. 3c). Because *CPG2* is an activity-regulated gene [46], we used a protocol with mild depolarizing KCl in the presence of the GABA receptor inhibitor picrotoxin (KCl + PcTX) to stimulate activity in the cultures. This protocol increased the relative Luciferase activity

◀ **Fig. 2** Human *CPG2* variants identified by deep-sequencing. Brain tissue gDNA was extracted from all patient and control subjects, and deep-sequenced in the *CPG2* locus to identify genetic variants. Common BD associated variants identified from GWASs [26, 33, 61, 62] were statistically tested for correlation with CPG2 protein expression levels. **a** The genomic position (GRCh37 assembly) of five SNPs identified as BD associated by GWAS mapped onto the *CPG2* region of *SYNE1* (dark blue vertical bars represent exons) shown in the context of previously published ChIP-sequencing data from human neurons identifying active promoter (green) and enhancer (purple) regions [43]. The five SNPs are rs4523096 (green*), rs7747960 (red*), rs9371601 (blue*), rs214972 (yellow*), and rs215006 (gray*). **b** The allele frequencies of the five BD associated SNPs were quantified for high and low CPG2 expression subjects. **c** Six LD proxies (rs9478332, rs12055686, rs4343926, rs4318888, rs7771568, and rs6908747) for the five BD SNPs map to the *CPG2* TSS flanking region (color-matched to origin SNPs). **d** The LD allele frequencies of the six SNP proxies were quantified for high and low CPG2 expression subjects. Four alleles (rs4523096[T], rs7747960[T], rs9478332[T] and rs4343926[C]) were trending towards higher allele frequency in low expressing subjects. **e** The frequency of having at least one of the four non-reference alleles was compared between high and low CPG2 expression subjects, and **f** between BD patients with low CPG2 and control subjects (Mann–Whitney binary tests). Note: rs4523096[T] and rs4343926[C] have high allele frequencies (>0.4) and were quantified for homozygous subjects in **e**, **f** and **g**. **g** CPG2 protein expression levels (from Fig. 1) are displayed on a continuum from low (red) to high (blue) expression, where each colored bar represents one subject and each of the four identified variants enriched in the low CPG2 population shown as dark gray bars. The threshold between high and low CPG2 expression (dashed red line) was defined as the mean CPG2 protein expression level of the BD group as displayed in Fig. 1c

promoted by the i16 construct (Two-way ANOVA: $F(1,56) = 19$, $^{****}p < 0.0001$, Tukey's post-hoc: $^{*}p = 0.011$, $n =$ 15 transfections, 5 cultures), but we found no effect of the rs4523096[T] i16 SNP allele, either on basal or activity-induced gene expression in our cell culture assay (Two-way ANOVA: $F(1,56) = 1.6$, $p = 0.22$, Tukey's post-hoc: control vs. rs4523096[T] $p = 0.89$ (basal), $p = 0.73$ (KCl + PcTx), $n = 15$ transfections, 5 cultures).

Recent studies suggest that risk genes containing common variants with low effect often also contain rarer variants with larger effect [63, 64]. In our limited sample set, we could not use statistical methods to identify rare variants. Instead, from our deep sequencing data of patient gDNA we identified several genetic variants within the putative *CPG2* promoter regions, and tested all observed non-reference alleles in our Luciferase reporter assay. One SNP allele, rs4530871[T], located within a mammalian conserved sequence of the 5′UTR *CPG2* promoter region significantly attenuated activity-induced gene expression (Two-way ANOVA: $F(1,56) = 7.0$, $^{*}p = 0.011$, Tukey's post-hoc: control vs. rs4530871[T] $p = 0.86$ (basal), $^{*}p =$ 0.022 (KCl + PcTx), $n = 15$ transfections, 5 cultures) (Fig. 3d).

Since the rs4530871[T] allele does not exist in our dataset without rs4523096[T], we tested the combined effect of the two alleles on relative Luciferase activity of the 5′UTR i16 promoter construct, which encompasses both SNP locations. We found that the combination of the two alleles significantly affects gene expression, even at basal activity levels of the cultured neurons (One-way ANOVA: $F(2,30) = 6.26$, $^{**}p = 0.0053$, Tukey's post-hoc: control vs. rs4523096[T] + rs4530871[T] $^{**}p = 0.0054$, $n = 11$ transfections, 3 cultures) (Fig. 3e).

