Somatic and germline CACNA1D calcium channel mutations in aldosterone-producing adenomas and primary aldosteronism

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Adrenal aldosterone-producing adenomas (APAs) constitutively produce the salt-retaining hormone aldosterone and are a common cause of severe hypertension. Recurrent mutations in the potassium channel gene KCNJ5 that result in cell depolarization and Ca\(^{2+}\) influx cause ~40% of these tumors. We identified 5 somatic mutations (4 altering Gly403 and 1 altering Ile770) in CACNA1D, encoding a voltage-gated calcium channel, among 43 APAs without mutated KCNJ5. The altered residues lie in the S6 segments that line the channel pore. Both alterations result in channel activation at less depolarized potentials; Gly403 alterations also impair channel inactivation. These effects are inferred to cause increased Ca\(^{2+}\) influx, which is a sufficient stimulus for aldosterone production and cell proliferation in adrenal glomerulosa. We also identified de novo germline mutations at identical positions in two children with a previously undescribed syndrome featuring primary aldosteronism and neuromuscular abnormalities. These findings implicate gain-of-function Ca\(^{2+}\) channel mutations in APAs and primary aldosteronism.

Aldosterone is normally produced in response to intravascular volume depletion (via angiotensin II signaling) or hyperkalemia. Aldosterone signaling maintains normal intravascular volume by increasing intestinal and renal Na\(^+\) and Cl\(^-\) absorption and reabsorption, respectively. Constitutive production of aldosterone (primary aldosteronism) results in hypertension, often associated with hypokalemia. About 5% of individuals referred to hypertension clinics (1 to 10 million people worldwide) have APAs. APAs are typically benign, well circumscribed and solitary; their removal cures or ameliorates hypertension. KCNJ5 mutations alter channel selectivity, allowing Na\(^+\) conductance. Na\(^+\) influx results in cell depolarization, the activation of voltage-gated Ca\(^{2+}\) channels, aldosterone production and cell proliferation. These mutations are inferred to be sufficient for APA formation because rare individuals with mendelian aldosteronism and massive adrenal hyperplasia have identical KCNJ5 mutations in the germline.

We performed exome sequencing of 14 APAs and matched germline DNA. All affected individuals had hypertension with elevated aldosterone concentrations, despite suppressed plasma renin activity (PRA), and a pathological diagnosis of APA (Supplementary Table 1). We added 4 previously sequenced APAs to subsequent analysis (for a total of 18 APAs). We sequenced samples to high coverage and called somatic mutations (Online Methods and Supplementary Table 2). The mean somatic mutation rate was 3.0 × 10\(^{-7}\) mutations per base, with means of 1.7 silent and 6.1 protein-altering somatic mutations per tumor (medians of 1 and 3.5, respectively; Supplementary Fig. 1). Five of these 18 APAs had disease-causing mutations in KCNJ5 (encoding p.Gly151Arg or p.Leu168Arg), and one had a known gain-of-function mutation in CTNNB1 (encoding p.Ser45Phe), previously found in adrenocortical tumors.

One gene, CACNA1D, had somatic mutations in more than one APA (missense mutations encoding p.Gly403Arg (NM_001128840.2: c.1207G>C) and p.Ile770Met (NM_000720.3: c.2310C>G and in a different isoform, NM_001128840.2: c.2250C>G)), both in tumors without KCNJ5 or CTNNB1 mutations (Fig. 1). Both mutations were previously undescibed (absent from >10,000 exomes in public and Yale databases), appeared to be heterozygous and were confirmed by

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direct Sanger sequencing (Fig. 1a). Both occurred in tumors with few protein-altering somatic mutations (four and two, respectively) (Supplementary Table 3) and no detected copy number variants (CNVs). CACNA1D encodes Ca\textsubscript{\alpha}1.3, the \( \alpha\)\textsubscript{t} (pore-forming) subunit of an L-type (long-lasting) voltage-gated calcium channel. The \( \alpha\)\textsubscript{t} subunits contain four repeated domains (I–IV) (Fig. 2), each with six transmembrane segments (S1–S6) and a membrane-associated loop between S5 and S6. S5, S6 and the interposed loop line the channel pore\textsuperscript{7}. The residues altered by the two CACNA1D mutations occur in similar positions near the cytoplasmic ends of the S6 segments of domains I and II (Figs. 1c and 2).

