ORIGINAL INVESTIGATION

CACNA1C SNP rs1006737 associates with bipolar I disorder independent of the Bcl-2 SNP rs956572 variant and its associated effect on intracellular calcium homeostasis

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Abstract

Objectives. Intracellular calcium (Ca\(^{2+}\)) dyshomeostasis (ICDH) has been implicated in bipolar disorder (BD) pathophysiology. We previously showed that SNP rs956572 in the B-cell CLL/lymphoma 2 (Bcl-2) gene associates with elevated B lymphoblast (BLCL) intracellular Ca\(^{2+}\) concentrations ([Ca\(^{2+}\)]\(_{i}\)) differentially in BD-I. Genome-wide association studies strongly support the association between BD and the SNP rs1006737, located within the L-type voltage-dependent Ca\(^{2+}\) channel α1C subunit gene (CACNA1C). Here we investigated whether this CACNA1C variant also associates with ICDH and interacts with SNP rs956572 on [Ca\(^{2+}\)]\(_{i}\) in BD-I. Methods. CACNA1C SNP rs1006737 was genotyped in 150 BD-I, 65 BD-II, 30 major depressive disorder patients, and 70 healthy subjects with available BLCL [Ca\(^{2+}\)]\(_{i}\) and Bcl-2 SNP rs956572 genotype measures. Results. SNP rs1006737 was significantly associated with BD-I. The [Ca\(^{2+}\)]\(_{i}\) was significantly higher in BD-I rs1006737A compared with healthy A allele carriers and also in healthy GG compared with A allele carriers. There was no significant interaction between SNP rs1006737 and SNP rs956572 on [Ca\(^{2+}\)]\(_{i}\). Conclusions. Our study further supports the association of SNP rs1006737 with BD-I and suggests that CACNA1C SNP rs1006737 and Bcl-2 SNP rs956572, or specific causal variants in LD with these proxies, act independently to increase risk and ICDH in BD-I.

Key words: CACNA1C; Bcl-2; SNP; bipolar disorder; intracellular calcium homeostasis

Introduction

Aberrant intracellular calcium (Ca\(^{2+}\)) signalling dynamics and intracellular Ca\(^{2+}\) dyshomeostasis (ICDH) have been implicated in the pathophysiology of bipolar disorder (BD; Warsh et al. 2004). Numerous molecular processes, signal transducing proteins and genes participate in the production, assembly and maintenance of the variety of cellular “toolkits” that modulate intracellular Ca\(^{2+}\) signalling and homeostasis in cells (Warsh et al. 2004; Kato 2008; Fisar and Hroudova 2010). Genetic variants of proteins within these toolkits have been implicated with increasing frequency in pathophysiological investigations of complex trait disorders (Gleichmann and Mattson 2011; Zundorf and Reiser 2011). Among plausible candidate genes and proteins implicated in ICDH in BD in recent studies, the alpha 1C subunit of voltage-dependent L-type Ca\(^{2+}\) channel (LTCC), Cav1.2, encoded by the CACNA1C gene on chromosome 12p13.3 (Casamassima et al. 2010; Bhat et al. 2012), and the antiapoptotic B-cell CLL/lymphoma 2 (Bcl-2), mapped to 18q21.3 (http://www.ncbi.nlm.nih.gov/gene/596), have come to the forefront based on genetic and/or molecular findings,
as well as their respective physiological roles in intracellular Ca\(^{2+}\) dynamics (Warsh et al. 2004; Kato 2008; Fisar and Hrouda 2010; Uemura et al. 2011; Soeiro-de-Souza et al. 2012).

The LTCCs are widely expressed in the central nervous system (CNS) and have been shown to influence neurotransmitter release and neuron excitability (Catterall 2000; Casamassima et al. 2010). LTCCs are formed by the Cav1 family that is comprised of four isoforms (Nowczyk et al. 1985; Ertel et al. 2000). Only Cav1.2 and Cav1.3 play a prominent role in the brain; Cav1.2 accounts for 75% of LTCCs in rat cerebral cortex and hippocampus (Striessnig et al. 2006; Calin-Jageman and Lee 2008). Cav1.2 is important in modifying the effects of synaptic activity on cell survival, synaptic plasticity, and gene expression processes thought to be altered in BD brain (Casamassima et al. 2010; Bhat et al. 2012).