We also tested the effect of another rare non-reference SNP allele rs924872285[G] on promoter activity of the −0.2 kb i16 construct. This allele was identified in a single BD patient with low CPG2 expression in our dataset, and is situated in a conserved enhancer region immediately upstream of the TSS. This allele too, is only present together with rs4523096[T]. When we tested the combination of both alleles in the context of the −0.2 kb i16 promoter construct, we saw a significant reduction in gene expression at basal activity levels as compared to controls (One-way ANOVA: $F(3,45) = 6.83$, $^{***}p = 0.0007$, Tukey's post-hoc: control vs. rs4523096[T] + rs924872285[G] $^{*}p =$ 0.015, $n = 13$ transfections, 3 cultures) (Fig. 3f).

The protein expression data, together with the identification of genetic variants in *CPG2* regulatory regions from BD patients that negatively affect gene expression, possibly implicate low CPG2 expression in susceptibility to BD.

Non-reference SNP alleles in the CPG2 coding region affect spine localization of the CPG2 protein

While low CPG2 levels could lead to dysregulation of synaptic glutamate receptor internalization [43, 47], synapse dysregulation could also derive from CPG2 protein variants with reduced function.

From public databases (1000 Genomes, gnomAD), we identified 12 common missense SNPs within the CPG2 coding region (Table 1). The reference alleles for ten of the SNPs in the dataset are conserved among mammalian species.

To gauge the extent to which individual coding SNPs could influence CPG2 cellular localization and/or function in the general population, we tested seven representative mutations (corresponding to the identified missense SNP non-reference alleles). We used a previously described knockdown (KD) [47] and replacement strategy [43], where endogenous CPG2 was knocked down in cultured cortical rat neurons and replaced with human reference or mutated CPG2. We have previously used this molecular replacement strategy to show that human CPG2 can replace endogenous rat CPG2, both in terms of its cellular localization to dendritic spines and its ability to regulate internalization of synaptic glutamate receptors [43]. The seven representative mutations were individually introduced into the full length human CPG2 coding sequence and cloned as GFP fusion