Figure 1  CACNA1D mutations in APAs and primary aldosteronism. (a) Sequences of tumor and blood genomic DNA and (where available) of tumor cDNA for CACNA1D codons 402–404 in APA37, APA31, APA65 and APA59 and for codons 769–771 in APA29. Mutations are present in only the tumor sample and are expressed in cDNA. Sequencing of cloned PCR products confirmed the presence of the identified mutations in APA31, APA65 and APA59. (b) Pedigrees of kindreds with germline CACNA1D mutations. A, affected individual; A IM A, affected individual with parents; Tumor Blood cDNA for CACNA1D, Tumor cDNA for CACNA1E. Repeat I Repeat II

Direct Sanger sequencing of the genomic regions of CACNA1D encoding all S6 segments in 46 additional APAs, including the highly similar alternatively spliced isoforms (containing alternative exons 8A and 8B (ref. 8)), identified three additional somatic mutations affecting these segments. Most notably, two mutations (in exon 8A) encoded the same p.Gly403Arg alteration found by exome sequencing, and one mutation (in exon 8B) produced the homologous p.Gly403Arg alteration. Further sequencing identified 16 additional tumors with KCN5 mutations (encoding p.Gly151Arg or p.Leu168Arg) and 1 additional CTNN1B mutation (encoding p.Ser45Pro). All CACNA1D mutations occurred in tumors without KCN5 or CTNN1B mutations.
Collectively, CACNA1D mutations were identified in 5 of 64 APAs (7.8%), including in 5 of 41 APAs without KCNJ5 or CTHNB1 mutations (12.2%). The probability of finding an identical somatic mutation at any base in the exome 3 times by chance in 64 tumors, given the observed somatic mutation rate, is $3.0 \times 10^{-9}$, including the tumor with somatic mutation at the homologous base in exon 8B, the probability of seeing either of these mutations by chance in 4 of 64 tumors is $2.2 \times 10^{-12}$ (Online Methods).

Gly403 and Ile770 are completely conserved in proteins encoded by orthologs from invertebrates to humans (Fig. 1c). Additionally, Ile770 is conserved in all proteins encoded by paralogs (Fig. 1c). Notably, Gly403 is conserved in related human channels that are activated by large depolarizing potentials (high voltage activated) but not in channels that are activated by small changes in membrane potential (low voltage activated) (Fig. 1c). The finding of recurrent and clustered somatic CACNA1D mutations strongly suggests a gain-of-function mechanism.

Although CACNA1H (Ca₃.2) has been implicated in cyclic potential oscillations of glomerulosa cell membranes and in aldosterone secretion, little is known about the role of Ca₁.3 (encoded by CACNA1D). Wild-type and mutant CACNA1D alleles were both expressed in tumor cDNA (Fig. 1a). Moreover, analysis of normal human adrenocortical RNA showed that CACNA1D was the most highly expressed Ca²⁺ channel subunit, followed by CACNB2 (encoding the β₂ subunit) and CACNA2D1 (encoding the α₂δ₁ subunit) (Supplementary Table 4). Additionally, immunohistochemistry with three antibodies to Ca₁.3 demonstrated expression in human zona glomerulosa (Fig. 3 and Supplementary Fig. 2). These findings support a role for Ca₁.3 in the normal adrenal cortex and APA.

