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A new homozygous *CACNB2* mutation has functional relevance and supports a role for calcium channels in autism spectrum disorder

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Research

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Abstract

Background

Diagnostic yield in patients with autism spectrum disorder (ASD) has improved over the last years, thanks to the introduction of whole genome arrays and next generation sequencing, but etiology is still unknown for the majority of cases. Among distinct cellular pathways, evidence implicating dysregulation of cellular calcium homeostasis in ASD pathogenesis has been accumulating, and specific mutations in voltage-gated calcium channels found in patients with autism were shown to be functionally relevant.

Methods

Whole exome sequencing and Sanger sequencing were performed to identify and confirm variants in a girl with ASD, global developmental delay and precocious puberty, born of first-degree cousins. Site-directed mutagenesis was used to generate a human $Ca_V\beta_{2d}$ calcium channel subunit carrying a *CACNB2* mutation. Whole-cell patch-clamp recordings were performed to reveal functional effects of mutant $Ca_V\beta_{2d}$ on Ba^{2+}-currents mediated by L-type ($Ca_V1.2$) calcium channels in transiently transfected HEK-293 cells.

Results

In an ASD patient, we identified a rare homozygous variant (p.Arg70Cys) in the *CACNB2* gene coding for the auxiliary $Ca_V\beta_2$subunit of voltage-gated calcium channels. In a recombinant system, the $Ca_V\beta_2$ variant, which was not previously associated to ASD, was found to alter $Ca_V1.2$ calcium channel function by significantly affecting activation and inactivation of whole-cell Ba^{2+}-currents.

Limitations

Although the evidence of *CACNB2* involvement in ASD is slowly accumulating, the number of reported patients is very limited. Deep clinical phenotyping and functional studies in larger sets of subjects will be instrumental to fully understand the penetrance and outcome of *CACNB2* variants.

Conclusions

The p.Arg70Cys variant in *CACNB2* shows functional consequences similar to other ASD-associated $Ca_V\beta_2$ mutations. These results support the idea of *CACNB2* variations contributing to the development of ASD and hint to a rare form of Mendelian recessive autism with possible specific comorbidities.

Background

Autism spectrum disorder (ASD) is a complex neurodevelopmental condition affecting about 1 in 60 individuals [1]. It is defined by dysfunctions in social interaction and communication, stereotypic behaviors and sensory integration problems. ASD can be isolated and, in such cases, predominantly

follows a polygenic pattern of inheritance caused by several weakly penetrant genetic variants that incrementally enhance susceptibility [2, 3]. Common genetic variants have been implicated by genome-wide association studies, with the identification of polygenic risk scores [4, 5]. On the other hand, ASD is a frequent feature of rare multisystemic neurodevelopmental disorders, where Intellectual Disability (ID) and epilepsy are common comorbidities [6]. A substantial subset of these patients show rare de novo copy number or single nucleotide variants (CNVs and SNVs) [7–9]. The contribution of recessive mutations has been less characterized and was addressed in a recent large-scale sequencing study, which demonstrated an excess of biallelic mutations justifying approximately 5% of ASD cases [10]. Monogenic “recessive” ASD is expected to be very rare but should be enriched in children of consanguineous marriages.

The introduction of next generation sequencing technologies offers the opportunity to test large cohorts of ASD patients and the results are helping to delineate the pathophysiological pathways which lead to this disorder. Hundreds of genes have been implicated in the pathogenesis of ASD, but the impacted pathways often cluster into three major functional groups: chromatin structure, transcription factors and calcium signaling [11].