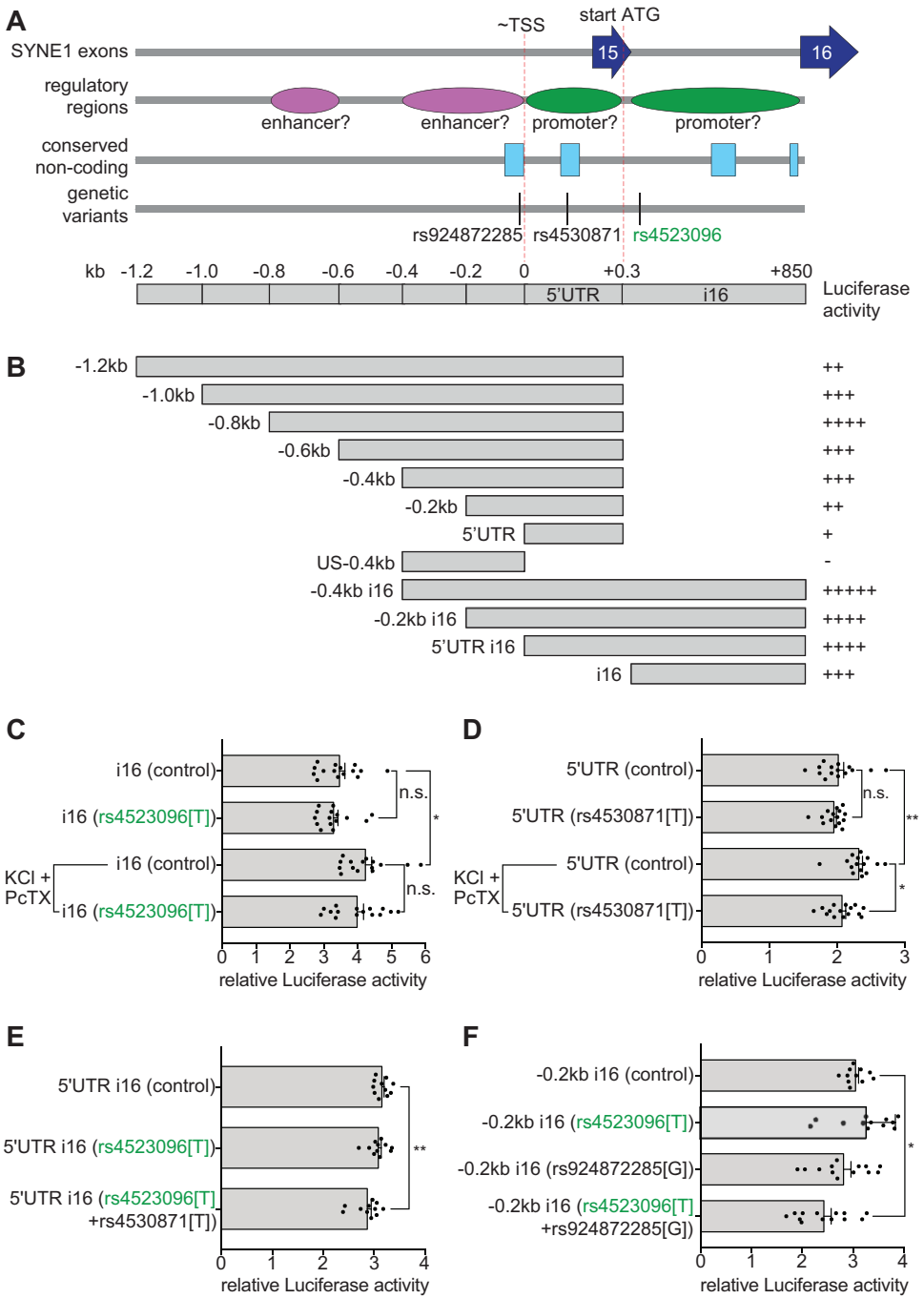


Fig. 3 Non-reference SNP alleles in the *CPG2* promoter region and their effect on gene expression. *CPG2* gene regions with promoter activity were identified using a Luciferase gene expression assay in cultured cortical neurons. **a** Schematic depiction of the *CPG2* promoter region in *SYNE1* (−1.2 kb to +850 kb from proposed *CPG2* transcription start site (~TSS) [43]). Dark blue arrows indicate *SYNE1* exons and red dashed lines mark the approximate ~TSS and the translation start site (start ATG). Green and purple ovals mark identified regulatory regions with promoter or enhancer activity, respectively. Light blue boxes indicate mammalian conserved intronic regions and black vertical lines mark identified variants within the region. **b** Overview of constructs with *CPG2* promoter region fragments and their relative activity in the Luciferase expression assay. **c** Effects of SNP allele rs4523096[T] on activity of the i16 promoter region with or without KCl + PcTX-induced neuronal stimulation. **d** Effects of SNP allele rs4530871[T] on activity of the 5′UTR region with or without KCl + PcTX-induced neuronal stimulation. The 5′ UTR SNP allele rs4530871[T] significantly attenuated activity-induced gene expression. **e** Effect of SNP alleles rs4523096[T] and rs4530871[T] combined, on relative Luciferase activity of the 5′UTR i16 region. The combination of the two non-reference SNP alleles significantly affects gene expression at basal neuronal activity levels as compared to control. **f** Effect of SNP alleles rs4523096[T] and rs924872285[G] combined, on relative Luciferase activity of the −0.2 kb i16 region. This combination of non-reference alleles also significantly affects gene expression as compared to control. (*$p < 0.05$, **$p < 0.01$, One-way or Two-way (KCl + PcTX) ANOVAs, Tukey's post-hoc tests)

Table 1 Genetic variants in CPG2 exons

Genome position (GRCh37) chr6:	SNP	Variant allele	MAF (1000G)	SYNE1 exon #	AA residue substitution in human CPG2	Effect on spine Localization	Effect on GluA2/ GluN1 internalization
152784621	rs9397509	A/G	$G = 0.004$	19	Q171R	No	No
152779933	rs34610829	C/T	$T = 0.011$	22	R359C[a]	n/a	n/a
152777095	rs17082709	T/G	$G = 0.043$	23	L401V[a]	n/a	n/a
152776571	rs76646638	G/A	$A = 0.002$	24	R477Q	No	Yes
152776571	rs201146062	C/T	$T = 0.001$	24	R477W	n/a	n/a
152774753	rs148346599	G/A	$A = 0.002$	25	E515K	n/a[b]	n/a[b]
152772264	rs214976	T/C	$T = 0.39$	26	V551A	Yes	No
152771967	rs141464488	T/C	$C = 0.001$	27	V579A	n/a	n/a
152768738	rs116939102	C/T	$T = 0.0002$	29	T691I	no	Yes
152768726	rs117461489	A/C	$C = 0.0008$	29	E695A	no	No
152762307	rs149109801	T/A	$A = 0.0002$	32	F885L	n/a	n/a
152757224	rs34028822	C/T	$T = 0.0029$	33	R904W	Yes	Yes

[a]Not conserved between rat and human

[b]Low expression in cellular assays

constructs into a lentiviral vector also expressing a previously validated rat *Cpg2*-specific small hairpin RNA (shRNA) [47]. These replacement viruses were used to infect cultured neurons, and mutated CPG2 localization was compared to human reference CPG2. As previously shown, uninfected neurons showed enriched CPG2 localization in dendritic spines (Fig. 4a), adjacent to the postsynaptic density protein PSD95 [43, 47]. Consistent with previous findings [43], neurons infected with the shRNA virus showed robust KD of CPG2 protein (Fig. 4b), and molecular replacement with plasmids co-expressing the *Cpg2*-specific shRNA and a shRNA-resistant *GFP-hCPG2* fusion construct showed a punctate anti-GFP staining pattern, resembling the spine localization of the endogenous protein (Fig. 4c).

