

The effect of psychosis associated CACNA1C, and its epistasis with ZNF804A, on brain function

Short title: The effect of CACNA1C on brain function

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Abstract

CACNA1C-rs1006737 and *ZNF804A*-rs1344706 polymorphisms are amongst the most robustly associated with schizophrenia (SCZ) and bipolar disorder (BD), and recently with brain phenotypes. As these patients show abnormal verbal fluency (VF) and related brain activation, we asked whether the latter was affected by these polymorphisms (alone and in interaction) – to better understand how they might induce risk. We recently reported effects on functional VF-related (for *ZNF804A*-rs1344706) and structural (for both) connectivity.

We genotyped and fMRI-scanned 54 SCZ, 40 BD and 80 controls during VF. With SPM, we assessed the main effect of *CACNA1C*-rs1006737, and its interaction with *ZNF804A*-rs1344706, and their interaction with diagnosis, on regional brain activation and functional connectivity (psychophysiological interactions - PPI). Using public data, we reported effects of *CACNA1C*-rs1006737 and diagnosis on brain expression.

The *CACNA1C*-rs1006737 risk allele was associated with increased activation, particularly in the bilateral prefronto-temporal cortex and thalamus; decreased PPI, especially in the left temporal cortex; and gene expression in white matter and the cerebellum. We also found unprecedented evidence for epistasis (interaction between genetic polymorphisms) in the caudate nucleus, thalamus, and cingulate and temporal cortical activation; and *CACNA1C* up-regulation in SCZ and BD parietal cortices. Some effects were dependent on BD/SCZ diagnosis. All imaging results were whole-brain, voxel-wise, and familywise-error corrected.

Our results support evidence implicating *CACNA1C* and *ZNF804A* in BD and SCZ, adding novel imaging evidence in clinical populations, and of epistasis – which needs further replication. Further scrutiny of the inherent neurobiological mechanisms may disclose their potential as putative drug targets.

1. Introduction

Schizophrenia (SCZ) and bipolar disorder (BD) are severe psychiatric diseases with a strong genetic component (a heritability of up to 80% in SCZ (Cardno *et al.* 1999) and 93% in BD (Kieseppä *et al.* 2004)). Recently, genome-wide association studies (GWAS) have identified *CACNA1C* and *ZNF804A* as significant risk genes for both SCZ and BD susceptibility (Gurung & Prata 2015). Nevertheless, how they induce risk for psychiatric illness remains relatively unknown.

CACNA1C encodes an alpha-1 subunit of the voltage dependent L-type calcium channel Ca_v1.2. This type of channels is widely expressed in the brain and involved in, for example, regulation of signalling pathways, neurotransmitter release, synaptic plasticity, neuron excitability and specifically modulates the effects of synaptic activity on cell survival (Uemura *et al.* 2015). The rs1006737 single nucleotide polymorphism (SNP) of the *CACNA1C* gene was identified through GWAS to be associated with risk for both BD (Ferreira *et al.* 2008) and SCZ (Green *et al.* 2010; Nyegaard *et al.* 2010). This risk allele adenine (A) of this SNP was also associated independently with: 1) increased *CACNA1C* mRNA expression (which might affect the receptor's activity (Bigos *et al.* 2010)) in induced human neurons; 2) increased density of Ca_v1.2-mediated currents (Yoshimizu *et al.* 2015); and 3) decreased expression in the human cerebellum (Gershon *et al.* 2014). This may

suggest that either an increase or decrease of calcium influx in excitable cells might be associated with SCZ or BD, as both could lead to changes in monoamine neurotransmitter synthesis and release (Gershon *et al.* 2014) - which has, indeed, been associated with other psychiatric disorders (Booij *et al.* 2003).

In terms of anatomy, the same *CACNA1C* rs1006737 risk allele, has been associated with increased total and fronto-limbic white matter volume (Frazier *et al.* 2014), albeit only after a few earlier negative findings (Kempton *et al.* 2009; Franke *et al.* 2010). Regarding white matter, after a reported association with reduced microstructural integrity in the right hippocampal formation in healthy Caucasians (Dietsche *et al.* 2014), we have published, for the first time using whole-brain tract-based spatial statistics, an association with reduced microstructural integrity. This effect was found within SCZ subjects (but not controls or BD), in portions of the left middle occipital and para-hippocampal gyri, right cerebellum, left optic radiation and left inferior and superior temporal gyri (Mallas *et al.* 2016a) - consistent with previous voxel-based findings (Woon *et al.* 2014). We also found the first evidence of an additive interaction of the *CACNA1C* and *ZNF804A* genotype on white matter microstructure (Mallas *et al.* 2016a). Both risk alleles' concomitant presence in BD was associated with decreased integrity in the body of the corpus callosum, the right superior and left anterior corona radiata, comparatively more than in healthy controls. This finding is consistent with the hypothesis that both these polymorphisms increase risk for psychosis.

In terms of brain function, healthy risk allele (A) carriers have shown: 1) a trend for increased left precuneus and left inferior frontal activation in healthy volunteers during semantic verbal fluency (Krug *et al.* 2010) and, 2) a trend for increased prefrontal activation during working memory (Bigos *et al.* 2010). Both frontal effects, given that performance level was controlled

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for, could be interpreted as lower efficiency – which is also found in SCZ relatively to controls (Gurung & Prata 2015). However, the latter was contested by another study that surprisingly found the reverse effect in healthy subjects: the risk allele homozygous showing *less* activity vs. G-allele carriers in the right dorsolateral prefrontal cortex (Paulus *et al.* 2014). Increased functional connectivity between that region and the bilateral hippocampal formations (dose-dependently) was also found, which, interestingly, mimics some *ZNF804A* rs1344706 risk allele's findings, suggesting a common downstream pathway for both risk variants (Gurung & Prata 2015). As replication is key to clarify cause-effect assumptions in correlational approaches, we asked whether we could re-produce the above pattern of findings for *CACNA1C*'s role on brain function – and help clarify inconsistencies.

Regarding the impact of *ZNF804A* rs1344706 genotype, the risk allele A has been extensively associated with alterations in connectivity, and, to a lesser extent, in brain activation (Gurung & Prata 2015). The risk allele A was recently associated in verbal fluency with decreased functional coupling between the left precentral gyrus/inferior frontal gyrus and both the left inferior frontal gyrus and the left posterior cingulate gyrus, encompassing the precuneus (Tecelão *et al.* 2018). This converges with findings showing intra- and inter-hemispheric prefrontal connectivity decrease (albeit not always) in other tasks (Gurung & Prata 2015), abnormal white matter microstructure (Mallas *et al.* 2016b), and with the disconnection hypothesis of SCZ (Gurung & Prata 2015). Finally, the risk allele A was also associated during verbal fluency with higher regional activation in BD, but the reverse in healthy controls, in the left inferior frontal gyrus, pars opercularis/triangularis (Tecelão *et al.* 2018), supporting a previous finding in healthy subjects during theory-of-mind (Gurung & Prata 2015).