Voltage-gated calcium channels (VGCCs) are heteromultimeric protein complexes consisting of up to four different subunits: a pore-forming α1 subunit ($Ca_V1.x$ – $Ca_V3.x$) and an auxiliary $\alpha_2\delta$- ($\alpha_2\delta_{1-4}$), β- ($Ca_V\beta_{1-4}$) and in some tissues γ-subunit (γ_{1-8}) [12, 13]. Among other functions, $Ca_V\beta$ subunits modulate the channel activation and inactivation [14, 15]. The first calcium channel gene that was reported as mutated in a form of monogenic syndromic autism was CACNA1C, which is the cause of Timothy syndrome, characterized by arrhythmia, hand/foot anomalies and high prevalence of ASD [16]. Since then other VGCC subunits have been associated with ASD (reviewed in [17]). Concerning to $Ca_V\beta$ subunits, CACNB2 variants showed genome-wide suggestive significance in siblings with autism [18]. Furthermore, CACNB2 was found as a risk locus for five major psychiatric disorders including ASD [19]. We previously reported three rare heterozygous CACNB2 missense variants in patients with autism, altering the kinetic parameters of recombinant L-type calcium channels similarly to the CACNA1C mutations associated with Timothy syndrome [20]. Furthermore, an N-terminal de novo missense mutation (p.Val2Asp) in CACNB2 was recently discovered in a whole-genome sequencing study of ASD individuals and it was considered clinically relevant [21].

In the present work, we report the identification of a CACNB2 homozygous rare variant in a girl with ASD, global developmental delay and precocious puberty via whole exome sequencing (WES). Functional characterization by whole-cell patch-clamp revealed a decelerated inactivation behavior of L-type calcium channels (LTCCs) similar to the previously described CACNB2 mutations suggesting a common feature among ASD-associated $Ca_V\beta_2$ mutations.

Methods

Clinical characteristics of the proband

An 11-year-old girl received a diagnosis of global developmental delay and ASD at the age of four. She is the first child of consanguineous parents (first-degree cousins) from Bangladesh. She was born at term after an uneventful pregnancy. Birth weight was 3255g. Motor development was normal, she had normal growth and no facial dysmorphisms. She underwent a neuropsychiatric evaluation at the age of four for absent speech. The Denver Scale showed a severe developmental delay, mainly affecting the areas of language and socialization. She was administered ADOS (Autism Diagnostic Observational Schedule) and CARS (Childhood Autism Rating Scale) and met diagnostic criteria for ASD. Brain imaging and EEG were normal. She also underwent a cardiac evaluation with ECG recordings, which did not show abnormalities of heart rhythm; she had normal hearing and normal ophthalmic evaluation. She had sleep disturbances with frequent awakenings during the night and a severe constipation.

Blood karyotype, FRAXA analysis, *MECP2* sequencing and MLPA, chromosomal microarray, urine and plasma metabolic investigations, were normal.

At the age of 6 years and 6 months, progression of breast development (B3 according to Tanner stages) and advanced bone age (8 years 10 months) were noticed. Pubertal response of LH (LH peak > 5 IU/L) to standard GnRH test and increased uterine length *(*40 mm) at ultrasound confirmed the diagnosis of central precocious puberty. MR imaging of hypothalamus-pituitary region was normal.

Therapy with GnRH analogues was started at the age of seven years and two months, and was interrupted after three years. One year after interruption (at 11), she had menarche.

At the last evaluation (11 years), speech was absent and the sleeping pattern was described as slightly more regular. She still showed relevant deficits in the areas of social interaction, communication, and range of interests and activities, confirming the diagnosis of ASD (severity level 3: "Requiring very substantial support") in association with a severe ID (DSM-5 criteria).

Parents do not show cognitive/behavioral problems and family history is unremarkable in this respect. Mother's menarche was at 13 years. The father was a heavy smoker, had a gastric lymphoma at 34 years and an acute myocardial infarction at 37: coronary angiography showed an occlusion of the left circumflex artery and he was subjected to primary angioplasty and placement of a bare-metal stent; ECG-holter was performed during follow-up and it was normal.

Whole Exome Sequencing

Genomic DNA was extracted from peripheral blood with the QIAamp DNA Blood Mini (Qiagen, Venlo, Netherlands). Targeted capture and enrichment was performed using the Nextera Rapid Capture Exome kit (Illumina Inc., San Diego, CA) and library was sequenced as 100-bp paired-end reads on Illumina HiScanSQ (Illumina).

Generated reads were treated following a general pipeline elsewhere described [22] including alignment with BWA [23] to the reference genome hg19, realignment and base quality score recalibration with GATK [24] and duplicate removal with Picard Tools (https://broadinstitute.github.io/picard). Alignment and

coverage statistics were collected with SAMtools and GATK. Variants were called and filtered by quality with GATK HaplotypeCaller and Variant Quality Score Recalibration, and then annotated with Ensembl Variant Effect Predictor (www.ensembl.org/info/docs/tools/vep/index.html).