Six of the seven tested GFP-hCPG2 protein variants were expressed at levels comparable to the human reference GFP-hCPG2 protein (Fig. 4d–i). Interestingly, expression of the E515K variant was very low and did not allow proper evaluation of spine localization (data not shown), suggesting this coding mutation could also impact CPG2 levels or protein stability. When compared to the reference GFP-hCPG2 protein, immunostaining with anti-GFP antibodies showed significantly decreased spine localization for the V551A and R904W GFP-CPG2 protein variants (One-way ANOVA: $F(6,193) = 3.6$, $^{**}p = 0.002$, Dunnett's post-hoc: reference vs. V551A $^{**}p = 0.0089$, reference vs. R904W $^{*}p = 0.044$, $n = 4$ independent experiments) (Fig. 4j). Significant differences in spine size was not observed for the tested variants (One-way ANOVA: $F(6,213) = 2.1$, $^{*}p = 0.049$, Dunnett's post-hoc: $p > 0.05$, $n = 4$ independent experiments) (Fig. 4k).

Non-reference SNP alleles within the CPG2 coding region affect glutamate receptor internalization

Rat CPG2 is known to localize to the endocytic zone of dendritic spines, where it regulates endocytosis of synaptic glutamate receptors [47, 65]. We have recently shown that human CPG2 is functionally equivalent to rat CPG2 in facilitating glutamate receptor internalization in cultured neurons, suggesting a conserved function for CPG2 in rat and human brain [43]. To test whether any of the six human CPG2 coding variants could affect the ability of human CPG2 to functionally replace endogenous rat CPG2, we used an internalization assay for biotinylated surface receptors. Consistent with previous data [43, 47, 65], we found that $6.0 \pm 0.9\%$ of GluA2 containing AMPARs were constitutively internalized after 30 min in uninfected neurons, and KD of CPG2 decreased GluA2 internalization by approximately half ($3.5 \pm 0.8\%$, $^{*}p < 0.05$) (Fig. 5a–f). Viral replacement with the GFP-hCPG2 reference protein rescued GluA2 internalization to levels comparable to endogenous CPG2 ($6.0 \pm 1.0\%$). When testing the six mutated CPG2 variants, we found significantly decreased GluA2 internalization rates for the R477Q (One-way ANOVA: $F(3,37) = 5.7$, $^{**}p = 0.0027$, Tukey's post-hoc: reference vs. R477Q $^{*}p = 0.014$, $n = 8$), T691I (One-way ANOVA: $F(3,35) = 5.6$, $^{**}p = 0.003$, Tukey's post-hoc: reference vs. T691I $^{*}p = 0.039$, $n = 7$) and R904W (One-way ANOVA: $F(3,34) = 7.2$, $^{***}p = 0.0008$, Tukey's post-hoc: reference vs. R904W $^{*}p = 0.012$, $n = 12$) variants (Fig. 5b, d and f).

Similar to its effect on GluA2 internalization, CPG2 KD significantly decreased constitutive internalization of GluN1