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Thus, in addition, in this study we assessed, for the first time, interaction between these polymorphisms (i.e. epistasis) in clinical samples of BD and SCZ. We inferred the main effect of *CACNA1C* rs1006737 genotype (or, rather, the linkage disequilibrium block it tags) and its interaction with *ZNF804A* rs1344706 genotype, on regional brain activations and functional connectivity, including that under psychophysiological interaction (PPI), during verbal fluency – across healthy volunteers, and SCZ and BD patients. We also tested for genotype associations that would be dependent on diagnosis. We used verbal fluency as we, using an overlapping sample to the present one (Prata *et al.* 2009a), and others, have shown that it is (Curtis *et al.* 1998, 1999, 2001; Fu *et al.* 2005) – as are its neural correlates (Krabbendam *et al.* 2005; Daban *et al.* 2006) - impaired in psychosis, especially in SCZ. *CACNA1C* risk allele A was expected to be associated with less efficient regional activation and with functional connectivity disruptions during verbal fluency. This is given previous evidence of its effect on regional activation (Bigos *et al.* 2010; Krug *et al.* 2010), and functional (Paulus *et al.* 2014) and structural (Dietsche *et al.* 2014; Woon *et al.* 2014; Mallas *et al.* 2016a) connectivity. We also expected that these individual effects of the risk allele might be augmented by the presence of the risk allele A of *ZNF804A* rs1344706 which we have recently found to have a putatively detrimental effect during the same task and sample as the present ones – i.e. of decreased left ipsilateral prefrontal functional connectivity across diagnoses (Tecelão *et al.* 2018). In other words, we predicted that the presence of both risk alleles would be associated with the most inefficient activation and/or disrupted functional connectivity – mimicking our abovementioned findings in white matter (Mallas *et al.* 2016a). To lend possible converging evidence to our neuroimaging findings, we further enquired, using an online public brain gene expression database, whether these SNPs affected gene

expression (i.e. were expression quantitative trait loci; eQTLs) in each of 10 post-mortem human brain areas. With a second database, we tested diagnosis-wise differences in these genes' expression in several brain areas (comparing SCZ, BP and healthy subjects).

2. Materials and Methods

2.1. Sample

Our sample consisted of 174 English native speakers, the majority (93%) Caucasian, including a control group comprised of 80 healthy volunteers (34 males, 39±13 y.o.) with no history, or first degree family history, of a psychotic spectrum disorder, 54 patients with established SCZ (42 males, 37±11 y.o.) and 40 with BD (16 males, 40±12 y.o., 75% of which with a history of psychosis). Patients were recruited from the South London and Maudsley (SLaM) NHS Trust. Diagnosis, according to the criteria of the Diagnostic and Statistical Manual of Mental Disorders (DSM) 4th Edition (American Psychiatric Association 1994), was ascertained by an experienced psychiatrist using a structured diagnostic interview with instruments detailed elsewhere (Prata *et al.* 2009b). All SCZ and BD patients were in a stable clinical state. Exclusion criteria applied to all participants were a history of significant head injury and current (last 12 months) substance dependency according to DSM-IV diagnostic criteria. The study was approved by the National Health Service (NHS) South East London Research Ethics Committee, UK (Project "Genetics and Psychosis (GAP)" reference number 047/04). All subjects gave written informed consent.

Genotyping for the CACNA1C rs1006737 and the ZNF804A rs1344706 SNPs was performed using standard genotyping techniques we previously described (Mallas *et al.* 2016a, 2016b). Possible genotype outcomes for CACNA1C were A homozygous (AA,

adenine-adenine), heterozygous (AG, adenine-guanine) and G homozygous (GG, guanine-guanine), and for ZNF804A were A homozygous (AA, adenine-adenine), heterozygous (AC, adenine-cytosine) or C homozygous (CC, cytosine-cytosine). Given the unbalanced frequency of allele counts in the Caucasian population (very low frequency of the allele A for the CACNA1C genotype and the allele C for the ZNF804A genotype), we grouped the CACNA1C risk allele A homozygotes with the CACNA1C heterozygotes (AA+AG) and the ZNF804A non-risk allele C homozygotes with the ZNF804A heterozygotes (AC+CC). Quality control-wise, the distribution of Caucasian genotype frequencies for the CACNA1C (0.18 AA, 0.42 AG, 0.40 GG) and the ZNF804A (0.46 AA, 0.39 AC, 0.15 CC) was consistent with Hardy-Weinberg Equilibrium, in patients (χ^2 (ZNF804A/CACNA1C)=1.60/1.69, df=1, p-value=0.21/0.19 and controls (χ^2 (ZNF804A/CACNA1C)=1.07/0.84, df=1, p-value=0.30/0.36). Sample size, in each diagnostic group, and for a ZNF804A and CACNA1C genotype-genotype combination were, respectively: 1) in healthy controls: 26 AA-[AA+AG], 14 AA-GG, 23 [AC+CC]-[AA+AG], and 17 [AC+CC]-GG; 2) in BD patients: 11 AA-[AA+AG], 6 AA-GG, 14 [AC+CC]-[AA+AG], and 9 [AC+CC]-GG; and 3) in SCZ patients: 16 AA-[AA+AG], 11 AA-GG, 16 [AC+CC]-[AA+AG], and 11 [AC+CC]-GG. The sample's demographics are described in detail in Supplementary Table 1.

Demographic differences between diagnostic and/or genotype groups were analysed using the R software (R Core Team 2016) using χ^2 tests for categorical variables and independent t-tests and analysis of variance (ANOVA) for continuous variables. There were no significant differences in age, years of education, ethnicity or handedness between the

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groups of diagnosis, genotypes or genotypes in each diagnosis. As expected, IQ significantly differed ($p<0.001$) between diagnoses, being significantly lower in SCZ compared to controls (or BD) – but there were no significant differences in IQ between genotype groups (of either gene). Diagnoses also significantly ($p<0.001$) differed in gender with more males in SCZ than in BD and more females in controls than in SCZ. The patient groups differed in chlorpromazine (CPZ) equivalents in medication ($p<0.001$) with SCZ having a higher load than BD, as expected given current treatment strategies.

2.2. Verbal Fluency Task and Image Acquisition

The verbal fluency task and image acquisition was performed as previously described elsewhere (Fu *et al.* 2002) (see Supplement 2 for details). Briefly, subjects were required to overtly generate a word starting with a visually displayed letter; or overtly read the word “rest” (control or “repetition” condition). Task difficulty, although not factored in the group analysis, was manipulated by presenting separate, and counterbalanced, sets of “easy” and “hard” letters (Fu *et al.* 2002).

2.3. Neuroimaging Analysis

Data preprocessing was performed using SPM software (University College London, UK) running under Matlab 8.3 (The Mathworks, Inc., USA). All volumes from each subject were realigned and unwarped (using the first slice as reference), with a separation of 4mm between the points sampled in the reference image, a 5 mm full width at half maximum (FWHM) isotropic Gaussian kernel applied to the images before estimating the realignment parameters, and 2nd degree B-spline interpolation. Normalization to the functional MNI template (EPI)

was then performed using a voxel size of 2x2x2mm and trilinear interpolation. Spatial smoothing was carried out with an 8 mm FWHM isotropic Gaussian kernel. The remaining realignment, unwarping, normalization and smoothing parameters corresponded to the default choices.

After the pre-processing steps, statistical analysis of regional responses in a subject-specific fashion was performed using SPM, by convolving each onset time with a synthetic haemodynamic response function (HRF) (Mechelli *et al.* 2008). The ensuing event-related (general linear) model comprised five experimental regressors: 1) easy; 2) repetition-easy; 3) hard; 4) repetition-hard; 5) incorrect responses. The latter was excluded from the group analysis so we could control for differences in task performance (and, as such, restrict our inferences to scans corresponding to correct responses). Data were high-passed filtered with a cut-off period of 128s using a set of discrete cosine basis function. Parameter estimates were calculated for all brain voxels using a general linear model, and contrast images for ‘verbal fluency (easy plus hard) > repetition (easy plus hard)’ were computed for each subject to test for a main effect of task. The second (between-subject or group) level inferences were made using the standard summary statistic approach. This involved entering the subject-specific contrast images for “verbal fluency (easy plus hard)>repetition (easy plus hard)” into a 3x2x2 full-factorial ANOVA (‘Diagnosis’x‘*ZNF804A*-genotype’x‘*CACNA1C*-genotype’). [A complementary analysis was performed where the levels of ‘Diagnosis’ were ‘healthy volunteers’ and ‘patients with psychosis’ (i.e. all SCZ plus 75% of the BD patients)]. Since the superior region of the prefrontal cortex was not scanned in a sub-group of subjects, it was automatically excluded from the group analyses. We tested the main effect of *CACNA1C* genotype and of its interaction with *ZNF804A* genotype and/or with diagnosis. The main

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effect of *ZNF804A* genotype is not reported herein, as it has already been reported in a previous study using the same sample (Tecelão *et al.* 2018), and the effect of task has also been described in a highly overlapping sample (Prata *et al.* 2009a). The main effect of diagnosis is reported as supplementary material, as it has been discussed using a subset of the present sample earlier (Prata *et al.* 2009a).