The first waves of genome-wide association studies (GWAS) of BD uncovered the potential involvement of the CACNA1C gene (Ferreira et al. 2008; Sklar et al. 2008). Using the STEP-UCL sample collections (1461 BD-I patients and 2008 controls) with the WTCCC dataset (1868 BD patients and 2938 controls), Sklar et al. (2008) showed the strongest consistent signal for association with BD at the single nucleotide polymorphism (SNP) rs1006737 (G→A) that is located in the intron 3 of the CACNA1C gene. In an expanded collaborative GWAS combining the WTCCC, STEP-UCL and ED-DUB-STEP2 datasets (4387 BD-I patients and 6209 controls), Ferreira et al confirmed CACNA1C SNP rs1006737 to be one of strongest associated regions (G→A; \(P = 7.0 \times 10^{-8}\)) with BD (Ferreira et al. 2008). Further confirmation followed in subsequent GWAS of increased sample sizes (Cichon et al. 2011; Psychiatric GWAS Consortium Bipolar Disorder Working Group 2011; Liu et al. 2011) and independent replications (Scott et al. 2009), leading to a strong consensus of support for this CACNA1C variant’s contribution to BD risk, but of small effect. That said, more recent GWAS findings indicate the association of CACNA1C variation with major depressive disorder and schizophrenia, suggesting it may exert a more general effect to increase risk for major psychiatric illness (Cross-Disorder Group of the Psychiatric Genomics Consortium 2013).

Potential links between CACNA1C SNP rs1006737 and some intermediate phenotypes strengthen its pathophysiological relevance. The CACNA1C risk variant (A-carrier) has been associated with psychopathologic symptoms (Erk et al. 2010; Roussos et al. 2011), brain anatomical changes (Kempton et al. 2009; Franke et al. 2010; Wang et al. 2011), and functional effects (Bigos et al. 2010; Erk et al. 2010; Wang et al. 2011). Additionally, Gershon et al. (2013) found that CACNA1C risk variant is associated with reduced expression of CACNA1C mRNA in human cerebellum and suggested that SNP rs1006737 is acting as a cis-association of CACNA1C expression. However, little is known of the specific molecular disturbances and mechanisms caused by this CACNA1C variant or whether and how it affects intracellular Ca\(^{2+}\) signalling and homeostasis implicated in the pathophysiology of BD. It is known that intracellular Ca\(^{2+}\) levels regulated by LTCC influence the cAMP response element-binding protein (CREB), in turn activating signalling cascades that modify expression of Ca\(^{2+}\)-dependent genes (West et al. 2001). One of these, Bcl-2, has been implicated as an important target of mood stabilizer action and as a risk gene for BD-I that impacts Bcl-2 expression and intracellular Ca\(^{2+}\) homeostasis (ICH; Kim et al. 2010; Uemura et al. 2011). Bcl-2 modulates ER-Ca\(^{2+}\) dynamics (Pinton and Rizzuto 2006), and is encoded in a putative BD susceptibility locus (Stine et al. 1995; Verheyen et al. 1999; McMahon et al. 2001). We have reported genetic variation in Bcl-2 SNP rs956572 that affects its expression and impacts ICH in BD-I (Uemura et al. 2011). Importantly, Bcl-2 SNP rs956572 affects left ventral striatum gray-matter volume in healthy subjects (Salvadore et al. 2009), and anterior cingulate cortical glutamate level in euthymic BD-I (Soeiro-de-Souza et al. 2013), findings that strengthen this Bcl-2 variant’s functional impact in CNS. Taken together, CACNA1C SNP rs1006737 and Bcl-2 SNP rs956572 are important contributing factors in the pathophysiology of BD. While it remains to be shown whether these variants themselves are causal in BD or in linkage disequilibrium (LD) with the specific causal variants in these genes, their association with functional effects in BD supports that they are suitable proxy markers to study the effects of CACNA1C on intracellular Ca\(^{2+}\) signalling and its potential interaction on Bcl-2 to disrupt ICH and thereby increase the associated risk of BD.