H^3M^2 [25] was used for the identification of ROHs from WES alignments. Candidate disease-causing variants were defined as variants with potential to alter the protein product (missense, nonsense, small insertion/deletions and splicing-affecting variants) with allele frequency lower than 0.01 and not seen in homozygous state in gnomAD database [26]. The selected variant in *CACNB2* was confirmed by Sanger sequencing and tested in parents.

DNA constructs and site-directed mutagenesis

For functional analysis, the p.Arg70Cys variant was introduced in human $Ca_V\beta_{2d}$ (NM_201596.2) by site-directed mutagenesis (Stratagene QuikChange Kit) and verified by sequencing. EGFP was used as reporter gene, which was co-expressed together with the β_2-subunit by the bicistronic pIRES2-EGFP vector (Clontech). Primer pairs for the mutagenesis were the following: p.Arg70Cys forward primer 5'-gccgaaccctggcaaacaaaactatttgaggtagtatca-3'; reverse primer 5'-tgatactacctcaaatagttttgtttgccagggttcggc-3'.

Since the N-termini of $Ca_V\beta_2$ vary in sequence and length among the different splice variants ($Ca_V\beta_{2a-e}$) and this has been shown to impact LTCC modulation [27,28], we used a $Ca_V\beta_{2d}$ backbone as in our previous study [20], thus comparing wild-type (WT) $Ca_V\beta_{2d\_WT}$ and mutant $Ca_V\beta_{2d\_R70C}$.

Cell culture and transfection

HEK-293 cells were grown in Petri dishes in Dulbecco's modified Eagle medium (Gibco Thermo Fisher, Waltham, MA, USA) supplemented with 10% FCS (Biochrom GmbH, Berlin Germany). Cells were routinely passaged twice a week and incubated at 37°C und 5% CO_2 growth conditions.

HEK-293 cells were transfected with human calcium-channel subunits and EGFP using a standard calcium phosphate method [29]. For whole-cell recordings, HEK-293 cells were transfected with a 1 : 0.5 : 1.5 ratio of $Ca_V1.2$ ($\alpha1c_{77}$) [30], either a WT or a mutant β_{2d}-subunit and an $\alpha_2\delta_1$-subunit [31].

Whole-cell patch-clamp recordings

Whole-cell recordings of EGFP-positive cells were obtained 48–72 h after transfection. Immediately prior to recording, cells kept in 35-mm culture dishes were washed at room temperature (19–23°C) with bath solution. The bath solution contained (in mM) 10 $BaCl_2$, 1 $MgCl_2$, 10 HEPES, 65 CsCl, 40 TEA-Cl, 10 Glucose (pH 7.3 with TEA-OH) and the pipette solution (in mM) 140 CsCl, 10 EGTA, 9 HEPES, 1 $MgCl_2$,4 MgATP (pH 7.3 with CsOH). Patch pipettes made from borosilicate glass (1.7 mm diameter and 0.283 mm wall thickness, Hilgenberg GmbH, Malsfeld, Germany) were pulled using a Sutter Instrument P-97 horizontal puller and fire-polished using a Narishige MF-83 microforge (Narishige Scientific Instrument

Lab, Tokyo, Japan). Pipette resistance was 3-5 MΩ. Currents were elicited by applying 500 ms long test potentials of -40 mV to +50 mV from a holding potential of -80 mV using Clampex software pClamp 10 and an Axopatch 200B amplifier (Molecular Devices, Sunnyvale, CA, USA). Currents were sampled at 10 kHz and filtered at 2 kHz.

Data were analyzed using Clampfit10.3 (Molecular Devices, Sunnyvale, CA, USA) and GraphPad 6 Prism software. For voltage dependence of activation data were fitted by combined Ohm and Boltzmann relation according to Karmazinova and Lacinova [32]. To obtain the time-course of activation (τ_{act}) a first order exponential function was used to fit to the current traces.