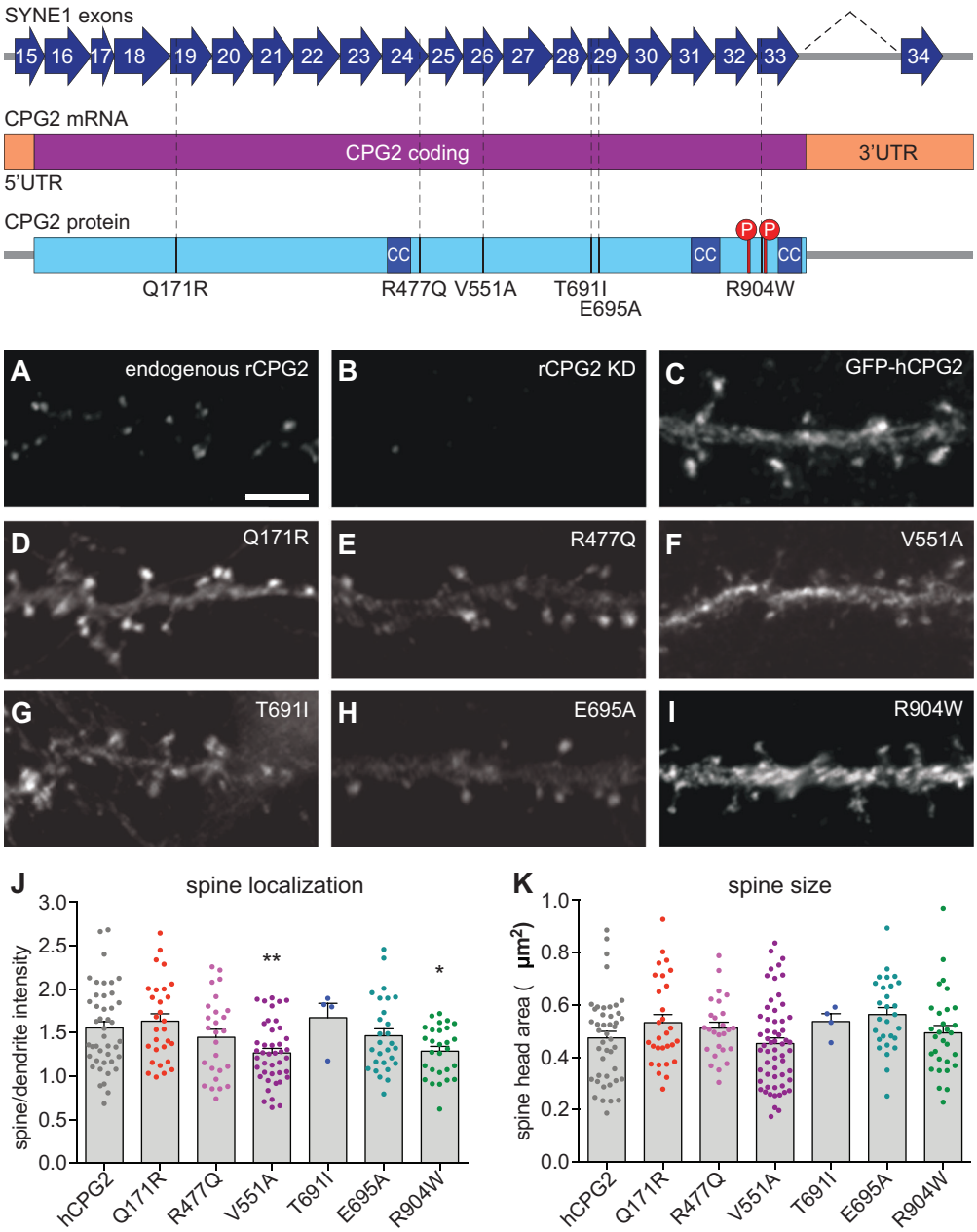


Fig. 4 Effect of human coding variants on CPG2 spine localization. Schematic depiction of the *CPG2* coding region in *SYNE1*. Dark blue arrows indicate *SYNE1* exons (note: the i34 intron is not spliced out in *CPG2*). Purple indicates the *CPG2* coding region and orange marks the 5′ and 3′UTRs in the *CPG2* mRNA. Light blue depicts the predicted CPG2 protein structure with dark blue regions indicating coiled-coil domains and red circles marking two PKA phosphorylation sites known to affect CPG2 protein function [65]. Black vertical lines label the positions of identified missense SNPs within the *CPG2* coding region with their amino acid residue exchange indicated. Cultured hippocampal neurons were lentivirus infected with shRNA for KD of endogenous rat CPG2 (rCPG2) and replaced with GFP-fused human CPG2 (hCPG2) reference protein or mutated variants. Representative images showing dendritic segments of neurons expressing endogenous rCPG2 (**a**) and shRNA rCPG2 KD (**b**) stained with anti-CPG2 monoclonal antibodies. **c**–**i** Representative images showing dendritic segments of neurons expressing GFP-hCPG2 control or mutated variants stained with anti-GFP antibodies. Scale bar: 2 µm. **j** Spine localization was quantified as the ratio between fluorescence intensity in spine regions and in 10 µm of adjacent dendrite. **k** Quantification of spine size defined as spine head area (µm^2). Spine localization of the V551A and R904W variants is significantly decreased while spine size is comparable to control. (*$p < 0.05$, **$p < 0.01$, One-way ANOVAs, Dunnett's post-hoc tests)

containing NMDARs (*$p < 0.05$), while viral replacement with human CPG2 also rescued internalization of GluN1 to control levels (Fig. S4A-F). GluN1 receptor internalization rates were significantly decreased for the same CPG2 variants that affected GluA2 internalization (One-way ANOVAs: $F(3,29) = 6.2$, **$p = 0.0022$ (R477Q); $F(3,37) = 5.7$,