For functional connectivity, we used the same subject and group-level models as above, this time using (instead of activation) coupling (i.e. time-correlated activation) between each subject-specific seed region and the remaining brain. Those seeds were defined, per subject, as the coordinates where the main effect of task was the highest, within a 6-mm radius sphere ROI centred on the group maximum (i.e. left precentral gyrus/inferior frontal gyrus, pars opercularis, tagged by its peak coordinates: -44 4 34). To test for condition-specific changes in connectivity we used a PPI analysis, using the same previous subject and group level models and the seed approach as above. By including an interaction between the physiological and the psychological (verbal fluency) regressors, we tested for the ensuing psychophysiological interaction. Effectively, this reflects the change in directed (effective) connectivity mediated by the task – as evaluated under a simple linear model of coupling between the seed region and the remaining brain. The PPI regressor was formed by multiplying the seed time-series with the HRF convolved task (using “verbal fluency (easy plus hard)>repetition (easy plus hard)” contrast). The resulting PPI vector was then used as a regressor in the subject-level analysis, with both the seed time-series and the HRF convolved task as covariates of no interest.

In addition to a whole-brain approach, we ran one additional analysis with selected regions-of-interest (ROIs) reported in two previous studies finding an effect of CACNA1C rs1006737 in semantic verbal fluency (Krug *et al.* 2010) and working memory (Bigos *et al.* 2010). These ROIs were derived from the automated anatomical atlas (AAL) (Tzourio-Mazoyer *et al.* 2002) and the Talairach Daemon database in Wake Forest University PickAtlas (Lancaster *et al.* 1997, 2000; Maldjian *et al.* 2003) (version 3.0.5). From the former (Krug *et al.* 2010) we derived a mask formed by the left precuneus and inferior frontal gyrus, and from the latter (Bigos *et al.* 2010), one comprising the Brodmann areas 9, 10 and 46. Additionally, the selected ROI masks were also defined using 10 mm spheres centred in their respective peak coordinates (obtained from the given studies). These post-hoc analyses allowed us to further clarify inconsistencies in the published literature.

Significant findings are reported as so, if they survive voxel-wise familywise rate error (FWE) correction for multiple comparisons at $p < 0.05$ across the whole brain (or within the ROI, for the ROI analyses), and at a cluster size ≥ 5 . All other results are considered ‘trends’. In order to assess how much of the inter-individual (+ error) variance in blood oxygen level-dependent activation on the voxel of peak effect of each reported effect was explained by genotype, we calculated the η_p^2 (partial eta squared) measure of effect size using R software (R Core Team 2016). Brain regions are labelled using an automatic-labelling atlas (Tzourio-Mazoyer *et al.* 2002) and confirmatory visual inspection of a manual book atlas (K. Mai *et al.* 2008). Post-hoc analysis exploring the driving force of the significant interaction effects between genotypes and/or diagnosis are contained as supplementary material. Finally, in order to ascertain that none of our extraneous variables confounded, or added significant

noise to our imaging results, extra analyses were performed as described in supplementary material 2.

2.4. Gene expression analyses

To test whether the *CACNA1C* rs1006737 risk variant (or other variants tagged by it in the same linkage disequilibrium block) affected any genes' mRNA expression level (i.e. was an eQTL), we used the publicly available Braineac database - which includes genotypic and microarray profiling of 10 brain regions of 134 neuropathologically normal individuals with European descent (Ramasamy *et al.* 2014) (cerebellar cortex, frontal cortex, hippocampus, medulla oblongata, occipital cortex, putamen, substantia nigra, temporal cortex, thalamus, and intralobular white matter). Expression levels from exon-specific probes and total transcripts (Winsorised mean over exon-specific levels) were used to determine the association between this SNP and the expression of mRNA of all genes distant less than 1MB (cis-eQTL analysis), considering its transcription initiation site. We focused on cis-eQTL associations as these are more likely to truly reflect direct effects of a genomic variant on gene expression (Bryois *et al.* 2014). More detailed information is described in the Braineac database (Ramasamy *et al.* 2014). The same approach was followed for *ZNF804A* rs1344706 in our recent paper regarding that gene (Tecalão *et al.* 2018).

For completeness, we also analysed Allen Brain Atlas data to define maps of *CACNA1C* expression in the human brain. Normalized log₂ expression data relative to 3 probes targeting *CACNA1C* mRNA were downloaded. The probe presenting higher variance was selected based on the fact that it may more accurately represent gene distribution across the brain

structures available. Mean-normalized z-scores were then calculated. Enriched areas were defined for a threshold of Z-score >1.

3. Results

3.1. Regional activation

Effect of genotype

3.1.1. Main effect of *CACNA1C*

Irrespective of diagnosis, the *CACNA1C* rs1006737 risk allele *A* was significantly associated (voxel-level FWE $p < 0.05$) with greater activation in the right (R) thalamus ($Z = 4.44$, $\eta^2_p = 2.95\%$), and the left (L) middle frontal gyrus ($Z = 4.32$; Figure 1; Table 1). At a trend level (i.e. with a cluster less than 5 voxels, $k < 5$), the same effect was found in the L thalamus ($Z = 4.27$, $\eta^2_p = 3.02\%$).

When inspecting each diagnostic group separately, we found that in the BD group alone, the above effect was also significant (whole-brain voxel-level FWE $p < 0.05$) in some of the above areas, plus others: the R thalamus ($Z = 4.89$, $\eta^2_p = 17.7\%$), the L middle ($Z = 4.71$ and $Z = 4.21$) and superior ($Z = 4.56$) frontal gyrus, the R superior ($Z = 4.53$) and middle ($Z = 4.47$ and $Z = 4.25$) temporal gyri and, as a trend, in the L calcarine sulcus (occipital gyrus; $Z = 4.28$ and $Z = 4.22$). The same genotype had an effect in another region of the R middle temporal gyrus ($Z = 4.25$) but associated with decreased deactivation. No other diagnostic group alone showed significant effects of *CACNA1C* genotype.

When inspecting only patients with a history of psychosis, we found that the risk allele *A* was associated as a trend with decreased deactivation in the R precuneus ($Z = 4.24$, $\eta^2_p = 9.61\%$).

3.1.2. *CACNA1C* by diagnosis interaction

The effect of increased activation associated with risk allele A was significantly (voxel-level FWE $p < 0.05$) higher in BD than in healthy volunteers in the superior temporal gyrus bilaterally ($Z=4.72$, $\eta^2_p=7.35\%$ and $Z=4.29$, $\eta^2_p=6.52\%$; Figure 2) and R middle temporal gyrus ($Z=4.53$). The same effect was found in the L occipital gyrus ($Z=4.67$), the L calcarine sulcus (occipital gyrus; $Z=4.34$ and $Z=4.30$) and L lingual gyrus ($Z=4.21$). Furthermore, this effect was found as a trend in the R angular gyrus ($Z=4.36$; in which it signified lower deactivation), and in the L middle frontal gyrus ($Z=4.24$). The same genotype effect was also higher as a trend in SCZ patients than in controls in the R inferior frontal gyrus, pars opercularis ($Z=4.31$, $\eta^2_p=7.41\%$). No significant interaction effects were found when contrasting BD and SCZ.

The effect of increased activation associated with the risk allele A mentioned above in the L calcarine sulcus (occipital gyrus; $Z=4.69$, $\eta^2_p=7.32\%$) and in the L middle frontal gyrus ($Z=4.30$), but not in the other regions, was significantly higher in psychotic patients as a whole than in healthy volunteers (voxel-level FWE $p < 0.05$).

3.1.3. *CACNA1C* by *ZNF804A* genotype epistasis

Irrespective of diagnostic group, there was no significant interaction between genotypes anywhere in brain.