Statistical analyses

Data are shown as mean ± SEM and were analyzed using Student's unpaired two-sided t-test. Differences were considered statistically significant if $p < 0.05$.

Results

The proband belonged to a cohort of 50 individuals, all children of consanguineous parents, affected by heterogeneous disorders. According to our local protocol for consanguinity, WES experiment was performed in the proband only. Mean coverage was 66.8 X with the 90.7% of the bases covered. No pathogenic/likely pathogenic variants remained after filtering, but a rare and potentially disruptive homozygous missense variant in *CACNB2* (NM_201597.2:c.208C>T:p.Arg70Cys) was selected for further investigation. It was confirmed by Sanger sequencing and parents were found to be heterozygous carriers (Supplementary Figures 1a-b). It is a very rare variant (only three alleles were reported in the gnomAD database, none in the South Asian population). The pathogenic computational verdict is supportive, because of six pathogenic predictions from DANN, GERP, MutationTaster, PROVEAN, FATHMM-MKL and SIFT vs three benign predictions from FATHMM, LRT and MutationAssessor. Some *CACNB2* heterozygous variants have been described in families with Brugada syndrome [33,34], while others were associated with ASD [20]. The present *CACNB2* variant was deposited in ClinVar with identification number 545669, as a variant of unknown significance according to the American College of Medical Genetics (ACMG) guidelines [35].

Since our study design was a "proband only" WES, we could not evaluate the presence of de novo variants. However, no pathogenic/likely pathogenic heterozygous variants (according to ACMG guidelines) were identified in genes known to cause neurodevelopmental disorders.

In order to investigate the effect of the p.Arg70Cys variant, we expressed human $Ca_V\beta_{2d\_WT}$ or $Ca_V\beta_{2d\_R70C}$ together with $Ca_V1.2$ and $Ca_V\alpha_2\delta_1$ in HEK-293 cells and performed whole-cell patch-clamp recordings. Exemplary traces of Ba^{2+}-currents are shown in Figure 1a.

Our analysis revealed no differences in current density (Figure 1b), but a significantly lower activation time constant (τ_{act}) at various test potential in the presence of $Ca_V\beta_{2d\_R70C}$ compared to $Ca_V\beta_{2d\_WT}$

(Figure 2a). Furthermore, time-dependent inactivation at 150 ms was significantly reduced by $Ca_V\beta_{2d\_R70C}$ (Figure 2b). Accordingly, the inactivation time constant (τ_{inact}) was increased for $Ca_V\beta_{2d\_R70C}$ compared to $Ca_V\beta_{2d\_WT}$ (not shown). In summary, the p.Arg70Cys mutation shows functional consequences on $Ca_V1.2$-mediated Ba^{2+}-currents similarly to other ASD-associated $Ca_V\beta_2$ mutations as we described previously [20].

Discussion

More than 100 genes and genomic regions have been associated with ASD [6], suggesting a genetic architecture with many genes involved, each accounting for a small fraction of cases. Several lines of evidence indicate calcium signaling as one of the most significant pathways for ASD [17,36,37] and mutations in at least one gene encoding a calcium channel (*CACNA1C*) have been linked to a syndrome with a high prevalence of ASD [16]. We provide further evidence that *CACNB2* variation may be involved in the pathogenesis of ASD: a young girl with ASD, child of consanguineous parents, was found to carry a rare homozygous missense variant in *CACNB2*, which was predicted pathogenic by software tools. We proved that this variant has a functional impact since it alters the kinetic parameters of currents carried by recombinant L-type calcium channels.

In a recent study on recessive ASD, *CACNB2* was among 409 distinct genes that harbored biallelic, damaging missense mutations in cases but not in controls. It is instructive to note that also in this study one single gene (*AMT*) was independently hit in multiple families, confirming how rare the involvement of each independent gene is [10]. Unfortunately, no additional clinical data were reported for the subject mutated in *CACNB2*, therefore it was not possible to know if relevant comorbidities were present.

