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A rare mutation of *CACNA1C* in a patient with Bipolar disorder, and decreased gene expression associated with a Bipolar-associated common SNP of *CACNA1C* in brain

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Abstract

Timothy Syndrome (TS) is caused by very rare exonic mutations of the *CACNA1C* gene that produce delayed inactivation of Cav1.2voltage-gated calcium channels during cellular action potentials, with greatly increased influx of calcium into the activated cells. The major clinical feature of this syndrome is a long QT interval that results in cardiac arrhythmias. However, TS also includes cognitive impairment, autism, and major developmental delays in many of the patients. We observed the appearance of Bipolar Disorder (BD) in a patient with a previously reported case of TS, who is one of the very few patients to survive childhood. This is most interesting because the common SNP most highly associated with BD is rs1006737, which we show here is a *cis*-expression quantitative trait locus (eQTL) for *CACNA1C* in human cerebellum, and the risk allele (A) is associated with decreased expression. To combine the *CACNA1C* perturbations in the presence of BD in this patient and in patients with the common *CACNA1C* SNP risk allele, we would propose that either increase or decrease in calcium influx in excitable cells can be associated with BD. In treatment of BD with calcium channel blocking drugs (CCBs), we would predict better response in patients without the risk allele, because they have increased *CACNA1C* expression.

Keywords

Bipolar disorder; Timothy syndrome; calcium channel blockers; *CACNA1C*; long QT syndrome

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Introduction

Timothy Syndrome (TS) comprises a range of symptoms, including long QT intervals, cognitive impairment, autism and major developmental delays, all of which appear to stem from one of four different mutations in the *CACNA1C* gene, which encodes the alpha-1 subunit of the voltage-gated calcium channel CaV1.2. Very few TS patients have survived childhood, but we observed a previously reported patient[1] who survived and went on to develop Bipolar disorder (BD) in his late teens. The occurrence of BD in a TS patient is interesting because common *CACNA1C* SNPs have been consistently and strongly associated with BD in genome-wide association studies (GWASs)[2-4]. We suggest that this patient's BD is associated with his TS mutation and that perturbation of calcium channel gating may be the mechanism underlying both this patient's BD and BD in patients with the common BD-associated SNPs. Furthermore, we propose BD patients with and without the risk alleles may have different responses to treatment with calcium channel blockers, based on differential gene expression of *CACNA1C*.

Case Summary

The patient is a European-American male with TS Type 2 (see below) diagnosed in childhood. At age 28, the patient was referred for research evaluation after diagnosis of BD by one of us (FO). This was confirmed by interview with the patient and his mother, using the Diagnostic Interview for Genetic Studies (DIGS), version 3.0.

The patient was born full-term in good health, and had an uneventful history during his first three years. At age 4, he suffered a cardiac arrest during a trampoline class[1, 5]. Diagnostic work-up identified long QT syndrome, with a QT interval of 550-600 ms. In molecular investigation of his case and one other, TS was described as a severe variant of TS, as discussed below [1] (See Supplementary Figure 1).

No neurological sequelae were observed after this episode of cardiac arrest. He later had other arrests, and a cardiac pacemaker was implanted when he was 8 years old. He suffered his most complicated arrest at age 10, which led to a prolonged hospitalization complicated by liver failure, coma, and anoxic brain injury. After this illness, he received an implanted cardioverter-defibrillator (ICD).

His cognitive abilities declined after this episode. He became more socially withdrawn and began attending special education classes with full-time aides. He showed left hemiparesis, which persisted, although not severely. He completed high school and worked for one year as a cleaner at a gym. He has never had a seizure.

At age 19, he had his first episode of major depression, and at 21 he had an episode of mania. Shortly afterwards[6], TS was described as a mutation in the voltage-gated calcium channel gene *CACNA1C*. A molecular diagnosis was made when he was 22, and he was treated with a calcium channel blocker (CCB) verapamil at 250 mg/d, which continues to the present time. Theoretically, this would counteract the effects of his mutation, which causes increased Ca^{++} cellular influx during its extended activation[5]. He had a marked decrease in

the number of ventricular fibrillation episodes, but no change in QT interval, and continued to have some episodes of a trial fibrillation.