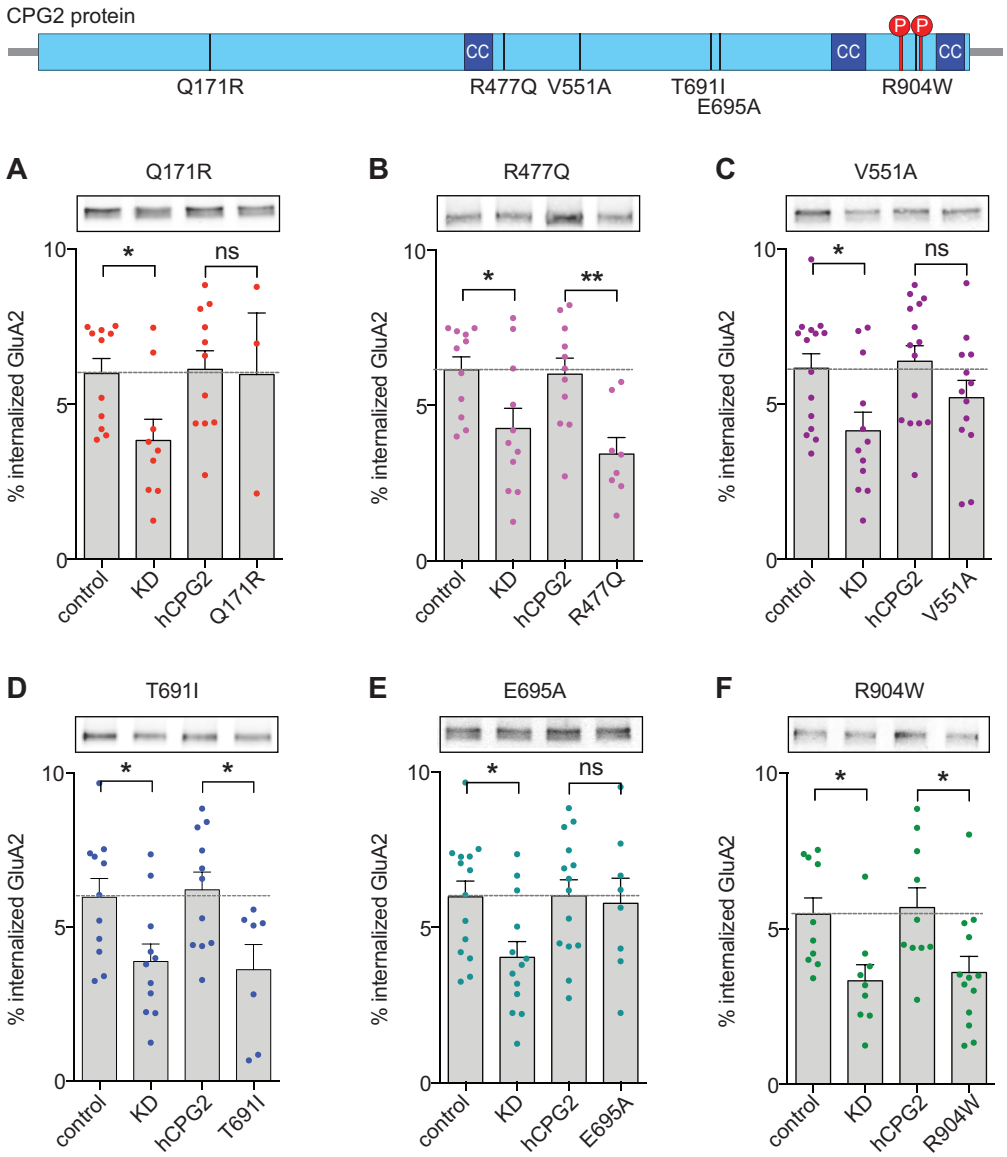


Fig. 5 Effect of human CPG2 coding variants on glutamate receptor internalization. *CPG2* coding variants were tested in an internalization assay for biotinylated surface receptors. Schematic depiction of predicted CPG2 protein structure with dark blue regions indicating coiled-coil domains and red circles marking two PKA phosphorylation sites. Black vertical lines label the positions of identified missense SNPs with their amino acid residue exchange indicated. **a–f** Cultured cortical neurons were either uninfected (control), infected with shRNA for KD of endogenous CPG2 (KD) or infected with shRNA for CPG2 KD together with GFP-fused human CPG2 reference protein (hCPG2) or mutated variants (as indicated). Representative Western blots showing the internalized fraction of biotinylated surface receptors probed with GluA2 antibodies. Quantification of internalized GluA2 is presented as percent internalization calibrated to levels of surface receptors. Human CPG2 replacement shows receptor internalization rates comparable to control. CPG2 variants R477Q, T691I, and R904W show significantly decreased GluA2 internalization. ($*p < 0.05$, $**p < 0.01$, One-way ANOVAs, Tukey's post-hoc tests)