When inspecting the healthy volunteers group alone, a significant 2-way genotype (at whole-brain voxel-level FWE $p < 0.05$) interaction was found (Table 1): *CACNA1C* risk allele carriers activated less than non-risk allele homozygotes, within the *ZNF804A* risk allele homozygotes group, but the reverse was seen for *ZNF804A* non-risk allele carriers. This effect was found bilaterally in the precuneus ($Z=5.05$, $\eta^2_p=15.39\%$ and $Z=4.73$), posterior cingulate gyrus ($Z=5.05$ and $Z=4.42$), calcarine sulcus (occipital gyrus; $Z=4.42$ and $Z=4.31$) and thalamus ($Z=4.75$, 4.64 and $Z=4.40$). This same effect was found as a trend ($k < 5$) in the L lingual gyrus ($Z=4.26$), R middle cingulate gyrus ($Z=4.24$) and R superior temporal gyrus ($Z=4.21$). (Note that, bilaterally in the precuneus and posterior cingulate gyrus and in the R calcarine sulcus (occipital gyrus) and superior temporal gyrus, the effect signified increased deactivation).

No other significant interactions between the *ZNF804A* and *CACNA1C* genotypes were found when inspecting the BD, SCZ alone or all patients with a history of psychosis groups as a whole.

3.1.4. *ZNF804A* by *CACNA1C* by diagnosis interaction

There were significant 3-way interactions between the *ZNF804A* genotype, *CACNA1C* genotype and diagnosis (at voxel-level FWE $p < 0.05$; Table 1). The above genotype interaction effect significant in healthy subjects, was reversed in BD in the anterior cerebellum (vermis; $Z=4.56$, $\eta^2_p=13.90\%$), the R thalamus ($Z=4.55$ and $Z=4.37$; Figure 3) and both hemisphere caudate nucleus ($Z=4.52$ and $Z=4.46$); and in SCZ in the L superior ($Z=4.65$, $\eta^2_p=8.82\%$; Figure 3) and middle ($Z=4.55$) temporal gyri. This means that, in their respective areas, in each patient group, the *CACNA1C* risk allele carriers activated more (which in the anterior cerebellum, for this task, signifies decreased deactivation) than non-

risk allele homozygotes, in the *ZNF804A* risk allele homozygotes group, but the reverse was seen for *ZNF804A* non-risk allele carriers.

When comparing both patient groups, this genotype interaction effect was found, as trend ($k < 5$), to be more pronounced in BD than in SCZ in the R medial caudate nucleus ($Z = 4.20$, $r_p^2 = 10.24\%$).

The previous genotype interaction was also found, at trend level, to be more pronounced in patients with a history of psychosis than in controls in the R anterior thalamus ($Z = 4.20$, $r_p^2 = 8.87\%$).

3.2. Psycho-physiological interaction connectivity

For the *CACNA1C* SNP, there was a significant (voxel-level FWE $p < 0.05$) genotype by diagnosis interaction in condition-specific connectivity between the seed region (L precentral gyrus/inferior frontal gyrus) and the L superior temporal gyrus ($Z = 5.07$; Figure 1), L middle temporal gyrus ($Z = 4.80$), whereby the risk allele carriers showed decreased connectivity versus non-risk allele homozygotes in SCZ, but not in controls (Table 1). In addition, this same interaction effect was found, as trend, in the L supramarginal gyrus ($Z = 4.29$), and, in the SCZ alone, in the L superior temporal gyrus ($Z = 4.36$). Inspecting the control group alone, we found increased connectivity between the seed region and the R precuneus ($Z = 4.51$).

No significant epistatic effects, or of diagnosis, were found.

3.3. Region-of-interest analysis

No significant genotype effects were found at voxel-level FWE $p < 0.05$ when using either a mask using the pre-selected Brodmann areas or spheres to restrict the analysis to previously implicated brain areas in the published literature.

3.4. Potentially confounding factors

We found no variable to have an effect (at $p < 0.01$, uncorrected) on brain activation in areas that we report as being under a genotype effect. We also found no relevant change in effect size or foci of activation of genotype effects when these variables were introduced in the SPM ANOVA. Thirdly, no variable correlated with the peak activations values retrieved from our genotype effect analyses.

3.5. Gene expression

Using the Allen Brain Atlas, we found *CACNA1C* rs1006737 risk allele A to be associated with reduced mRNA levels of *CACNA1C* in total transcript levels ($p > 0.05$, FDR-corrected) in the cerebellum and trends for exon-specific probes in the cerebellum and white matter (Supplementary Table 6). *CACNA1C* enriched areas were identified in the thalamic nuclei, dentate gyrus, frontal and occipital poles. Detailed information is presented in supplementary material 6.

4. Discussion

In summary, we assessed the main effect of *CACNA1C* rs1006737 genotype and, unprecedentedly, its epistatic interplay with *ZNF804A* rs1344706 - and whether these effects

were altered in SCZ and BD groups - in regional brain activation and functional connectivity during verbal fluency – a task which engages brain regions and cognitive processes impaired in the two disorders. We found the *CACNA1C* genotype to modulate both brain activation and task-dependent effective connectivity – as assessed with PPI. We also found some of the genotype effect in some brain areas to be particularly pronounced in SCZ, BD or compared to health. In addition, we found an interaction effect of *CACNA1C* and *ZNF804A* genotypes on regional brain activation.

We found *CACNA1C* rs1006737 SNP to be associated with inefficient activation (i.e. increased activation when only correct trials were analysed, as we did) in prefrontal regions, which are typically implicated in SZ and BD. The superior temporal gyri bilaterally, the R middle temporal gyrus, the L occipital gyrus (whether or not within the calcarine sulcus area), and the L lingual gyrus were under a significant genotype x diagnosis interaction, whereby the presence of the risk allele increased inefficient activation in BD patients much more than in controls. Furthermore, this same effect was present, as trend, in the L middle frontal gyrus and R angular gyrus. In fact, in most of these areas, the genotype effect was significant in BD alone. The same interaction effect was also found as trend when considering SCZ versus controls, in the adjacent R inferior frontal gyrus, pars opercularis. When all psychotic patients were grouped together against controls, the interaction effects survived in the L middle frontal gyrus and in the L occipital gyrus within the calcarine sulcus area.

Our above findings support previous studies implicating the same polymorphism in semantic verbal fluency (Krug *et al.* 2010) and working memory (Bigos *et al.* 2010) neural correlates (even though not consistently (Paulus *et al.* 2014)). However, while these studies showed this in healthy volunteers - not having tested a clinical population - we show it to be significantly

stronger in BD and SCZ, for the first time. As mentioned, given that task performance has been controlled for, increased activation in the risk genotype group could be interpreted as lower neuronal efficiency. This is compatible with the same observation of inefficiency, in an ill group, being found (as well as lower performance), for verbal fluency, in SCZ and, albeit less severely, of BD (Curtis *et al.* 2001, 2007; Costafreda *et al.* 2011; Gurung & Prata 2015). The rationale is that once there is impaired prefrontal capacity (provided by a risk genotype or illness), additional activation of local neuronal resources may be needed in order to maintain a good-enough task performance. No areas showed the opposite effect, i.e., over-activation in the protective genotype group.

Sub-cortically, the thalamus showed greater activation, bilaterally (albeit as a trend in the L thalamus), in risk allele carriers, irrespective of diagnosis (with the effect in the R thalamus also being significant in BD patients on their own). The thalamus plays a critical role in the coordination of information as it passes between several brain regions (Kruger 1986). A disruption of that information flow may give rise to some of the cardinal symptoms of SCZ and BD (Cronenwett & Csernansky 2010), as suggested by previous studies showing: 1) altered thalamic volumes in BD and SCZ patients (Radenbach *et al.* 2010; Smith *et al.* 2011); 2) reduced neuronal density in post-mortem thalamic samples of SCZ patients (Blennow *et al.* 1996); 3) altered thalamic glutamate receptor expression and elevated dopamine in thalamic sub-regions (Meador-Woodruff *et al.* 2003); 4) emergence of SCZ-like syndromes when illnesses, such as stroke, selectively damage the thalamus while sparing the rest of the brain (Crail-Melendez *et al.* 2013).