As a first test of this hypothesis, we sought to first confirm whether CACNA1C SNP 1006737 variation is associated with BD-I in a BD-I subject data set in which we identified reduced Bcl-2 expression and elevated basal intracellular Ca\(^{2+}\) concentrations ([Ca\(^{2+}\)]\(_{b}\)), a biomarker of ICDH, in association with Bcl-2 SNP rs956572 in BD-I (Uemura et al. 2011), and, second, to determine whether this CACNA1C variant interacts genetically with the Bcl-2 SNP rs956572 variant on BD-I and/or [Ca\(^{2+}\)]\(_{b}\) in BD-I.
Materials and methods

Subjects

Patients and age- and sex-matched healthy subjects were recruited from the greater Toronto area as previously described (Emamghoreishi et al. 1997, 2000). Psychiatric diagnoses were confirmed using the Structured Clinical Interview for DSM-IV Axis I Disorders (SCID-I), patient edition (First et al. 1995b). Healthy subjects had no past or present history of psychiatric illness as determined by SCID-I–Non-patient Edition (First et al. 1995a). The study was approved by the Human subjects Review Board of CAMH and all subjects provided written informed consent after a detailed explanation of the study.

B lymphoblast cell lines

Blood was drawn from each subject into anticoagulant containing tubes and the leukocyte fraction isolated and transformed with Epstein–Barr virus as previously described (Emamghoreishi et al. 1997).

Ca\(^{2+}\) mobilization assay

The [Ca\(^{2+}\)]\(_{B}\) was determined in BLCLs at 13–17 passages using a standard ratiofluorimetric assay and the Ca\(^{2+}\)-sensitive dye fura-2 AM (Invitrogen, Burlington, Ontario, Canada), as previously described (Emamghoreishi et al. 1997). After loading with 1 \(\mu\)M fura-2 AM and washing, cells were resuspended (1 \(\times\) 10\(^6\) cells/ml) and equilibrated (3 min, 37°C) in a temperature-controlled cuvette chamber of a LS50B spectrofluorophotometer (PerkinElmer, Beaconsfield, UK). Free and Ca\(^{2+}\)-bound fura-2 fluorescence intensities were determined at excitation wave lengths of 380 and 340 nm, respectively, and an emission wave length of 500 nm.

DNA isolation

Genomic DNA was extracted from BLCL aliquots of approximately 5 \(\times\) 10\(^6\) cells using the QIAamp\textsuperscript{®} DNA Blood Mini Kit (Qiagen, Mississauga, Ontario, Canada) according to the manufacturer’s instructions. The DNA concentrations were determined spectrophotometrically (absorbance ratio 260/280 nm) using standard methods, DNA samples were stored at –70°C until use.

SNP analysis and allele determination

Genotypes were determined by TaqMan\textsuperscript{®} SNP allelic discrimination assay in a 96-well format and by the same conditions as used for the Bcl-2 SNP rs956572 genotyping (Uemura et al. 2011). To each well, 5 \(\mu\)l of PCR master mix (TaqMan\textsuperscript{®} Genotyping Master Mix), 60 ng of DNA template, 0.25 \(\mu\)l of commercially pre-designed 40×Taqman\textsuperscript{®} SNP Genotyping Assay were added. The amplification reaction was carried out on an ABI PRISM\textsuperscript{®} 7300 Sequence Detection System (Applied Biosystems, Inc., Foster City, CA, USA) using the following cycle parameters: initial denaturation 95°C for 10 min, followed by 40 cycles of denaturing at 95°C for 15 s, and annealing and primer extension at 60°C for 1 min. In addition to three no genomic DNA samples as negative controls, three randomly selected subject samples with known genotyping information (AA, AG, GG) were assayed as positive controls in each 96-well plate. After amplification, end point plate reading to discriminate alleles was performed using the Sequence Detection System software (Applied Biosystems). To confirm genotyping data, direct sequencing (Silva et al. 2001) was performed using six randomly selected subject samples with homozygous alleles (three each of AA and GG samples) as determined by the allele discrimination assay.