Ten months after starting the medication, he was referred to a neurologist, who found depression and prescribed citalopram. The patient then had significant improvement in mood and behavior.

By age 30 he had experienced 10 manic episodes and 3 hypomanic episodes. These were characterized by periods of up to two weeks duration when his sleep decreased markedly, and he would become aggressive with his siblings and would start fights and verbal altercations that were a departure from his usual behavior. Restlessness, talkativeness and difficult-to-follow speech were also present. Ability to focus was impaired. At age 27, the most extreme manic episode occurred, when, along with the usual symptoms, there were hallucinations and paranoid delusions.

Lithium was then given for mood stabilization, but was not well tolerated, with complaints of increased lethargy and irritability. He was restarted on his antidepressant and has been stable for the three years of observation since then. We note that this is not a typical course of BD, and that further observation will be needed to fully define his course.

Timothy Syndrome (TS) molecular pathophysiology

TS is caused by missense mutations in the *CACNA1C* gene, which encodes the alpha-1 subunit of the L-type calcium channel $Ca_v1.2$. All these mutations occur in one of the pore-forming S6 trans-membrane helix segments of the protein. The G406R mutation in exon 8A causes TS1[6], while TS2 is produced by one of two mutations in the alternate splice form exon 8, G406R or G402S (as in this patient)[1], and a recently reported mutation in exon 38 causes TS3[7]. Our patient is the only known living carrier of the exon 8 G402S mutation, for which he is a mosaic[1]. We confirmed his reported mutation by sequencing in our own lab (data not shown).

It is still not completely clear how TS mutations lead to altered function of CaV1.2 channels, but it is known that multiple aspects of channel function are affected. Voltage-dependent inactivation is decreased[8], action potentials are longer, and calcium flux through Cav1.2 channels is increased[9-11]. It is also not known how the delayed inactivation leads to long QT intervals, ventricular fibrillation, or any of the neurological traits associated with the syndrome. In neuronal induced pluripotent stem cells (iPSCs) derived from TS patients, there are multiple changes in gene expression, including increased tyrosine hydroxylase (TH) activity and increased production of norepinephrine and dopamine[12]. These changes are most likely related to the role of calcium influx as a second messenger indirectly regulating transcription. Conceivably, these changes in catecholamines are related to the observed arrhythmias and neuropsychiatric changes.

The L-type channel blocker nimodipine failed to reverse excess expression of TH in these neuronal cells[12]. However, treatment of the cells with roscovitine, an experimental drug in cancer treatment that inhibits cyclin-dependent kinases and also blocks the calcium channel,

specifically enhanced (normalized) inactivation of the L-type channel of the Cav1.2 protein[13], and caused a 68% reduction in the proportion of TH-positive neurons.

CACNA1C knockout mice cardiac effects

Two studies of transgenic mice with knockout (KO) of *CACNA1C* reported opposite effects on heart function. Rosati *et al*.'s heterozygous KO mouse showed a 58% reduction of *CACNA1C* mRNA and a 21% reduction in *CACNA1C* protein, but no change in L-type calcium channel (LTCC) current or in gross cardiac phenotype[14]. The Goonasekera study[15] had a graded knockdown heterozygote with cardiac protein levels of *CACNA1C* reduced by approximately 40%, and roughly 25% less whole-cell LTCC current measured in freshly isolated adult ventricular myocytes. These mice had a pronounced cardiac stress-induced phenotype, but the effects of verapamil were actually detrimental to cardiac function, despite a reduced LTCC current[15]. The differences are difficult to resolve. Both studies had a KO cassette maintained on a C57BL/6 background, but there were some differences in the cassette construction. Another mouse study, in which LTCC activity was increased by over-expressing the β2a subunit of the LTCC, found that increased LTCC activity produced a phenotype similar to that of the Goonasekera, leading to cardiac hypertrophy and early death[16]. This suggests that it may be a perturbation in calcium influx, rather than specifically an increase or decrease, that leads to a disease phenotype in the mouse models.