We also report, for the first time, *CACNA1C* and *ZNF804A* epistases on brain activation. We predicted, and found, that their respective GWAs-implicated SNPs would interact in an

additive manner, with the most inefficient activation occurring when both risk alleles were present (compared to just one or the other being present). This interaction effect was also significantly stronger in the SCZ and BD groups when contrasted individually against the control group. In SCZ, this was seen in the L superior and middle temporal gyrus and in BD, in the anterior cerebellum (vermis), the R thalamus and the caudate nucleus (an area specifically implicated in psychosis (Hannan *et al.* 2010)). When the psychotic patients were contrasted against controls, the epistatic effect was stronger, at trend level, in the R anterior thalamus.

The abnormal thalamic responses above are quite consistent with thalamus-based explanations for the ‘cognitive dysmetria’ of SCZ that has been proposed to underlie cognitive and fluency effects in the illness (Andreasen *et al.* 1998); cognitive dysmetria being a special case of functional dysconnection. On a more general note, our results speak to the disconnection hypothesis of SCZ (Friston *et al.* 2016) at a number of levels. First, the polymorphisms we have shown to affect condition-specific connectivity affect the regulation of synaptic efficacy (and plasticity) thought to underlie the dysfunctional integration in syndromes like SCZ. In brief, these aberrant (usually inefficient, disinhibited) responses to (cognitive) task-induced processes are thought to reflect a failure of gain control, synaptic excitation inhibition balance or, in the context of predictive coding, precision control in hierarchical message passing in the brain.

In line with the caudate nucleus being especially implicated in positive symptoms of psychosis, we found this area to show an additive effect of the risk alleles, which was stronger in SCZ than BD in the R caudate nucleus at trend level. This region belongs to the

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striatum, which has been repeatedly implicated in the positive (i.e. psychotic) symptoms of SCZ (Laruelle & Abi-Dargham 1999; Kirschner *et al.* 2018) and with abnormal dopamine levels (Laruelle & Abi-Dargham 1999; Breier *et al.* 1997; Abi-Dargham *et al.* 1998; Laruelle *et al.* 1999). These findings are consistent with the hypothesis that both these polymorphisms increase risk for psychosis. The two-SNP additive interaction was not seen independently of diagnosis, nor was the opposite direction of effect seen anywhere in the brain. The former suggests that the existence of other factors specific to SCZ, BD or psychosis make subjects more susceptible to the potential detrimental effects on brain function of the simultaneous presence of both the risk variants of these genome-wide associated polymorphisms.

In terms of task-specific effects on connectivity, we have also found a significant genotype by diagnosis interaction: the risk allele was associated with an intra-hemispheric connectivity decrease between the L precentral gyrus/inferior frontal gyrus, pars opercularis and the ipsilateral superior temporal gyrus, middle temporal gyrus and supramarginal (as trend) gyrus in SCZ but not in controls. In the first area, the decrease was indeed found as a trend in SCZ alone. These cortical effects are particularly consistent with our recent results showing this risk variant to be associated with decreased microstructural white matter integrity also in the L inferior and superior temporal gyri, and also found in SCZ only (Mallas *et al.* 2016a). Further support comes as well from reduced white matter integrity findings from others, also specifically in SCZ patients and in the same hemisphere and cortical areas: L temporal lobe (Woon *et al.* 2014) (more precisely in the L inferior and superior temporal gyrus (Mallas *et al.* 2016a)) and L parietal lobe (Woon *et al.* 2014). Our results are also consistent with previous independent findings in emotional face processing whereby the risk allele is

associated with amygdalar functional connectivity with the L fronto-temporal areas (Wang *et al.* 2011).

Importantly, the above effects on functional and structural connectivity are further consistent with our gene expression findings: a novel association of the *CACNA1C* rs1006737 risk allele with *reduced* mRNA levels of *CACNA1C* in white matter. This has also been independently found in the superior temporal gyrus (Eckart *et al.* 2016), an area typically affected in BD and SCZ (Ratnanather *et al.* 2013). Nevertheless, other studies with the dorsolateral prefrontal cortex (Bigos *et al.* 2010) and human induced-neurons (Yoshimizu *et al.* 2015), suggest the risk allele may also *increase* *CACNA1C* transcription at least in other areas - which may reflect a very finely tuned regulation of this gene in the brain.

The risk allele association with reduced gene expression was also found in the cerebellum – which is a direct replication of a previous independent work (Gershon *et al.* 2014). Indeed, we found this area to be recruited in ‘verbal fluency’ compared to ‘repetition’ (control) trials (Tecalão *et al.* 2018), as has been implicated by others using this task (Peterburs *et al.* 2010). Further studies using specific cerebellum-recruiting paradigms (i.e. sensorimotor tasks) will allow a clearer examination of this polymorphism’s impact on cerebellar function.

Finally, we provide a brain region- and structure-based map of *CACNA1C* mRNA distribution in the human brain. We identified the thalamic nuclei, the dentate gyrus, and the frontal and occipital poles as areas enriched in *CACNA1C* mRNA expression. Although limited by the possible discordance between mRNA and protein levels, this is the most detailed map so far published of the putative distribution of *CACNA1C* in the human brain. The data gathered may improve the interpretation of both future pharmaco-imaging and imaging genetics endeavours exploring the role of this channel in the human brain, based on

the fact that if positive findings could be achieved it is more likely that they appear in areas where the channel is most expressed and presumably more important from a functional point of view.

As a limitation of our ANOVA interaction tests, we note that the size in each of the smallest homogeneous groups (or “cells” in the parametric design matrix) which combine the diagnostic group, the *ZNF804A* rs1344706 and the *CACNA1C* rs1006737 genotype, is modest, albeit the vast majority (10 in 12 groups) is over 10 subjects and up to 26 subjects (see Materials and Methods). Although the sample size we used herein compares well with that of contemporary functional imaging genetic studies of these and other SCZ- and BD-risk polymorphisms (Gurung & Prata 2015), we recommend future independent and meta-analytical evidence is gathered to confirm these genes’ role, and their interplay, at the systems brain level.

5. Conclusions

We have shown an effect of *CACNA1C* rs1006737 on brain activation, task-dependent functional connectivity and gene expression. We have also found unprecedented evidence of epistasis of *CACNA1C* and *ZNF804A* genotypes on brain activation during verbal fluency. Several of these effects were highly dependent on both BD or SCZ diagnosis. Taken together, our results support genetic and neuroimaging genetics evidence implicating *CACNA1C* and *ZNF804A* polymorphisms in BD and SCZ. Although current evidence on the clinical efficacy of calcium channels blockers in the treatment of psychosis (i.e. BD mania) is insufficient to support its use in the clinical practice (Levy & Janicak 2000), further studies scrutinizing the neurobiological mechanisms by which dysregulation of *CACNA1C* may

affect neuronal function and, as such, increase the risk for psychosis should be encouraged. These studies will be critical for our understanding of the pathophysiological mechanisms of these disorders and, from there, putatively derive new drug targets to improve their clinical management.