To assess the effect of CACNA1D mutations on channel function, we expressed wild-type and mutant Ca₁.3 together with the β₂ (β₂₄ isoform) and α₂δ₁ subunits in HEK293T cells. Representative whole-cell patch-clamp recordings are shown in Figure 4a. Upon depolarizing voltage steps, wild-type Ca₁.3 showed fast activation, followed by a slow decay caused by voltage-dependent (VDI) and calcium-dependent (CDI) inactivation (Supplementary Fig. 3). Compared to wild-type channels, the Gly403Arg and Ile770Met Ca₁.3 mutants both showed maximum current amplitudes at less depolarized potentials (Fig. 4b). Wild-type Ca₁.3 exhibited half-maximal activation voltage ($V_{1/2}$) at $-9.2 \pm 1.0$ mV (s.e.m.; $n = 7$), whereas $V_{1/2}$ values for Gly403Arg and Ile770Met Ca₁.3 were $-25.6 \pm 2.5$ mV ($n = 7$; $P < 0.001$ versus wild type) and $-31.7 \pm 1.1$ mV ($n = 6$; $P < 0.001$ versus wild type), respectively, indicating that the mutations facilitated channel opening (Fig. 4b). Current densities are shown in Supplementary Figure 4.

The p.Gly403Arg alteration also markedly impaired the inactivation of wild-type Ca₁.3 (Fig. 4a), suggesting a role for sustained activation with this alteration. The Ile770Met Ca₁.3 mutant showed inactivation shifted to more hyperpolarized potentials (Fig. 4a,c and Supplementary Fig. 3).

By analogy to studies of KCNJ5, we considered possible germline CACNA1D mutations. We sequenced the CACNA1D genomic regions encoding the S6 segments of domains I and II in 100 unrelated subjects with unexplained early-onset primary aldosteronism. Notably, we found germline mutations altering the same amino acids mutated in APAs in two subjects: one (1441-1) had a mutation in exon 8B encoding p.Gly403Asp and another (1555-1) had a mutation encoding p.Ile770Met (Fig. 1b). Neither mutation has been seen previously in SNP or exome databases. Both were de novo mutations, as they were absent in the parents, for whom the genotyping of nine highly polymorphic short tandem repeat markers provided unequivocal evidence of maternity and paternity (Supplementary Table 5). The probability of finding two de novo mutations by chance in any of the three CACNA1D codons mutated in APAs in 100 test cases is $3.8 \times 10^{-11}$ (Online Methods), implicating these de novo mutations in disease pathogenesis. Electrophysiology of the germline mutation encoding p.Gly403Asp (Gly403Asp Ca₁.3) demonstrated activation at less depolarizing potentials ($V_{1/2} = -32.4 \pm 2.3$ mV; $n = 5$; $P < 0.001$ versus wild type) and markedly impaired inactivation, similar to the p.Gly403Arg alteration (Fig. 4).

Subject 1441-1 was diagnosed with hypertension at birth (blood pressure of 119/78 mm Hg (systolic/diastolic); >99th percentile) with biventricular hypertrophy, a ventricular septal defect, pulmonary hypertension and second-degree heart block. Aldosterone levels were elevated (128.6 ng/dl) with low PRA (0.78 ng/ml/h) and a high aldosterone/renin ratio (ARR; 165; >30 is consistent with primary aldosteronism). The clinical course of this subject was notable for uncontrolled hypertension with hypokalemia (serum K⁺ concentration of 3.3 mM; normal range of 3.5–5.6). Notably, treatment with a calcium channel blocker, amlodipine, normalized blood pressure, and biventricular hypertrophy was resolved. Other features included a seizure disorder, apparent cerebral palsy, cortical blindness and complex dysembryoplastic neuroepithelial tumor (DNT) (Fig. 4).
neuromuscular abnormalities. There was no family history of early-onset hypertension or seizures (Supplementary Note).