Statistical analysis

Data are presented as means±SD. Hardy–Weinberg Equilibrium (HWE) analyses were examined using the Hardy–Weinberg Equilibrium Calculator (http://www.oege.org/software/hwe-mr-calc.shtml) (Rodriguez et al. 2009). Differences in the proportion of males to females in each diagnostic group, disease association of genotype, and allele and risk genotype frequencies compared with healthy subjects were assessed using two-tailed Chi-square tests. Potential differences in the clinical features among BD-I genotypes were analysed by Fisher’s exact test. Differences in age and age at onset (AAO) among diagnostic groups were analysed by univariate analysis of variance (ANOVA) with post-hoc Tukey’s HSD comparisons of means. The effects of diagnosis and CACNA1C variant on Ca\(^{2+}\) mobilization were analysed statistically by two-way ANOVA with post-hoc Bonferroni corrected pairwise comparisons of group means. The gene to gene interaction between CACNA1C SNP rs1006737 and Bcl-2 SNP rs956572 on intracellular Ca\(^{2+}\) homeostasis was tested within a three-way ANOVA, with SNP rs1006737, SNP rs956572 and diagnosis as factors with post-hoc Bonferroni corrected pairwise comparisons of group means. Statistical analyses were performed using SPSS version 17.0 (SPSS Inc., Chicago, IL, USA). Statistical power analysis was performed using SNP tools (http://www.bioinformatics.org/snp-tools-excel)
for risk genotype frequencies (Chen et al. 2009) and by G*Power 3.1.9.2 for ANOVAs (Faul et al. 2007). Differences with P-values < 0.05 were considered statistically significant.

Results

Study samples

The study sample set is the same as that of our previous Bcl-2 SNP rs956572 investigation, and demographic characteristics of the subjects are as previously reported (Uemura et al. 2011). Briefly, there were no significant differences in age or sex among comparison groups; age at onset (AAO) was earlier in BD-II patients (18.1 ± 7.5 years) compared with MDD patients. Selected clinical features including age, AAO, types of psychiatric comorbidity, family history of mood disorders (first-degree relatives), history of psychosis and grouped antidepressant medication at time of interview, were analysed among CACNA1C SNP rs1006737 genotypes in BD-I patients (Total, n = 150; AA + AG genotype, n = 93; GG genotype, n = 57) by Fisher’s exact test. However, no differences among these clinical variables achieved statistical significance (Supplementary Table I available online at http://informahealthcare.com/doi/abs/10.3109/15622975.2015.1019360).

Test of association of CACNA1C SNP rs1006737 with mood disorders

BLCL derived genomic DNA was available from a total of 245 patients with primary mood disorders (BD-I, n = 150; BD-II, n = 65; MDD, n = 30) and 70 healthy subjects for whom BLCL [Ca^{2+}]_{B} had been determined in the course of earlier studies (Emamghoreishi et al. 1997, 2000; Uemura et al. 2011). The CACNA1C SNP rs1006737 genotype frequencies for BD-I ($P = 0.18$), BD-II ($P = 0.07$), MDD patients ($P = 0.52$) and healthy subjects ($P = 0.08$) did not deviate significantly from Hardy–Weinberg equilibrium. Although no significant differences were observed in the genotype frequencies compared with healthy subjects among MDD patients ($P = 0.33$) or BD-II ($P = 0.89$), CACNA1C SNP rs1006737 was significantly associated with BD-I in genotype frequency ($P = 0.035$) (Table I). However, allele frequencies were not significantly different in BD-I ($P = 0.15$; Odds ratio (OR) = 1.38, 95% confidence interval (CI) 0.89–2.13), BD-II ($P = 0.59$; OR = 1.15, 95%CI 0.69–1.93) or MDD patients ($P = 0.39$; OR = 0.73, 95%CI 0.36–1.48) compared with healthy subjects (Table I). Due to the small number of AA genotype subjects and to estimate risk genotype

| Genotype/Allele frequencies of CACNA1C SNP rs1006737 in mood disorders and comparison healthy subject groups. |
|---|
| Genotype | AA | AG | GG |
| BD-I patients | 150 | 16 (10.7) | 77 (51.3) | 57 (38.0) |
| | | 57 (9.8) | 23 (38.0) | 23 (38.0) |
| | | 10 (15.4) | 22 (33.8) | 25 (40.8) |
| | | 21 (33.8) | 35 (56.7) | 35 (56.7) |
| | | 0.08 | 0.24 | 0.21 |
| | | 2.23 | 0.33 | 0.18 |
| | | 0.07 | 0.89 | 0.035 |
| | | 1.38 | 0.36–1.48 | 0.36–1.48 |

Data presented as number of subjects and percentage.
frequencies of \textit{CACNA1C} SNP rs1006737 in mood disorders and healthy subject groups, we compared A risk-allele carriers (AA+AG) and GG genotype in this sample set. Risk genotype frequencies were not significantly different in BD-II (P = 0.68; OR = 1.15, 95\%CI 0.59–2.26) or MDD patients (P = 0.82; OR = 0.91, 95\%CI 0.38–2.15) compared with healthy subjects. Interestingly, \textit{CACNA1C} SNP rs1006737 was significantly associated with BD-I in risk genotype frequency (P = 0.023; Supplementary Table 2 available online at http://informahc.com/doi/abs/10.3109/15622975.2015.1019360) and the odds ratio was 1.94 (95\%CI 1.09–3.44), however, the statistical power was 0.62 in the analysis of risk genotype frequencies.