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Figure legends

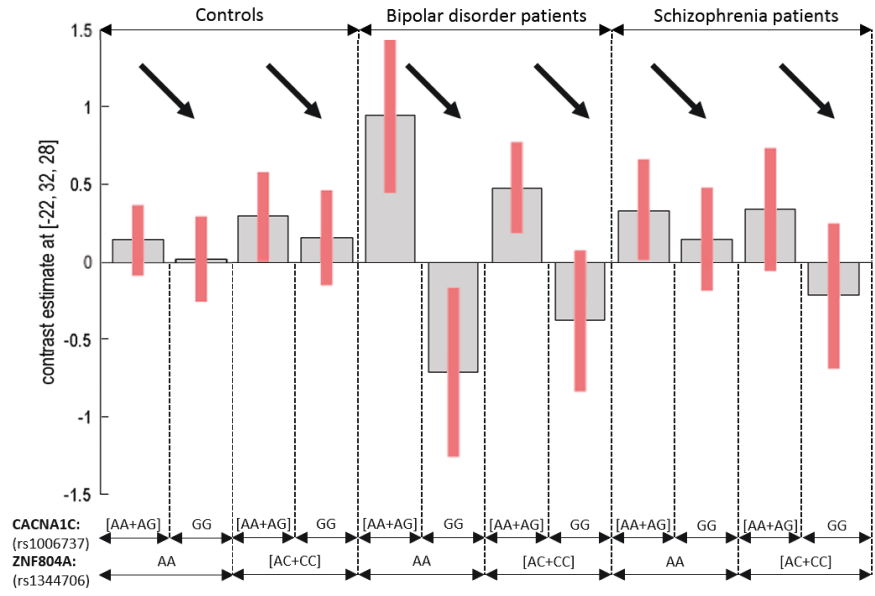
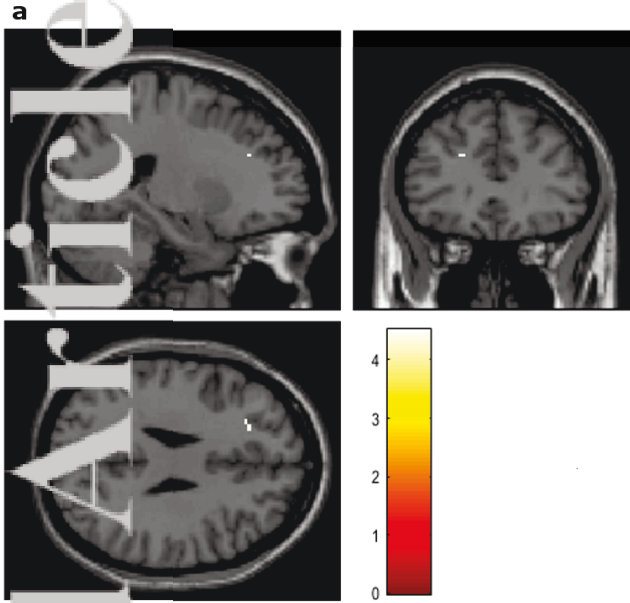
Figure 1 – Effects on “verbal fluency > repetition” brain activation (Part A) and on psychophysiological interaction (PPI, i.e. task-dependent effective connectivity) with the seed L precentral gyrus/inferior frontal gyrus, pars opercularis (Part B) (the area most recruited for verbal fluency) at whole-brain voxel-level FWE $p < 0.05$. **Part A:** Main effect of *CACNA1C* rs1006737 genotype in the L Middle frontal gyrus (plotted), where risk allele (A) carriers

activated more than G homozygotes, particularly so in BD patients. **Part B:** Interaction of *CACNA1C* rs1006737 genotype and SCZ diagnosis on PPI, where the risk allele A carriers show decreased connectivity between the seed and L superior and middle temporal gyrus (plotted) in SCZ patients but the opposite in healthy controls.

Figure 2 – Interaction of *CACNA1C* rs1006737 genotype with diagnosis on “verbal fluency > repetition” brain activation, where the risk allele (A) was associated, at whole-brain voxel-level FWE $p < 0.05$, with increased activation in BD patients but the opposite in healthy controls, in the L superior temporal gyrus (plotted) as well as in its R homologue.

Figure 3 – Three-way interactions between the *ZNF804A* rs1344706, *CACNA1C* rs1006737 genotype and diagnosis on “verbal fluency > repetition” activation. Among the *CACNA1C* risk allele (A) carriers, *ZNF804A* risk allele A homozygotes activated more than their counterparts, whereas the opposite applied in *CACNA1C* non-risk allele (G) homozygotes, at whole-brain voxel-wise FWE $p < 0.05$. **Part A:** Interaction, where BD and controls were contrasted, in the R thalamus (plotted) and caudate nucleus bilaterally. **Part B:** Interaction, where SCZ and controls were contrasted, in the L superior and middle temporal gyrus (plotted).

Main effect of *CACNA1C* rs1006737 genotype on regional brain activation



CACNA1C rs1006737 genotype by diagnosis interaction on psychophysiological interactions

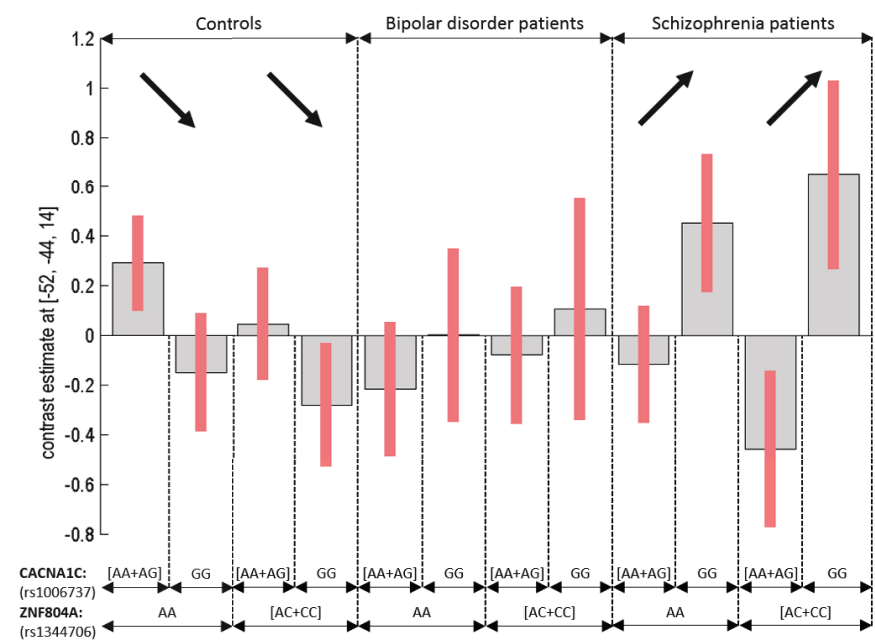
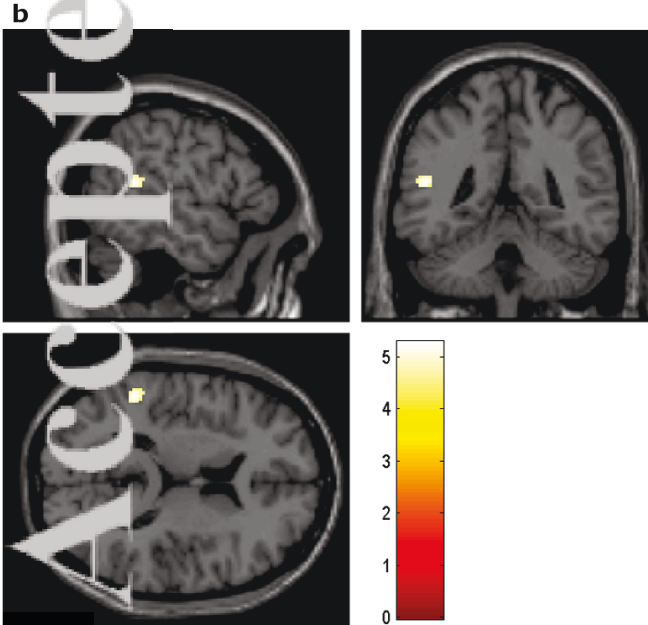