Our female patient showed ID, which is the most common associated feature in ASD patients, and precocious puberty. An early timing of puberty is a specific feature of some disorders characterized by ID [38], such as imprinting disorders and chromosome defects [39,40]. Our proband did not have features evocative of an imprinting defect (such as IUGR and small stature) and chromosomal microarray excluded CNVs. Moreover, she had normal brain imaging and no familiarity for early onset of puberty (maternal age of menarche was 13 years). To our knowledge, no variants in calcium channels have ever been associated to precocious puberty. However, given that calcium signaling is involved in essential cellular functions that span multiple tissues and physiological systems, including the reproductive axis through gonadotropin-releasing hormone neurons [41], *CACNB2* variants may indeed play a role in puberty progression. Only the identification of further patients carrying homozygous mutations in *CACNB2* will give the opportunity to clarify this issue.

$Ca_V\beta$ subunits are the only cytosolic subunit of high voltage-activated (HVA) VGCCs and serve, besides other functions, as main modulator of activation and inactivation of the channel [14,15,42]. They consist of variable N- and C-termini and conserved SH3- and GK-domains, separated by a variable HOOK-domain [43]. HOOK-domain and N-terminus have a major impact on voltage-dependent inactivation of HVA VGCCs [27,44–47]. The herein described p.Arg70Cys mutation significantly attenuates inactivation of

LTCCs, similar to the previously described ASD-associated mutations p.Gly167Ser and p.Ser197Phe [20]. Given these data, it is tempting to speculate that ASD-associated mutations in Ca_Vβ-subunits cause similar functional effects, i.e. enhanced calcium influx due to lowered channel inactivation, reminiscent of *CACNA1C* mutations in the Ca_V1.2 subunit of LTCCs causing Timothy syndrome [16].

Timothy syndrome is characterized by abnormalities of heart rhythm and a high co-occurrence of ASD. Some mutations were identified in patients presenting only with non-syndromic long QT syndrome [48], whereas no *CACNA1C* mutations were reported, to our knowledge, in patients with an isolated neurodevelopmental disorder. The dissimilarities in genotype-phenotype correlation for calcium-channel disorders might be determined by diverse factors, ranging from the impact of the mutation on channel activity (gain of function vs loss of function vs a dominant-negative effect), to the extensive alternative splicing, with many distinct transcripts exhibiting different biophysical properties and expression profiles. For instance, the classical Timothy syndrome phenotype results from the presence of the p.Gly406Arg pathogenic variant in exon 8A, an exon contained in a specific splice variant of *CACNA1C* found in approximately 20% of all cardiac mRNAs [16]. Two individuals with atypical Timothy syndrome had been reported with pathogenic variants (p.Gly402Ser and p.Gly406Arg) in exon 8 of an alternate splice form that represents 80% of all cardiac mRNAs [49].

Dominant mutations in *CACNB2* have been identified in a small subset of individuals with Brugada syndrome, a familial cardiac arrhythmia [33,34]; none of the reported patients had autism or other neurodevelopmental disorders. Data from the Genotype-Tissue Expression (GTEx) project indicate that the p.Arg70Cys variant in exon 2a of *CACNB2* is present in a transcript with higher expression in brain than in heart tissue, whereas the transcript with highest heart tissue expression does not include exon 2a (Supplementary Figure 1c). This expression pattern suggests a possible explanation for the absence of heart rhythm abnormalities in the present family.

As the expansion of personalized medicine proceeds, with increasing potential for interpretation of genomic data, the early identification of molecular and genetic defects can lead to a better clinical refinement and the possibility of applying timely targeted therapies. Most importantly, it is crucial to carry out the functional characterization of proteins and mutations, to make progress from gene identification to function [50]. Therefore, developing models for specific mutations allows to better define the underlying molecular defects, exemplified in the present case by a significantly lower current activation time of a calcium channel.

Limitations

The major limit of this study is that the analysis was performed on a single subject. The evidence of *CACNB2* involvement in ASD is slowly accumulating, but the number of reported patients is very limited. Although knowledge on recessive causes of ASD is lacking, they are expected to be rare, and the majority of large-scale studies evaluated polygenic inheritance or de novo variants. Deep clinical phenotyping and

functional studies in larger sets of subjects will be instrumental to fully understand the penetrance and outcome of *CACNB2* variants.