CAC1NAC in Bipolar disorder

The marker most significantly associated with BD in a recent meta-analysis of genome-wide association studies is an intronic SNP within *CACNA1C*, rs1006737[17]. Although the variant with biological effect is not necessarily the associated SNP, it must be in LD with the functional variant, so the biology of the associated SNP is of some interest. One of us (CL) is leading an ongoing study of expression Quantitative Trait Loci (eQTLs) in brain, using the Stanley Medical Research Institute postmortem brain collections. For the current paper, we tested rs1006737 for association with *CACNA1C* expression levels in human brain tissue.

Materials and methods for CACNA1C expression study

Samples

In our ongoing study, we obtained 164 cerebellum and parietal cortex brain samples from two collections of the Stanley Medical Research Institute (SMRI)[18-20]. We used the Norgen DNA purification kit to extract high molecular weight DNA from tissue blocks. The DNA was resuspended in low EDTA TE buffer. The DNA concentration and A260/A280 ratio were determined on a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE). Only intact DNA samples showing a major band at approximately 10-20 kb on a 1% agarose gel were genotyped.

RNA preparation and QC

We used the RN easy Mini kit (Qiagen, Valencia, CA) to extract total RNA from brain tissue blocks. The ratio of 28S to 18S rRNA and RNA Integrity Number (RIN) were measured using an RNA LabChip kit on the Agilent 2100 Bioanalyzer (Agilent

Technologies, Santa Clara, CA). To avoid use of seriously degraded samples, only RNA samples with a RIN > 7 were used for expression profiling.

Genotyping was performed using Affymetrix GeneChip Mapping 500K Array at TGen by Dr. David Craig (www.tgen.org). Genotypes were called using the BRLMM-p algorithm (Affymetrix). SNPs with call rates ≥99%, Hardy-Weinberg equilibrium (HWE) p values ≥ 0.001 and minor allele frequencies (MAF) ≥ 10% were included in the association tests. Pairwise identity-by-state was calculated using PLINK to verify unrelatedness. We used MACH (http://www.sph.umich.edu/csg/abecasis/MACH/index.html) to impute 852,963 SNPs.

Gene expression profiling was performed using Affymetrix's GeneChip Human Gene 1.0 ST Array, by the NIH Neuroscience Microarray Consortium at Yale University. Raw data in CEL files were summarized by Affymetrix Expression Console software EC1.1, using our customized library files to remove probes that have cross-hybridization to multiple genomic regions, or contain common SNPs. Batch effects were removed by ComBat[21]. All covariates were removed by SVA[22] before the SNP-expression association tests.

SNP-expression association tests were performed for *cis*- regulation. *Cis*-association refers to correlation of genes and SNPs within 1 Mb of before or after those genes. We used mach2qtl to perform the association tests, and permutation correction. Permutation was used to correct for multiple testing: region-wide significance corrected for the number of SNPs tested; phenotype-wide significance corrected for the number of phenotypes tested as well. No *trans*-associations (with SNPs outside this region) were found with *CACNA1C*.

Results of *CACNA1C* expression study

We detect a significant *cis*- association of *CACNA1C* expression in cerebellum for SNP rs1006737 that survives correction for region-wide multiple tests, where the risk allele (A) is associated with reduced expression (Table 1). The association is present in multiple exons as well as transcripts of this gene in cerebellum but not in parietal cortex (see Supplementary Table 2). This finding is also present for rs1024582, the *CACNA1C* SNP recently associated by Smoller et al.[3] with multiple psychiatric disorders. Rs1024582 is in nearly complete LD with rs1006737 ($R^2 = 0.94$), and its risk allele (A) is also associated with significantly decreased cerebellar expression of the same *CACNA1C* probes (data not shown).

Discussion

Cerebellum and psychiatric disorders

In a landmark review in 2005, Konarski et al.[23] advanced the paradigm-shifting hypothesis that, based on functional associations evident through cerebellar stimulation, lesions, and functional and morphometric imaging, cerebellar abnormalities play a crucial role in several psychiatric disorders, including schizophrenia, depression, and bipolar disorder. In a 2008 review, Andreasen and Pierson[24] remark that “The tentorium was once the Maginot Line of the brain. Supratentorial regions governed “higher cortical functions,” while the humble subtentorial cerebellum performed “lower” functions unrelated to cognition.” They then