Figure 1.tif

CACNA1C rs1006737 genotype by diagnosis interaction on regional brain activation

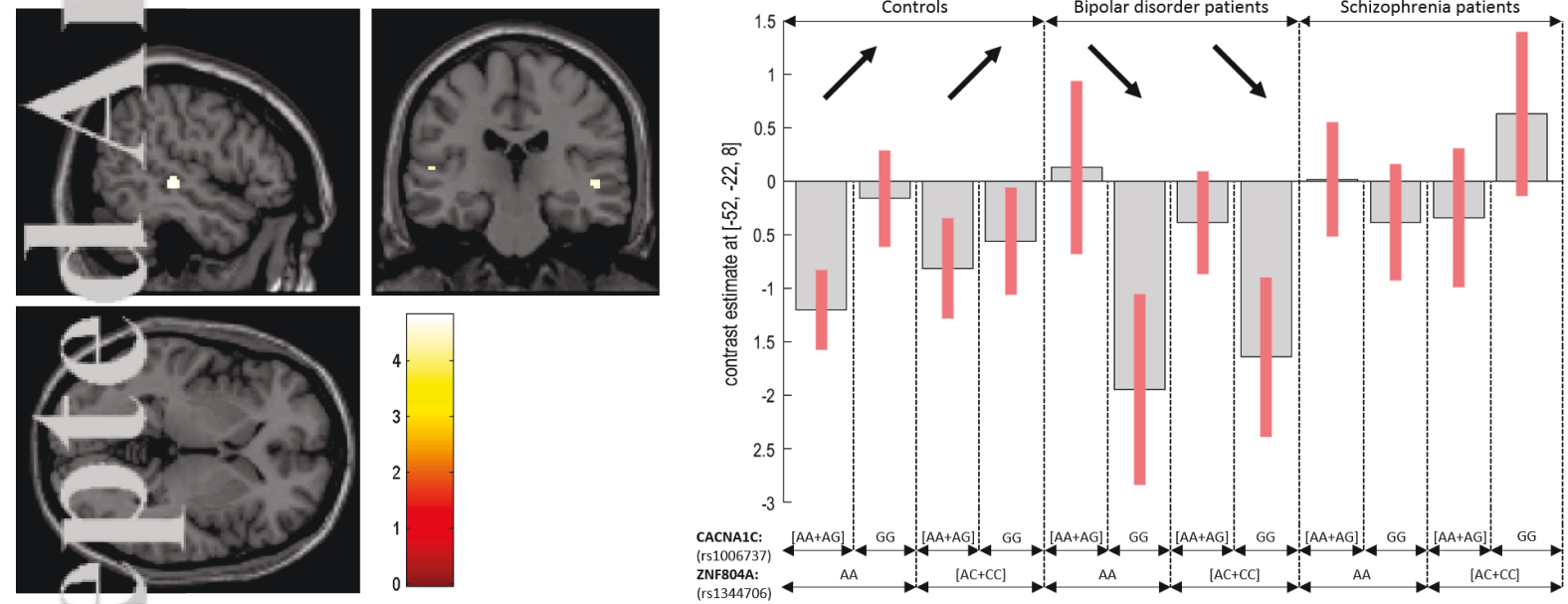


Figure 2.tif

CACNA1C rs1006737 and ZNF804A rs1344706 genotype by diagnosis interactions on regional brain activation

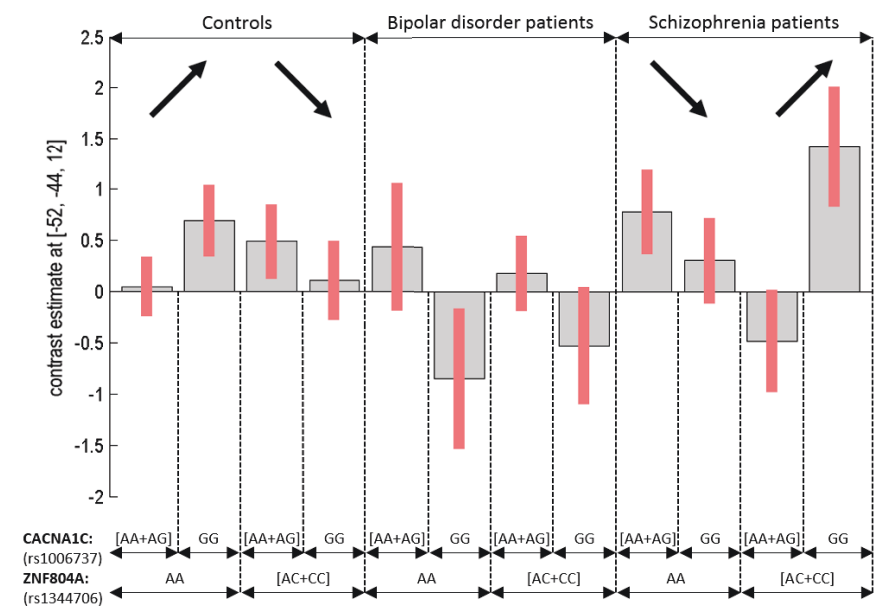
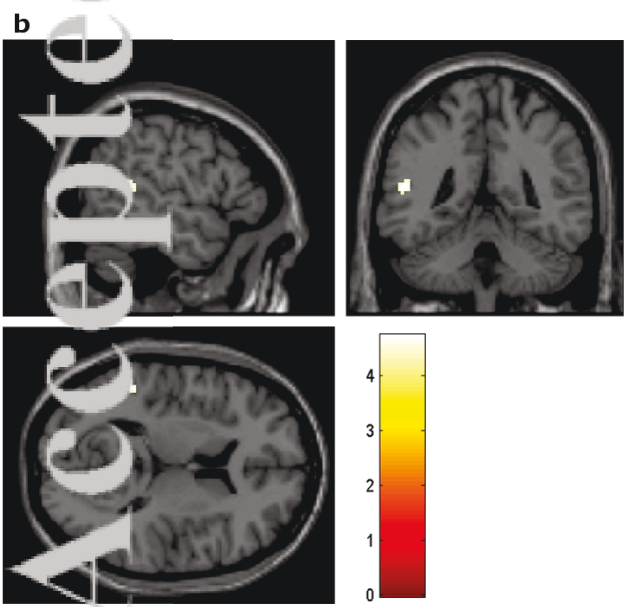
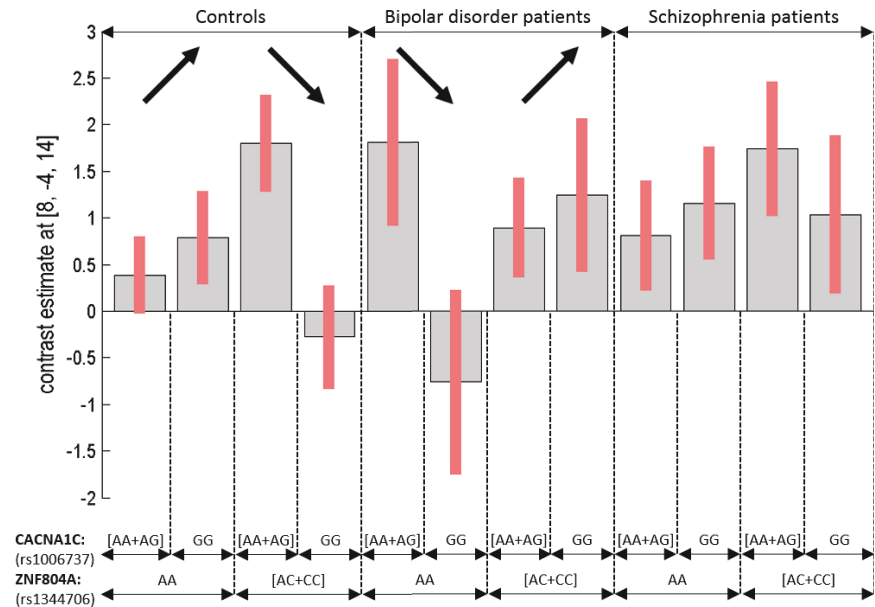
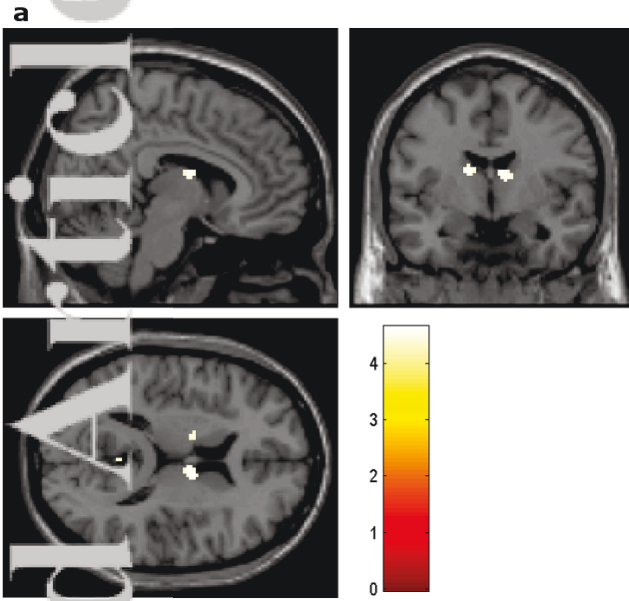