\textbf{Effect of \textit{CACNA1C} SNP rs1006737 variants on intracellular Ca\textsuperscript{2+} homeostasis}

In this derivative analysis, there was a significant effect of \textit{CACNA1C} SNP rs1006737 gene variant (comparing A risk-allele carriers with GG subjects) on mean [Ca\textsuperscript{2+}]\textsubscript{B} \( [F(1,216) = 6.725, P = 0.010] \) (Figure 1) in addition to the main effect of diagnosis on mean BLCL [Ca\textsuperscript{2+}]\textsubscript{B} \( [F(3,307) = 6.036, P = 0.0005] \) attributable to the significantly higher [Ca\textsuperscript{2+}]\textsubscript{B} in BD-I patients (61.9 ± 9.1 nM, n = 150; \( P = 0.00078 \), post-hoc Bonferroni test) compared with the healthy subjects (57.4 ± 7.4 nM, n = 70) as reported previously (Uemura et al. 2011). Mean BLCL [Ca\textsuperscript{2+}]\textsubscript{B} was modestly lower in \textit{CACNA1C} A risk-allele carriers (59.8 ± 8.7 nM, n = 125) compared with \textit{CACNA1C} GG subjects (61.4 ± 9.0 nM, n = 95). A statistically significant diagnosis \times gene variant interaction was also found \( [F(1,216) = 5.374, P = 0.021] \). Bonferroni pairwise comparisons to elaborate the basis for this interaction revealed significantly higher mean BLCL [Ca\textsuperscript{2+}]\textsubscript{B} in healthy \textit{CACNA1C} GG subjects (60.2 ± 7.6 nM, n = 38) compared with healthy \textit{CACNA1C} A risk-allele carriers (54.1 ± 5.3 nM, n = 32; \( P = 0.003 \)), and also significantly higher mean BLCL [Ca\textsuperscript{2+}]\textsubscript{B} in BD-I \textit{CACNA1C} A risk-allele carriers (61.8 ± 8.8 nM, n = 93) compared with healthy \textit{CACNA1C} A risk-allele carriers (\( P = 0.000016 \)). However there was no effect of genotype on mean [Ca\textsuperscript{2+}]\textsubscript{B} within BD-I.

\textbf{Test of interaction between \textit{CACNA1C} SNP rs1006737 and Bcl-2 SNP rs956572 on intracellular Ca\textsuperscript{2+} homeostasis}