summarize evidence cortical circuitry connecting the cerebellum and cortex, and conclude that through its role modulating cognition the cerebellum appears to play a crucial role in Schizophrenia. More recent evidence showed, and changes in gene expression of NMDA receptor subunits in cerebellum in Schizophrenia [25], and differential expression of genes encoding neuronal ion-channel subunits in cerebellum in several psychiatric disorders including bipolar disorder [26]. In a whole-genome expression analysis, Chen et al.[27] demonstrated two gene co-expression modules associated with Bipolar Disorder and Schizophrenia in multiple data sets. One of the modules was associated with these diagnoses in both cerebral cortex and cerebellum. This module included metallothioneins (MT) and metal binding site functions, which are involved in oxidative stress and other cellular processes. In a related finding, myelination and oxidative stress alterations were observed in the cerebellum of the G72/G30 transgenic schizophrenia mouse model [28]. In view of all these findings, we would conclude that it is no longer valid to consider the cerebellum as a brain region that cannot play a decisive role in a mental disorder.

Role of common SNPs in brain expression of CACNA1C

It is logical to test for an association between the *CACNA1C* SNP rs1006737 genotype and *CACNA1C* expression levels, given the strength and scope of the associations between that SNP and risks of multiple psychiatric diseases, as well as numerous behavioral and cognitive endophenotypes. We report such an association in human cerebellum, but not in parietal cortex. These findings have not yet been replicated in a second dataset; while similar genotype-expression datasets exist, they differ from the current data in multiple ways (See Supplemental Table 1 for summary). In particular, none included parietal cortex and only one included cerebellum.

For the CACNA1C, there is reason to expect different results from different brain regions, because there are differences in relative expression of L-type calcium channels across brain regions. In a mouse model, Schlick *et al*. found that the ratio of CaV1.2 to CaV1.3 expression was about 1:1 in cortex and hippocampus, while in cerebellum it was 4:1[29]. Detection of expression differences in CaV1.2 may thus be more feasible in cerebellum than in cortical regions.

The genome-wide mapping of brain expression and methylation QTLS by Gibbs et al.[30] is the one comparable study that included cerebellum. They reported no association between *CACNA1C* expression and rs1006737 genotype in cerebellum or in the other three regions tested. However, the Illumina expression platform they used had one probe for all of *CACNA1C*. Our study and Kang et al.'s[31] QTL mapping of human brain both used an Affymetrix platform with much better *CACNA1C* coverage. The increased number of probes and regions studied dramatically increased the multiple testing burden in the Kang study, which, combined with the Kang study's small sample size (N=57), left it with low statistical power. None of the probes studied met their criteria for statistical significance of association of SNPs with a gene (gene-wide Bonferroni correction followed by genome-wide Q<0.1) in any of the 16 brain regions they tested, which did not include cerebellum or parietal cortex.

Bigos *et al*.[32] studied the association between *CACNA1C* genotype and expression in dorsolateral prefrontal cortex, with data that are also included in a later and broader

publication by the same group[33].Using another SNP rs2159100 as proxy for rs1006737, since the two are in complete LD (R^2=1.0), they reported that that the risk allele is associated with increased *CACNA1C* expression as measured by probe 28032. To review the group's findings, we used BrainCloud (http://braincloud.jhmi.edu/) to retrieve their cis-eQTL data for *CACNA1C*. There were actually six expression probes for this gene. Two (28032, 36147) showed nominally significant association with rs2159100 (P=0.022 and 0.028 respectively), with the risk allele (A) having increased expression relative to the non-risk allele. However, the expression probe near exon 8a (37564) and the other three probes were not even nominally associated with rs2159100 genotype. In addition, when the Bonferroni correction for multiple testing is applied (0.05/6 tests =0.008), none of the probes reach gene-wide signficance.

BD in Timothy Syndrome

TS includes a range of neurological, cognitive and psychiatric symptoms, including autism[1, 6, 34], and it would appear from this case that BD is one of them. A possible "confluence of rare/uncommon and common genetic variation on the same genetic [disease] loci" has been noted in GWASs[35], which would fit variations in *CACNA1C* associated with BD. However, we must consider other possibilities: the patient's anoxic brain injury might be considered as an alternative causes. Also, if his symptoms are due to a gain-of-function calcium channel defect, verapamil might be expected to have prevented or treated this defect, as it partially did in his cardiovascular system. Third, BD is a common disease, and could occur in the same patient independently of a rare disease.