Ba^{2+} is not the physiological charge carrier trough L-type VGCCs, but it was used here for two main reasons: (1) Ca^{2+}-dependent effects on channel gating, like calcium-dependent inactivation, might be difficult to discriminate from other Ca^{2+}-independent effects, e.g. voltage-dependent inactivation; (2) using Ba^{2+} allows for comparison with findings from our previous study [20].

Conclusions

In conclusion, biallelic *CACNB2* variants may define a rare form of recessive ASD, which is comorbid with Intellectual Disability and, possibly, precocious puberty. The electrophysiological phenotype is similar to that of other $Ca_V\beta_2$ variants described earlier and thus suggests common features of ASD-associated $Ca_V\beta_2$ mutations, reminiscent of Timothy syndrome mutations in *CACNA1C*.

List Of Abbreviations

- ADOS: autism diagnostic observational schedule
- ACMG: American College of Medical Genetics
- ASD: autism spectrum disorder
- CARS: childhood autism rating scale
- CNV: copy number variant
- HVA: high voltage-activated
- ID: intellectual disability
- LTCC: L-type calcium channel
- SNV: single nucleotide variant
- VGCC: voltage-gated calcium channel
- WES: whole exome sequencing

Declarations

Ethics approval and consent to participate

Parents provided consent according to the IRB protocol 3206/2016 at Policlinico S. Orsola-Malpighi (Bologna, Italy).

Consent for publication

Consent to participate included consent for publication.

Availability of data and materials

The datasets analysed during the current study are available from the corresponding authors on reasonable request.

Competing interests

The authors declare no competing interests.

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Authors' contributions

CG conceived and designed the study, analyzed the data, and wrote the paper. PD and JM performed the electrophysiological studies and contributed to the writing of the paper. FP, TP, FI performed the sequencing and bioinformatic analyses. GS, AP, AC provided the patient data. EB supervised the research and co-wrote the paper. All authors read and approved the final manuscript.