Subject 1555-1 was diagnosed at birth with cerebral palsy, spastic quadriplegia, mild athetosis, severe generalized intellectual disability and complex partial and generalized seizures. At 5 years of age, the subject was markedly hypertensive (blood pressure of 132/90 mm Hg; >99th percentile), with persistently elevated blood pressure and polydipsia recognized retrospectively. The subject had hypokalemia (serum K\(^+\) concentration of 2.8 mM) and metabolic alkalosis (bicarbonate concentration of 30 mM). Serum aldosterone levels were high (36 ng/dl), despite suppressed PRA (0.15 ng/ml/h; ARR of 240). Computed tomography (CT) scan showed no adrenal abnormality, and echocardiogram showed mild left ventricular hypertrophy. This individual was treated with clonidine and later with spironolactone. There was no family history of early-onset hypertension or seizures (Supplementary Note).

These results implicate recurrent gain-of-function mutations in \(\text{CACNA1D}\) in \(\sim 8\%\) of APAs and in a new mendelian syndrome featuring primary aldosteronism associated with seizures and neuromuscular disease. The finding of de novo germline mutations at the same positions as somatic mutations found in APAs is consistent with these single mutations being sufficient for the development of APA, analogous to findings with \(\text{KCNJ5}\) mutations\(^4\). These findings indicate that APAs are commonly caused by single mutations and have implications for other hormone-producing tumors. Moreover, they underscore the discovery of previously unknown biology in a range of benign tumors\(^1,11\).

Electrophysiological studies of mutant Ca\(_{\text{v}}\)1.3 channels implicated increased Ca\(^{2+}\) influx in disease pathogenesis (Supplementary Fig. 5). Mutant channels activate at membrane potentials closer to the glomerulosa resting potential (about \(-82\) mV (ref. 9)); moreover, inactivation is impaired with the Gly403Arg and Gly403Asp Ca\(_{\text{v}}\)1.3 mutants. Spontaneous glomerulosa membrane potential oscillations\(^5\) may contribute to the activation of these mutant channels. Although increased current densities were seen with the Ile770Met and Gly403Asp mutants, such an increase was not seen for the Gly403Arg mutant (Supplementary Fig. 4). Because high Ca\(^{2+}\) influx may increase cell lethality, one must interpret differences in current density with caution. Further studies of the properties of single channels and the impact of these mutations in engineered animal models will be of interest.

Increased Ca\(^{2+}\) influx due to \(\text{CACNA1D}\) mutations phenocopies the consequence of \(\text{KCNJ5}\) mutations, which cause increased Ca\(^{2+}\) influx by depolarizing glomerulosa cells. This similarity suggests increased intracellular Ca\(^{2+}\) as a final common pathway in APA formation. These findings have implications for other hormone-secreting tumors and endocrinopathies in which hormone secretion is regulated by Ca\(^{2+}\). Whereas the mutations described here result in gain of function, homozygous loss-of-function mutations affecting \(\text{CACNA1D}\) in mice and humans result in deafness, bradycardia and arrhythmia\(^8,12\).

Notably, mutations affecting the S6 segments of other L-type calcium channels cause disease by similar gain-of-function effects\(^13\). Germline mutations in \(\text{CACNA1F}\) altering the positions homologous to Gly403 and Ile770 cause congenital stationary night blindness type 2 (refs. 14,15); similarly, mutation affecting the position homologous to Gly403 causes Timothy syndrome\(^16\) (Fig. 1c). Finally, gain-of-function mutations in \(\text{CACNA1A}\) affecting S6 segments cause familial hemiplegic migraine\(^17,18\).

Although only a small number of APAs with \(\text{CACNA1D}\) mutations have been identified, tumors with \(\text{CACNA1D}\) mutations were significantly smaller than those with \(\text{KCNJ5}\) mutations (13.4 ± 4.8 mm versus 21.9 ± 7.4 mm, s.d.; \(P = 0.01\)), and there was a trend toward older age at tumor presentation (53.8 ± 9.0 versus 43.6 ± 10.7 years; \(P = 0.06\)). Unlike \(\text{KCNJ5}\) mutations\(^1,19\), \(\text{CACNA1D}\) mutations did not show a strong female bias (identified in 3/30 males versus 2/34 females).