Mania can arise after traumatic brain injury (TBI), but has only rarely been reported after anoxic brain injury[36]. Jorge *et al*. found a 9.1% incidence of mania after TBI within the first 12 months in 66 patients; but there is little data showing association of mania with a very long interval after head injury, such as the 11 years in this case[37, 38]. Most experts conclude that the longer the latency, the more the attribution of mania to the TBI may be questioned.

If the patient's *CACNA1C* mutation is responsible for his BD, one might expect that verapamil would also have prevented or treated that condition, as it succeeded in treating his heart condition. However, as noted above, verapamil does not correct the depolarization deficit in TS, or change the intracellular neurotransmitter abnormalities of TS in iPSC cells.[12] Also, the doses used for the management of cardiac conditions are lower than those needed to attain effects in the brain [39], because verapamil has low penetration into the brain, particularly in males [40].

Implications for BD

If the same disease is produced by the TS gain-of-function *CACNA1C* mutation (increased Ca^{++} flux) as is associated with the common *CACNA1C* BD risk polymorphism, and there is loss of function from the risk allele of the common polymorphism (decreased Ca^{++} flux from reduced gene expression), this would suggest that Ca^{++} flux that strays in either direction from normal can produce deficits in CNS function in humans, possibly as a result of changes in monoamine neurotransmitter synthesis and release. Our own human brain data, with the largest number of *CANA1C* expression probes so far examined, demonstrates

that individuals with the common polymorphic risk allele have decreased *CACNA1C* expression in at least one brain region (Table 1). These patients may have a degree of decreased Ca^{++} flux due to haploin sufficency, by analogy with the *CACNA1C* heterozygous graded KO mice of Goonasekera.[15]

This would suggest that efficacy of CCB treatment in BD patients would differ in patients with and without the risk allele. Because the *CACNA1C* risk allele is expected to produce loss of calcium channel function, we would expect BD patients without the risk allele to preferentially respond to CCB treatment. A 2000 review of treatment studies of CCBs in BD[41] concluded that CCBs had not been adequately evaluated as a BD treatment, but evidence that they were generally effective was not present. The reviewed trials had treated BD patients as a single population, but now patient groups can be subdivided according to *CACNA1C* genotype.

There is already evidence that *CACNA1C* genotype can affect response to CCBs in treatment of hypertension[42, 43]. A CCB medication which has less of a blood-brain gradient than verapamil, and which, like roscovitine, succeeds in reversing the TH abnormality in TS iPSC cells might be the preferred choice for a clinical trial.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Table 1

Association of SNP rs1006737 bipolar risk allele (A) with decreased expression of *CACNA1C* in human cerebellum

Probe type	Probe ID	Frequency (A allele)	Effect	CHISQ	P-value	Corr_p
exon	7953041	0.36	-0.47	14.67	1.28E-04	0.026
exon	7953044	0.36	-0.52	17.65	2.66E-05	0.006
exon	7953049	0.36	-0.61	24.46	7.59E-07	0.001
exon	7953050	0.36	-0.52	17.67	2.63E-05	0.004
exon	7953066	0.36	-0.54	19.73	8.91E-06	0.004
exon	7953068	0.36	-0.55	20.18	7.04E-06	0.002
exon	7953075	0.36	-0.61	24.50	7.43E-07	0.001
exon	7953076	0.36	-0.52	17.73	2.54E-05	0.004
exon	7953079	0.36	-0.50	16.30	5.41E-05	0.014
exon	7953081	0.36	-0.49	16.10	6.00E-05	0.026
exon	7953093	0.36	-0.67	29.47	5.67E-08	0.001
exon	7953095	0.36	-0.55	19.84	8.43E-06	0.004
transcript	7953040	0.36	-0.60	23.73	1.11E-06	0.001
transcript	7953094	0.36	-0.46	14.28	1.58E-04	0.048

Notes: EFFECT: Beta regression coefficient of expression on SNP after correcting for covariates; CHISQ: Test statistic; P-value for test statistic; Corr_p: regionwide-corrected p-value. Computation by MACHqtl (http://www.sph.umich.edu/csg/abecasis/MACH/download/).