$**p = 0.0026$ (T691I); $F(3,36) = 6.7$, $**p = 0.001$ (R904W), Tukey's post-hoc tests: reference vs. R477Q $*p = 0.035$, $n = 8$; reference vs. T691I $*p = 0.036$, $n = 7$; reference vs. R904W $*p = 0.025$, $n = 12$, respectively) (Fig. S4 B, D and F). Neither CPG2 KD, nor replacement with reference or mutated human CPG2 significantly affected the constitutive internalization of transferrin receptor (TfR) ($p > 0.05$) (Fig. S3G-L). Our data suggest that low frequency but relatively common CPG2 coding variants (MAF > 0.001 present in the population) can have significant effects on CPG2 synaptic function.

Discussion

Recently, large consortium GWAS studies have shed new light on the genetics of neuropsychiatric disorders. However, genetic variants identified so far account for only a

fraction of disease liability, a phenomenon generally characteristic of complex genetic traits. Purely genomic approaches such as GWAS and linkage studies are only the first step in elucidating the complex neurobiology of BD. They need to be followed and complemented with molecular and cellular studies of defective neuronal function to obtain a more complete understanding of disease etiology.

Identification of risk loci for BD has firmly implicated dysregulated excitatory neurotransmission as a key component of BD etiology [24, 25, 34–36]. Given the role of CPG2, a *SYNE1* gene product, in facilitating glutamate receptor internalization and regulating excitatory synaptic strength [43, 47, 65], this gene might be an important player in the neurobiological underpinnings of BD. Here, we show that low levels of the CPG2 protein are more prevalent in BD patients as compared to controls and other patient groups, and use multiple strategies to identify genetic variants within CPG2 regulatory or protein-coding regions that negatively affect gene expression or disrupt protein function, respectively.

Our finding that CPG2 protein levels are significantly lower in postmortem prefrontal cortex from BD patients as compared to control subjects, a phenomenon not shared with schizophrenia or major depression patients, supports specificity of *CPG2* perturbation in BD. This is consistent with GWAS findings that genetic variation in *SYNE1* is more closely associated with risk for BD [23, 26]. Low CPG2 levels across brain regions of BD patients suggests it is likely to derive from genetic causes. The clustering of common genetic variants in *CPG2* regulatory regions identified in the low CPG2 expression BD subjects further supports this notion, although we cannot exclude additional causes such as region-specific reduced activity in BD brains.

It is often noted that common risk variants identified by GWAS overwhelmingly reside in large introns and in sequences immediately upstream of the implicated genes [66]. This suggests that disease-associated variation may derive not mainly from disrupted protein function, but from dysregulated gene expression. For example, the largest number of disease associations found by GWAS in schizophrenia are in regulatory regions, such as promoter or enhancer sequences [67, 68]. Studies of expression quantitative trait loci (eQTLs) in human tissues [69, 70] also show that disease implicated genetic variants tend to associate with quantitative differences in expression levels of the same genes, especially when gene expression is measured in the tissue relevant to the disease [68, 69]. Unfortunately, we were not able to find eQTL data for the *CPG2* gene region in public databases (GTEx, ExSNP). Progress in the genome-scale analysis of chromatin states now reveals hundreds of thousands of sites across the genome that contain dynamic chromatin marks suggestive of tissue-specific promoter or enhancer activity, with the ability to regulate the expression of nearby genes in specific tissues [52, 71]. The regulatory elements mainly found in introns are often less conserved between humans and rodents, as well as across all of evolution. Interestingly, the CPG2 promoter region is highly conserved among mammalian species as compared to other intronic regions within the gene (see supplementary table ST4 and sequence alignment for quantification), suggesting that this region has a conserved activity-dependent regulatory function in mammalian brains.