Potential gene to gene interaction between \textit{CACNA1C} SNP rs1006737 and Bcl-2 SNP rs956572 on intracellular Ca\textsuperscript{2+} homeostasis was assessed in BD-I patients compared with healthy subjects by three-way ANOVA combining the Bcl-2 SNP rs956572 genotype data obtained from the same subjects reported on previously (Uemura et al. 2011). This analysis was restricted to the BD-I patients and healthy comparison groups as the small sample sizes of the MDD and the BD-II groups would have been too underpowered to adequately test for potential differences within genotypes. As with the two-way analysis, the \textit{CACNA1C} SNP rs1006737 variants exerted a statistically significant effect on mean BLCL [Ca\textsuperscript{2+}]\textsubscript{B} \( [F(1,208) = 4.134, P = 0.043] \): BLCL [Ca\textsuperscript{2+}]\textsubscript{B} was lower in \textit{CACNA1C} A risk-allele carriers (\( N = 125 \)) compared with GG carriers (\( n = 95 \)). However this effect was less robust compared with the main effect of diagnosis on mean BLCL [Ca\textsuperscript{2+}]\textsubscript{B} \( [F(1,208) = 9.473, P = 0.0024] \) in BD-I patients (\( n = 150 \)) versus healthy subjects (\( N = 70 \)) or that of Bcl-2 SNP rs956572 genotype on mean [Ca\textsuperscript{2+}]\textsubscript{B} \( [F(2,208) = 6.103, P = 0.0027] \), accounted for by significantly lower mean BLCL [Ca\textsuperscript{2+}]\textsubscript{B} in Bcl-2 AA (54.4 ± 7.3 nM, n = 30) compared with Bcl-2 AG (60.6 ± 8.3 nM, n = 117; \( P = 0.0007 \)) and Bcl-2 GG genotypes (62.7 ± 9.2 nM, n = 73; \( P = 0.000012 \)). A statistically significant diagnosis \times \textit{CACNA1C} SNP rs1006737 variant interaction was found \( [F(1,208) = 5.291, P = 0.022] \). Bonferroni pairwise
comparisons revealed significantly higher mean BLCL [Ca\(^{2+}\)]\(_B\) in healthy CACNA1C GG subjects (n = 38) compared with healthy CACNA1C A risk-allele carriers (n = 32; P = 0.008), and also significantly higher mean [Ca\(^{2+}\)]\(_B\) in BD-I CACNA1C A risk-allele carriers (n = 93) compared with healthy CACNA1C A risk-allele carriers (P = 0.000043). The statistically significant diagnosis \(\times\) Bcl-2 SNP rs956572 genotype interaction \(F(2,216) = 3.055, P = 0.049\) was also evident as reported earlier (Uemura et al. 2011). Bonferroni pairwise comparisons revealed significantly higher mean BLCL [Ca\(^{2+}\)]\(_B\) in BD-I Bcl-2 GG (65.3 \(\pm\) 9.1 nM, n = 52) compared with BD-I Bcl-2 AA (55.3 \(\pm\) 7.9 nM, n = 20; \(P = 0.000047\)) and BD-I Bcl-2 AG (61.4 \(\pm\) 8.5 nM, n = 78; \(P = 0.048\)) and healthy Bcl-2 GG subjects (56.4 \(\pm\) 6.0 nM, n = 21; \(P = 0.000052\)). Moreover, mean BLCL [Ca\(^{2+}\)]\(_B\) was significantly higher in the BD-I Bcl-2 AG compared with the BD-I Bcl-2 AA subjects (\(P = 0.009\)). Statistical power estimates for the analysis of these main and interaction effects ranged from 0.95 - 0.62 as determined using the G*Power statistical power application. However, there was no significant interaction effect between CACNA1C SNP rs1006737 variant and Bcl-2 SNP rs956572 on mean [Ca\(^{2+}\)]\(_B\) among comparison groups \(F(2,208) = 0.299, P = 0.742\) or among diagnosis, CACNA1C SNP rs1006737 variant and Bcl-2 SNP rs956572 genotype \(F(2,208) = 0.391, P = 0.677\) (Figure 2), but the power for testing these three-way interaction effects was very low (<0.15) for the available sample set analysed.

**Discussion**

The principal findings of this study are, first, confirmation of association of CACNA1C SNP rs1006737 with BD-I in this small but independent sample data set and, second, an effect of CACNA1C on ICH in healthy subjects and among CACNA1C A risk-allele carriers. Notable in its absence, CACNA1C SNP rs1006737 did not exert an independent or interactive effect on ICH in BD-I patient samples, in contrast to the effect of Bcl-2 SNP rs956572 which is associated with reduced BLCL [Ca\(^{2+}\)]\(_B\) in a diagnostic specific manner in BD-I. The implication of these findings, if they can be extrapolated to the CNS, is that CACNA1C and Bcl-2 variation each independently influence the risk for BD-I but the effect of Bcl-2 variation on its expression may be sufficient, in and of itself, to promote disruption of ICH in BD-I patients harboring the rs956572 risk variant.

Our results concur with those of recent GWAS supporting the association between the CACNA1C SNP rs1006737 and BD (Ferreira et al. 2008; Sklar et al. 2008; Scott et al. 2009; Cichon et al. 2011; Liu et al. 2011; Cross-Disorder Group of the Psychiatric Genomics Consortium 2013). Although...
allele frequencies were not significantly different in BD-I patients compared with healthy subjects, CACNA1C SNP rs1006737 was significantly associated with BD-I in genotype frequency ($P = 0.035$) in our study sample. Interestingly, CACNA1C SNP rs1006737 was also significantly associated with BD-I in risk-allele carrier frequency ($P = 0.023,$ Supplementary Table II available online at http://informahealthcare.com/doi/abs/10.3109/15622975.2015.1019360) compared with healthy subjects. These observations support that genetic variation at CACNA1C SNP rs1006737 associates with the pathophysiology of BD-I.