The subjects with de novo activating germline \(\text{CACNA1D}\) mutations represent a previously undescribed mendelian syndrome featuring primary aldosteronism. Although additional cases will be required to fully define the extra-adrenal manifestations of this syndrome, the complicated neuromuscular histories of the affected individuals indicate a multisystem disease, consistent with \(\text{CACNA1D}\) expression in neurons and heart\(^8\). The absence of adrenal hyperplasia by CT scan in subject 1555-1 at 9 years of age is noteworthy. Different germline mutations at the same position in \(\text{KCNJ5}\) are associated with aldosteronism with or without adrenal hyperplasia owing to variable Na\(^+\) conductance resulting in variable cell lethality; individuals with mutations causing high cell lethality never develop adrenal hyperplasia\(^9\). Further work will be required to establish whether the absence of hyperplasia with germline \(\text{CACNA1D}\) mutation is related to high cell lethality.

Recently, recurrent mutations in \(\text{ATTPA1}\) (encoding the \(\alpha\) subunit of a Na*/K*/Ca\(^{2+}\) ATPase) and \(\text{ATPP2B3}\) (encoding a plasma membrane Ca\(^{2+}\) ATPase) that account for 5.2% and 1.6% of APAs, respectively, were reported\(^20\). We sequenced these positions in our cohort and found the described mutations in three subjects (4.7%; Supplementary Table 1).

Lastly, the apparent response to treatment with a calcium channel blocker of subject 1444-1 raises the possibility of specific treatment for
individuals with APAs and CACNA1D mutations. Approved calcium channel blockers are weak antagonists of wild-type Ca\textsubscript{v}1.3, although potent and specific Ca\textsubscript{v}1.3 inhibitors have been identified\textsuperscript{21}. Such compounds or others that are selective for recurrent mutations may have particular promise in the treatment of a subset of individuals with CACNA1D mutations.

Note added in proof: Following acceptance of this manuscript, further exome sequencing of the APA cohort identified two additional tumors with somatic mutations in CACNA1D, encoding p.Phe767Val and p.Val1373Met. Mutations in CACNA1F and CACNA1A, respectively, affecting homologous positions are implicated in autosomal dominant congenital stationary night blindness\textsuperscript{22} and familial hemiplegic migraine\textsuperscript{23}. These findings bring the frequency of somatic CACNA1D mutations in our APA cohort to 11%.

METHODS

Methods and any associated references are available in the online version of the paper.

Accession codes. All somatic mutations found by exome sequencing are deposited in dbSNP under batch accession 1059250.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

A.L.F., L.F.S., J.W.K., N.M., R.B., J.R.S., E.A.H., N.M., M.R.B., T.B., J.R.S., E.L., U.I.S., R.P.L., and C.N.-W. prepared DNA and RNA samples and maintained sample archives. A.L.F., R.K., L.F.S., U.I.S., P.B. and C.N.-W. performed targeted DNA and RNA sequencing. U.I.S., G.G., G.S., C.F. and P. Hidalgo analyzed targeted DNA and RNA sequencing results. U.I.S. and R.K. performed immunohistochemistry. U.I.S., G.S., R.C.d.O., C.F. and P. Hidalgo made constructs and performed and analyzed electrophysiology. U.I.S., G.G., G.S., C.F. and P. Hidalgo wrote the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Subjects. Matched APA and venous blood DNA samples were obtained from individuals undergoing adrenalectomy for hypertension with primary aldosteronism and adrenocortical tumor at Yale New Haven Hospital, Uppsala University Hospital, Montefiore Medical Center and Albert Einstein College of Medicine and were evaluated as previously described\(^1\). Additional APAs and matched normal samples were obtained from paraffin-embedded samples from the Yale Department of Pathology. Additionally, individuals with unexplained early-onset aldosteronism, including parents in selected cases, were also studied. Research protocols were approved by local institutional review boards (IRBs), and informed consent was obtained from all research participants. The investigators were not blinded to allocation during experiments and outcome assessment.