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Figures

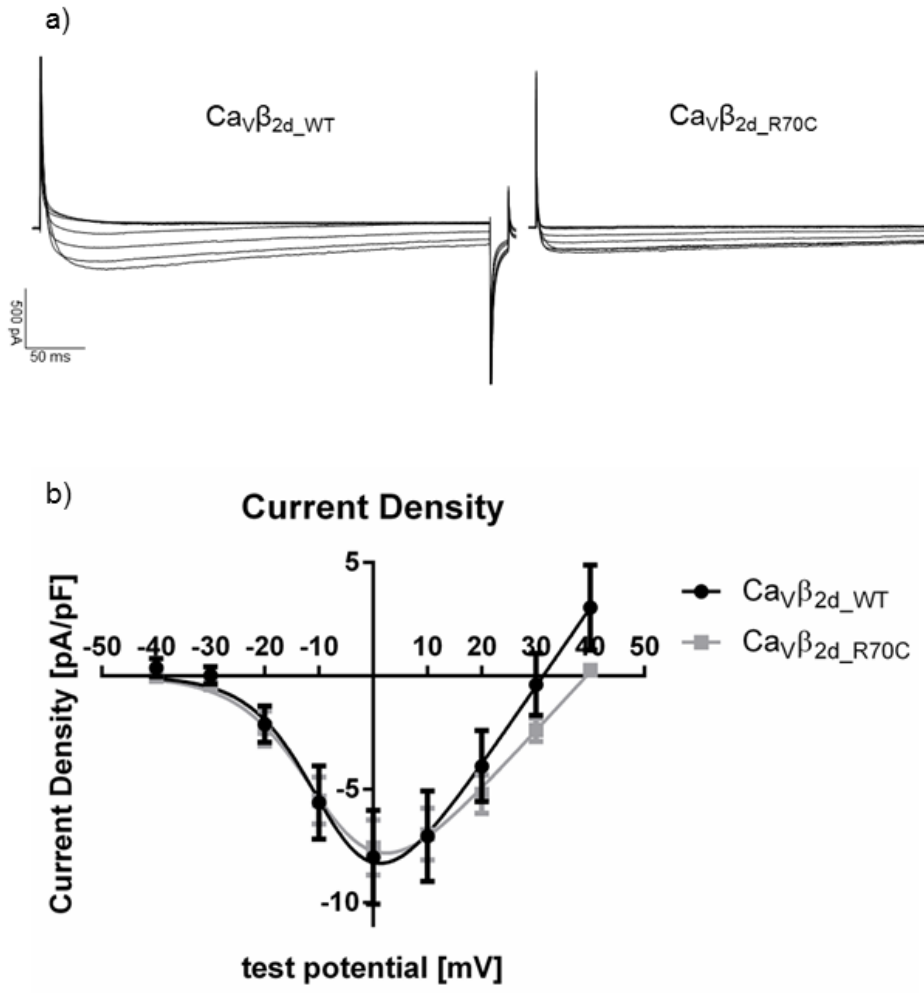

Figure 1

(a) Representative whole-cell Ba2+-currents recorded of HEK-293 cells expressing CaV1.2/α2δ1 and either CaVβ2d_WT (left) or the ASD-associated CaVβ2d_R70C (right). (b) I-V curve of CaVβ2d_WT (n=8) and CaVβ2d_R70C (n=13). Currents were elicited from -40 to +50 mV test potentials in 10 mV increments (Holding potential: -80 mV; charge carrier: 10 mM Ba2+).

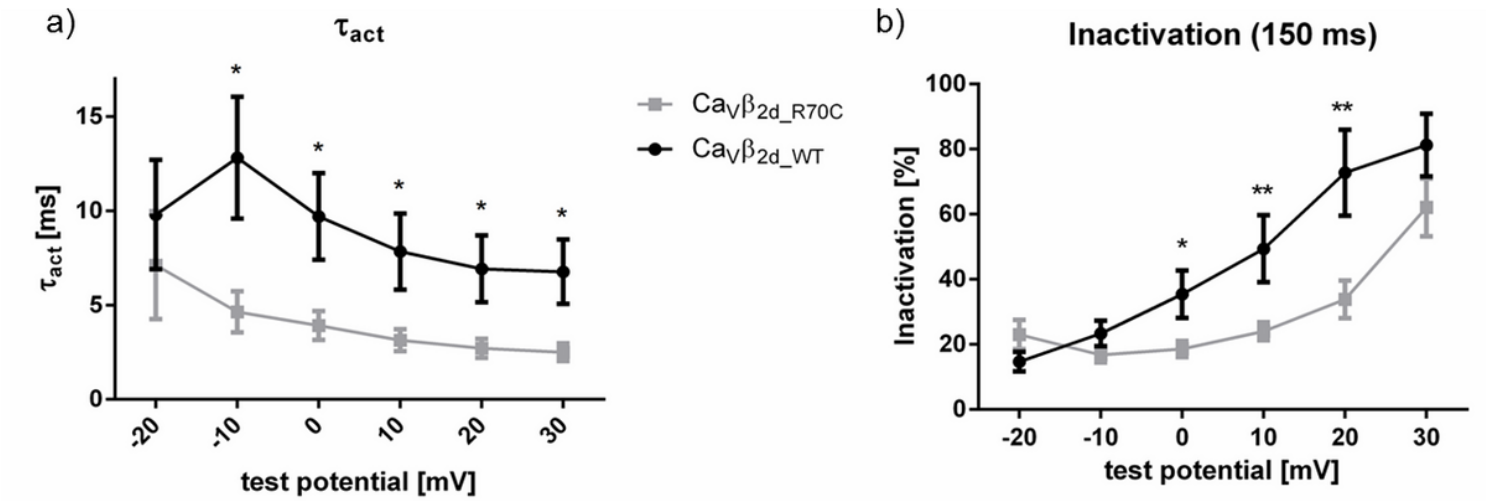

Figure 2

(a) Activation time constants (τact) showed a significant reduction for CaVβ2d_R70C (n=10) compared to CaVβ2d_WT (n=6) at various test potentials. (b) Time-dependent inactivation was analyzed as the remaining fraction of whole-cell current that has not been inactivated after 150 ms of depolarization. CaVβ2d_R70C (n=12) displayed a significant reduction at positive test potentials compared to CaVβ2d_WT (n=7) (Holding potential: -80 mV; charge carrier: 10 mM Ba2+). *: $p<0.05$ in Student's t-test.