Some potential links between CACNA1C SNP rs1006737 and intermediate phenotypes have been reported in recent studies. The CACNA1C risk variant (A-carrier) has been associated with (1) higher psychopathologic symptoms such as depression, anxiety, and/or obsessive-compulsive thoughts in healthy individuals (Erk et al. 2010; Roussos et al. 2011), (2) reduction of bilateral hippocampal activation during an episodic memory task in healthy volunteers (Erk et al. 2010), and (3) modulation of bilateral hippocampal activity during an emotional imaging task (Bigos et al. 2010). We examined clinical features such as age, AAO, selected types of psychiatric comorbidity, family history of mood disorders (first-degree relatives), history of psychosis and grouped antidepressant medication at time of interview, among genetic variants of CACNA1C SNP rs1006737 in BD-I patients. However, none of the examined clinical features further distinguished association of CACNA1C SNP rs1006737 variants in BD-I patients.

Contrary to our hypothesis, we found no significant interaction between CACNA1C SNP rs1006737 and Bcl-2 SNP rs956572 on ICH in BD-I patients compared with healthy subjects in our samples. While this may reflect that these ICH-impacting risk genes work through entirely independent processes, another consideration is the possibility that the functional effects of the CACNA1C SNP rs1006737 BD risk variant are only expressed in a cell type specific manner in neurons and/or astroglial cells and not B lymphoblasts. Such a possibility might be addressed using neural precursor or reprogrammed neuronal/astroglial cells from BD patients and controls.

That CACNA1C SNP rs1006737 variants exhibit higher mean BLCL $[\text{Ca}^{2+}]_\text{i}$ in healthy GG as compared with healthy A risk-allele carriers, but not in BD-I GG as compared with BD-I A allele patients, suggests the homozygous GG is associated with mechanisms that influence ICH differentially from the effect of Bcl-2 SNP rs956572, though the possibility that this is a spurious finding cannot be discounted. We also found that CACNA1C SNP rs1006737 variants exhibit higher mean BLCL $[\text{Ca}^{2+}]_\text{i}$ in BD-I A risk-allele compared with healthy A risk-allele carriers, but not in BD-I GG patients as compared with healthy GG allele subjects. Psychiatric illnesses including BD are complex traits caused by combinations of polygenic, gene-environment interactions and, genetic heterogeneity (Wolffe and Matzke 1999; Labrie et al. 2012). Gene expression is affected not only by changes at the DNA sequence level but also by DNA modifications mediated by epigenetic effects under the influence of the environment (Jaenisch and Bird 2003). The genetic variation on CACNA1C SNP rs1006737 was found here to impact mean BLCL $[\text{Ca}^{2+}]_\text{i}$ in healthy subjects. Interestingly, alteration of the mean BLCL $[\text{Ca}^{2+}]_\text{i}$ in BD-I A risk-allele compared with healthy A risk-allele carriers occurs in the absence of changes in DNA sequence. These findings may indicate that CACNA1C SNP rs1006737 variants may be vulnerable to gene–environment interactions and contribute to ICDH in the pathophysiology of BD not only as a genetic factor but also as an epigenetic factor.

A number of SNPs in the CACNA1C gene which have been linked to psychiatric illness are concentrated within the large 328.5-kb intron 3 (Soldatov 1994; Bhat et al. 2012), where CACNA1C SNP rs1006737 resides within the middle of this intron. Introns are regions of DNA that are transcribed into premessenger RNA but are removed during splicing to generate a mature mRNA. Despite being dismissed as “junk” DNA for many years, introns are known to have potential functions such as: (1) carriers of transcriptional regulatory elements, (2) contributing to alternative splicing, (3) generating non-coding RNAs that have gene regulatory functions, and (4) expressing nested genes that are usually located within one intron of a host gene and which regulate the respective expression of that host gene (Yu et al. 2005; Buretic-Tomljanovic and Tomljanovic 2009; Barrett et al. 2012; Rogozin et al. 2012).