DNA preparation, genotyping and exome sequencing. Genomic DNA was prepared from venous blood, tumor and surrounding normal tissue using standard procedures. For formalin-fixed, paraffin-embedded samples, DNA was prepared using the QIAamp DNA FFPE Tissue kit (Qiagen) according to the manufacturer’s instructions.

Targeted capture of exome sequences was performed using 2.1M NimbleGen Exome reagent, and sequencing was performed on an Illumina platform as previously described\(^1\). Somatic mutations were called on the basis of the significance of differences in read distributions between matched tumor and blood samples\(^1\). Calls were further evaluated by manual inspection of read alignments. The somatic mutation rate was calculated from the observed number of somatic mutations and the number of coding bases in exome capture.

Tumor purity was calculated from the mean minor allele frequency (MAF) of all high-quality SNPs in regions of loss of heterozygosity (LOH). For tumors with no detectable region of LOH, purity was estimated from the mean MAF of inferred heterozygous somatic variants.

CNVs were called from comparisons of the normalized ratio of coverage depth for tumor and normal samples and changes in MAF at informative SNPs.

Statistical tests. Two-tailed \( t \) tests were used for the statistical analysis of tumor characteristics. \( P \) values for the probabilities of observed mutations occurring by chance were calculated as binomial probabilities from the number of occurrences of specific mutations, the number of tumors studied, the observed somatic mutation rate and the total number of exonic bases. For statistical analysis of the voltage dependence of activation for wild-type and mutant channels, \( V_{1/2} \) values were tested for equality of variances and normality (Shapiro-Wilk method) and compared using pairwise multiple-comparison procedures (Holm-Sidak method).

Sanger sequencing of genomic DNA. Direct bidirectional Sanger sequencing of \( CACNA1D \) from genomic DNA in tumor-blood pairs was performed following PCR amplification using specific primers. All \( CACNA1D \) regions encoding \( S6 \) segments were sequenced in all APAs; the genomic regions encoding the \( S6 \) segments of domains I and II were sequenced for germline mutations in individuals with early-onset hypertension. Positions with recurrent mutations in \( KCNJ5 \), \( CTNNB1 \), \( ATP1A1 \) and \( ATP2B3 \) were sequenced in APAs following amplification by PCR using gene-specific primers. Primer sequences are listed in Supplementary Table 6.

Genotyping of parent-offspring trios. Nine highly polymorphic markers (loci CSFIP1, D13S317, D16S539, D18S51, D7S820, D8S1179, TH01, TPOX and D195433) were genotyped in parent-offspring trios of kindreds 1444 and 1555 by PCR amplification using marker-specific primers followed by direct Sanger sequencing. Genotypes were scored by the number of repeats present on each allele, as independently assessed by two investigators; genotype calls were concordant for all markers. Paternity and maternity indices were separately calculated for each putative parent\(^2\) using allele frequencies determined in 258 African Americans or 302 individuals of European descent\(^23\).

TOPO cloning of PCR products. DNA was amplified using the G403R_F and G403R_R primers, and PCR products were cloned using the TOPO TA Cloning kit (Invitrogen). Plasmid DNA from individual colonies was amplified by PCR, and Sanger sequencing was performed using the G403R_F primer.

cDNA synthesis and sequencing. Total RNA was prepared from fresh-frozen tissue using the AllPrep DNA/RNA/Protein Mini kit (Qiagen) or TRIzol reagent (Invitrogen), and column purification and DNase digestion were carried out using the RNeasy Mini kit (Qiagen). Reverse transcription of 100 ng of RNA was performed with Oligo(dT)\(_{12-18}\) priming (Invitrogen) using Superscript III Reverse Transcriptase (Invitrogen). cDNA was amplified using forward-spanning primers G403RTF/R and I770RTF/R and was sequenced with forward and reverse primers.