Figure 3.tif

Table 1 – Regions under an effect of *CACNA1C* rs1006737, the risk allele being allele A. All inferences correspond to results corrected for whole-brain voxel-wise FWE multiple comparisons correction at $p < 0.05$. Cluster size (k) is given only for the peak of each cluster.

1. Regional activations			
1.1. Effect of <i>CACNA1C</i> genotype			
Contrasts	Regions	Coordinates (x y z)	Z-score (Z), voxel-wise FWE corrected p-value (p), cluster size (k)
AA + AG > GG	R Thalamus	24 -16 0	Z=4.44, p=0.019, k=8
	L Middle frontal gyrus	-22 32 28 ^a	Z=4.32, p=0.031, k=5
	L Thalamus [*]	-14 -8 -6 ^a	Z=4.27, p=0.038, k=1
AA + AG > GG in BD	R Thalamus	24 -16 2	Z=4.89, p=0.003, k=50
	L Middle frontal gyrus	-26 26 30 ^a	Z=4.71, p=0.007, k=25
		-28 40 22 ^{*,a}	Z=4.21, p=0.047, k=3
	L Superior frontal gyrus	-18 32 28 ^a	Z=4.56, p=0.012
	R Superior temporal gyrus	52 -28 -2	Z=4.53, p=0.014, k=28
	R Middle temporal gyrus	52 -30 -2	Z=4.47, p=0.017
		42 -48 20 ^{a,b}	Z=4.25, p=0.041, k=7
	L Calcarine sulcus (occipital gyrus) [*]	2 -78 -6	Z=4.28, p=0.037, k=2
-2 -96 10		Z=4.22, p=0.046, k=2	
(AA + AG > GG) & (BD >	R Superior temporal gyrus	50 -26 -2 ^a	Z=4.72, p=0.006, k=49
	R Middle temporal gyrus	52 -28 -4	Z=4.53

CON)	L Superior temporal gyrus	-52 -22 8	Z=4.29, p=0.036, k=6
	L Occipital gyrus	-2 -96 8	Z=4.67, p=0.008, k=12
	L Calcarine sulcus (occipital gyrus)	-20 -68 8	Z=4.34, p=0.029, k=45
		-6 -72 10	Z=4.30, p=0.034
	L Lingual gyrus	0 -72 8	Z=4.21, p=0.047
	R Angular gyrus ^{*,b}	42 -66 38 ^a	Z=4.36, p=0.027, k=1
	L Middle frontal gyrus [*]	-32 48 20 ^a	Z=4.24, p=0.043, k=2
(AA + AG > GG) & (SCZ > CON)	R Inferior frontal gyrus, pars opercularis [*]	60 16 14	Z=4.31, p=0.032, k=3
AA + AG > GG in PSYCH	R Precuneus ^{*,b}	14 -50 14 ^a	Z=4.24, p=0.042, k=1
(AA + AG > GG) & (PSYCH > CON)	L Calcarine sulcus (occipital gyrus)	-20 -66 10	Z=4.69, p=0.007, k= 53
	L Middle frontal gyrus	-32 48 18 ^a	Z=4.30, p=0.033, k=10
1.2. Effect of <i>CACNA1C</i> x <i>ZNF804A</i> genotype interaction			
(AA + AG < GG) & (AA > AC + CC) in CON	L Precuneus ^c	-2 -52 20	Z=5.05, p=0.001, k=223
	R Precuneus ^c	2 -52 20	Z=4.73, p=0.006
	L Posterior cingulate gyrus ^c	-2 -50 20	Z=5.05, p=0.001
	R Posterior cingulate gyrus ^c	2 -44 16 ^a	Z=4.42, p=0.021
	L Calcarine sulcus (occipital gyrus)	-2 -58 12	Z=4.42, p=0.021
	R Calcarine sulcus (occipital gyrus) ^c	2 -58 14	Z=4.31, p=0.033
	R Thalamus	8 -8 10	Z=4.75, p=0.005, k=237

		2 -20 2	Z=4.64, p=0.009
	L Thalamus	-2 -20 4 ^a	Z=4.40
	L Lingual gyrus [*]	-8 -36 2 ^a	Z=4.26, p=0.040, k=3
	R Middle cingulate gyrus [*]	-2 -28 26 ^a	Z=4.24, p=0.043, k=2
	R Superior temporal gyrus ^{*,c}	64 -22 16	Z=4.21, p=0.048, k=1
(AA + AG > GG) & (AA > AC + CC) & (BD > CON)	Anterior Cerebellum (Vermis) ^b	2 -50 10	Z=4.56, p=0.012, k=24
	R Thalamus	8 -4 14	Z=4.55, p=0.013, k=63
		4 -14 18 ^{a,d}	Z=4.37, p=0.026
	L Caudate nucleus	-14 -4 16 ^a	Z=4.52, p=0.015, k=26
	R Caudate nucleus	12 -2 14 ^a	Z=4.46, p=0.018
(AA + AG > GG) & (AA > AC + CC) & (SCZ > CON)	L Superior temporal gyrus	-52 -44 12 ^a	Z=4.65, p=0.008, k=45
	L Middle temporal gyrus	-54 -44 10 ^a	Z=4.55, p=0.012
(AA + AG > GG) & (AA > AC + CC) & (BD > SCZ)	R Caudate nucleus [*]	12 -2 16 ^e	Z=4.20, p=0.049, k=1
(AA + AG > GG) & (AA > AC + CC) & (PSYCH > CON)	R Thalamus [*]	6 -14 14 ^{a,d}	Z=4.20, p=0.050, k=1

2. Psychophysiological interaction with L Precentral gyrus/inferior frontal gyrus, pars opercularis (seed corresponding to peak of main effect of task)			
2.1. Effect of <i>CACNA1C</i> genotype			
(AA + AG > GG) & (SCZ < CON)	L Superior temporal gyrus	-52 -44 14 ^a	Z=5.07, p=0.002, k=60
	L Middle temporal gyrus	-52 -46 14 ^a	Z=4.80, p=0.006
	L Supramarginal gyrus [*]	46 -40 32 ^a	Z=4.29, p=0.044, k=2
AA + AG < GG in SCZ	L Superior temporal gyrus [*]	-52 -44 14 ^a	Z=4.36, p=0.034, k=3
AA + AG > GG in CON ^f	R Precuneus	14 -62 34 ^a	Z=4.51, p=0.018, k=15

^{*} Trend results: clusters with less than 5 clusters.

^a Peak localized in the nearby white matter.

^b Region associated with decreased deactivation.

^c Regions associated with increased deactivation.

^d Anterior part of the thalamus.

^e Medial part of the caudate nucleus.

^f Only present in the ANOVA comprising controls and patients experiencing psychosis.

AA, adenine-adenine; AG, adenine-guanine, GG, guanine-guanine; BD, bipolar disorder; SCZ, schizophrenia; PSYCH, patients with a history of psychosis; R, right; L, left.