How variation at CACNA1C SNP rs1006737 might modulate ICH is unknown. GENSCAN (http://genes.mit.edu/GENSCAN.html) predicted a novel nested gene that consists of two exons spanning an approximately 5.9-kb region on the A risk allele; but the G allele could not be predicted by this novel gene. Of note, the CACNA1C SNP rs1006737 (A; risk allele) locates within the second exon of this novel gene, implying that this nested gene might regulate the expression of its host gene, that is, CACNA1C, only in A risk-allele carriers. In favour of this possibility, Gershon et al. (2013) found that CACNA1C risk variant is associated with reduced expression of CACNA1C mRNA in human cerebellum, and we found that CACNA1C SNP rs1006737
variants exhibit lower mean BLCL \([\text{Ca}^{2+}]_{\text{p}}\) in healthy A risk-allele carriers as compared with healthy GG carriers. Another possibility is that the alleles may be located in a carrier region of transcriptional regulatory elements. The SNP Function Portal (http://brainarray.mbn.edu/Brainarray/Database/SearchSNP/snpfunc.aspx) predicts this locus as a putative transcription factor binding site designated HSSGMCNF_04 and 03 with a match score of 100% for the A allele, but not the G allele. GM-CSF (granulocyte/macrophage colony stimulating factor) is a hematopoietic cytokine produced by peripheral immune cells and central astrocytes (Davignon et al. 1988; Guillemin et al. 1996; Hercus et al. 2009). It may exert a neuroprotective effect by prompting microglia to adopt a dendritic cell-like state (Fischer and Reichmann 2001; Santambrogio et al. 2001; Schermer and Humpe1 2002), thereby facilitating the release of BDNF (Hayashi et al. 2009) and increasing the expression of anti-apoptotic proteins such as Bcl-2 (Huang et al. 2007; Kim et al. 2009). Which of the aforementioned possibilities are likely to be involved in the molecular mechanisms by which CACNA1C increases risk for BD merits direct testing in future investigations.

The findings of this study must be considered within the context of some notable limitations. First, the study sample size is small relative to GWAS standards limiting the power of the test of association. That said, the sample size is comparable to that in reports examining gene association with specific cellular functional analyses. Second, the study design lacks controls to minimize effects of ethnicity and population structure, although the sample was comprised primarily of Caucasian subjects of European ancestry. The small number of subjects harbouring the CACNA1C SNP rs1006737 AA genotype and the lack of significant differences in allele frequencies in mood disorders patient groups compared with healthy subjects limited examining CACNA1C SNP rs1006737 AA and AG genotypes individually. As the AA and AG individuals do not seem equally associated with BD or BLCL \([\text{Ca}^{2+}]_{\text{p}}\), it would be of interest to test these allele individuals further in future studies examining suitably large data sets for this purpose. Though not the primary focus of this study, the small sample sizes of the MDD and BD-II groups also limited our ability to test the hypothesis that CACNA1C also associates with MDD and BD-II as might be expected based on results of GWAS findings. Fourth, although aberrant Ca\(^{2+}\) responses are consistent findings in peripheral blood cell (platelet, mononuclear leukocyte and BLCL) preparations from BD patients compared with controls (Warsh et al. 2004) and, CACNA1C is expressed in lymphocytes (Quinn et al. 2010), it remains to be confirmed that CACNA1C SNP rs1006737 exerts similar effects in a neuronal cell phenotype derived from BD-I patients and in brain, and/or affects its expression. These have not been addressed in this study. Fourth, while potential functions of CACNA1C SNP rs1006737 were suggested by in silico analysis, which, if any, of these predictions is tenable requires validation by direct testing.

In summary, our study provides further supportive evidence of the association between CACNA1C SNP rs1006737 with BD-I and demonstrates that CACNA1C SNP rs1006737 and Bcl-2 SNP rs956572 variant differentially influence the risk for BD-I. But the effect of Bcl-2 variation on its expression may be sufficient in and of itself to promote disruption of ICH in BD-I patients. Further functional analysis of CACNA1C and Bcl-2 are needed to better understand their respective roles in the pathogenesis of BD.

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Statement of Interest

None to declare.

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Supplementary material available online

Supplementary Table I. Demographic characteristics of BD-I patients.

Supplementary Table II. Risk genotype frequencies of CACNA1C intronic SNP rs1006737 in mood disorders and comparison healthy subject groups.