Immunohistochemistry. Sections (5 \( \mu \)m) from formalin-fixed, paraffin-embedded normal human adrenal cortex (two individuals) were obtained from the Yale Pathology Tissue Service. Sections were deparaffinized, and epitope retrieval was performed by heating samples in 10 mM sodium citrate in a microwave. Endogenous peroxidase activity was quenched with 0.3–3% \( \text{H}_2\text{O}_2 \), and samples were permeabilized in 1% SDS in TBS or 0.2% Triton X-100 in PBS. After blocking, slides were incubated with antibody to \( CACNA1D \) (HPA020215, Sigma; 1:100 dilution), Dab\(_2\) (sc-13982, Santa Cruz Biotechnology; 1:100 dilution), L-type Ca\(^{2+}\) CP\(_{\text{α1D}}\) (sc-32071, Santa Cruz Biotechnology; 1:50 to 1:100 dilution) or Ca\(_{\text{α1,3}}\) (ACC-005, Alomone; 1:100 to 1:250 dilution) for 12 h. HRP-conjugated secondary antibody to rabbit or goat and 3,3′-diaminobenzidine (DAB) were used to visualize signal. Where signal was low, a biotinylated secondary antibody was used, followed by the addition of HRP-conjugated streptavidin. For antibodies from Alomone and Santa Cruz Biotechnology, immunizing peptides were tested for their ability to block antibody labeling of tissues following the manufacturers’ protocols; incubations with and without immunizing peptides were processed in parallel. Sections were counterstained with hematoxylin. Images were captured using a Zeiss Axio Imager M2 microscope and processed using AxioVision software (Rel. 4.8) and Adobe Photoshop.

Molecular cloning. The canonical isoform of human \( CACNA1D \) in pCMV6-XL6 was obtained from Origene (SC309716; NM_000720.2). This isoform contains exon 8B. Other isoforms (NM_001128840.2 and NM_001128839.2) encode the same domain structures but vary in the presence of exons 11, 32 and 44 in extracellular and intracellular segments.

Site-directed mutagenesis (QuickChange, Stratagene) was performed to introduce the mutations encoding p.Gly403Arg, p.Ile770Met and p.Gly403Asp alterations into \( CACNA1D \). Each construct was validated by sequencing of the complete coding region. The Ca\(_{\text{β2c}}\) coding region (Swiss-Prot accession V8GC3–2) was fused at its 5′ end to sequence encoding mCherry and subcloned into the pcDNA3.1 vector. pIRE5-dsRed vector encoding Ca\(_{\text{δ2}}\) was kindly provided by F. Lehmann-Horn. Plasmids were prepared using the HiSpeed Plasmid Maxi kit (Qiagen).