Our data suggest that no single genetic variation is associated with the low CPG2 protein expression found in BD patient subjects. This is in accordance with the pleiotropic and polygenic nature of BD, and other neuropsychiatric disorders, where many genetic risk factors each contributing a small effect cumulatively add up to a larger disease susceptibility. Common regulatory variants can also result in milder phenotypes that reflect tissue-specific or cell type specific gene expression. For example, a common variant in a *CACNA1C* regulatory region associated with approximately 15% increased risk of schizophrenia and BD, has no apparent association to cardiac or immune phenotypes [66]. By analogy, common regulatory variants influencing expression of brain-specific *CPG2* transcripts from the *SYNE1* gene would confer increased risk for developing BD, without significant impact on muscular phenotypes associated with other *SYNE1* transcripts.

GWAS and genetic linkage studies, with the intrinsic requirement for very large sample sizes, are best suited to identify relatively common genetic risk variants. We found that when tested alone, the common BD associated i16 SNP allele (rs4523096[T]) [61] had no apparent effect on gene expression. A suggested explanation for some of the "missing heritability", not yet accounted for in the genetics of complex diseases, is that common disease-associated SNPs act as sentinels for other genetic risk factors adjacent within the same region with more penetrant effects [63]. From our deep sequencing data of patient gDNA, we identified several genetic variants within the putative *CPG2* promoter and enhancer regions. One SNP allele (rs4530871 [T]), situated within a mammalian conserved sequence of the 5'UTR *CPG2* promoter region significantly attenuated activity-induced gene expression when tested alone. Interestingly, (rs4523096[T] and rs4530871[T]) in combination, reduced basal gene expression. Likewise, another SNP allele, rs924872285[G] identified in a putative *CPG2* enhancer, also reduced basal gene expression when combined with rs4523096[T]. This exemplifies the common notion for complex genetic diseases that cumulative effects of low penetrant variants can combine to a greater effect size. Further, the differential effect of specific SNP alleles

on basal versus activity-dependent gene expression suggests that within the general population, there are low frequency but relatively common genetic variants in the *CPG2* promoter region that influence neuronal activity-dependent gene expression without apparent effect on basal level gene expression. The activity-dependent requirement for gene expression could potentially provide one explanation for the influence of environmental "stressors" on development of BD associated psychiatric symptoms.

While low CPG2 levels have already been shown to disrupt synaptic glutamate receptor internalization [43, 47], synapse dysregulation could also derive from CPG2 protein variants with reduced function. Thus, some patients with apparently normal CPG2 levels might suffer from risk for BD due to coding region mutations. In a recent report, six nonsynonymous SNPs were identified in the *CPG2* region of *SYNE1* using high-resolution melt analysis [72], but only predicted effects were described. The four most common of the missense SNPs overlap with our findings, namely rs34610829[T] (R359C), rs17082709[G] (L401V), rs148346599[A] (E515K), and rs214976[C] (V551A), of which the former two are not conserved across evolution and were therefore not tested here. The two latter were predicted by Polyphen and SIFT analyses to be tolerated in other *SYNE1* products and likely damaging to CPG2, and benign to both gene products, respectively [72]. This agrees with our findings that E515K had an apparent effect on CPG2 expression or stability, and V551A had a small effect on CPG2 spine localization with no apparent effect on glutamate receptor internalization. We found three additional SNPs with significant negative effects on glutamate receptor internalization, rs76646638[A] (R477Q), rs116939102[T] (T691I), and rs34028822[T] (R904W), of which the latter also affected spine localization. The R904W variant is situated at the C-terminal of CPG2 in between two reported protein kinase A (PKA) phosphorylation sites important for CPG2 spine localization and protein function [65]. We speculate that the bulky tryptophan in the minor allele variant might interfere with PKA binding and CPG2 phosphorylation, and thereby disrupt CPG2 protein function. The two other functionally disruptive variants R477Q and T691I do not have apparent effects on spine localization. They are situated far from the CPG2 C-terminal f-actin binding site important for spine localization [73] but might affect functional protein domains in ways that allows normal spine localization but disrupt binding to the endocytic machinery, which facilitates receptor internalization [47].

In this study, we present protein functional data from a statistically significant and replicated BD associated risk locus. We show that levels of the brain-specific *SYNE1* product CPG2 are lower specifically in BD patients as compared to controls as well as schizophrenia and depression patients. We identify genetic variants in the *CPG2* promoter region with negative effects on gene expression, as well as low frequency coding variants in *CPG2* that significantly affect CPG2 protein function. Taken together, our data fit a genetic architecture of BD, likely involving clusters of both regulatory and protein-coding variants, whose combined contribution to phenotype is an important piece of a puzzle containing other risk and protective factors influencing BD susceptibility. The ultimate goal is to allow a more scientifically informed, evidence-based approach to measure, differentiate and treat neuropsychiatric disorders, preferably with the aid of genetic and other non-invasive biomarkers.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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