Transient transfection and electrophysiological recordings. HEK293T cells (The European Collection of Cell Cultures) were grown to 80% confluency on 10-cm dishes in DMEM supplemented with 10% FBS, 1-glutamine (2 mM), penicillin G (100 U/ml) and streptomycin (10 mg/ml) and incubated in a 5% CO\(_2\) humidified atmosphere. Cells were cotransfected with 1 \( \mu \)g of plasmid encoding \( \text{B}_{\text{2a-MCherry}} \), 1 \( \mu \)g of plasmid encoding \( \text{G}_{\text{δ2}} \) and 2 \( \mu \)g of plasmid encoding wild-type, Gly403Asp, Gly403Arg or Ile770Met Ca\(_{\text{β2c}}\) using Lipofectamine 3000 (Life Technologies). Currents were recorded from transfactions with plasmids from at least two preparations of each \( CACNA1D \) construct. Cells were split 24 h after transfection. Currents were recorded on a HEKA EPC 10 amplifier (HEKA Elektronik) using Patchmaster software. Borosilicate pipettes with resistances of 1–3 MΩ were pulled on a Sutter P-97 puller (Harvard Apparatus) and fire polished using a Narishige MF–900 microforge. Extracellular solution contained 5 mM Ca\(_{\text{Cl2}}\), 125 mM TEA-Cl, 10 mM HEPES and 15 mM Mannitol, \( \text{pH} \) 7.4. Pipette solution contained 100 mM CsCl, 5 mM TEA-Cl, 3.6 mM Mg\(_{\text{Cl2}}\), 10 mM EGTA, 5 mM Mg-ATP, 0.2 mM Na-GTP and 10 mM HEPES, \( \text{pH} \) 7.4 (titration with CsOH). Currents were elicited by voltage pulses of 1–2 \( \text{mV} \) ranging from −70 to +20 mV in 10–mV increments from a holding potential of −80 mV (ref. 26). No visible calcium currents were recorded in cells transfected with the plasmids encoding the \( \text{B}_{\text{2a-MCherry}} \) and \( \text{G}_{\text{δ2}} \) subunits alone. Only recordings with distinct currents in the presence of external Ca\(^{2+}\) or Ba\(^{2+}\) that stayed stable over the recording duration were used for analysis.

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Data were filtered at 3 kHz. Peak current amplitudes depended on the voltage dependences of the open probability and the unitary current. Because calcium channels exhibit a constant unitary conductance under our experimental conditions, the voltage dependences of activation were obtained by fitting a plot of the current-voltage relation according to

\[ I(V) = G \times (V - V_{rev}) \times \frac{1}{1 + e^{-\frac{V - V_{1/2}}{k}}} \]  

with \( I \) being the macroscopic current, \( G \) the maximum conductance, \( V \) the voltage, \( V_{rev} \) the reversal potential, \( V_{1/2} \) the half-maximal activation and \( k \) the slope. Plots of the relative open probabilities of activation were calculated by dividing peak current voltage plots by the normalized maximum conductance \((I_{peak}/(V - V_{rev}))\) and were fitted with the Boltzmann function according to

\[ P_{open}(V) = \frac{1}{1 + e^{-\frac{V - V_{1/2}}{k}}} \]  

with \( P_{open} \) being the open probability at the desired voltage.

The time course of inactivation was determined by fitting the current decay after activation with a biexponential function

\[ I(t) = A_0 + A_1 \times e^{-\frac{t}{\tau_1}} + A_2 \times e^{-\frac{t}{\tau_2}} \]  

with \( A \) being the amplitude of the individual components, \( t \) the time and \( \tau \) values the corresponding time constants. The residual amplitude \( A_0 \) corresponds to the steady-state current at the given potential. The voltage dependence of the ratio of \( A_0 \) by \( I_{peak} \) provides the steady-state inactivation curve that was fitted with a Boltzmann function.

Data were analyzed in FitMaster (HEKA Elektronik) and SigmaPlot (Jandel Scientific).

**Orthologs and paralogs.** Proteins encoded by orthologs or close paralogs of CACNA1D in vertebrate and invertebrate species were identified by a BLAST search. GenBank accessions for these included NP_001122311.1 (human isoform c), NP_000711.1 (human isoform a), NP_001077085.1 (mouse), NP_990365.1 (chicken), XP_002938148.2 (frog), NP_982351.1 (zebrafish) and XP_002123864.2 (tunicate). For human \( \alpha_1 \) subunit paralogs, the GenBank accessions for the encoded proteins included NP_000060.2 (CACNA1S), NP_95630.2 (CACNA1C), NP_001734.2 (CACNA1F), NP_000059.3 (CACNA1A), NP_000709.1 (CACNA1B), NP_00119222.1 (CACNA1E), NP_061496.2 (CACNA1G), NP_066921.2 (CACNA1H) and NP_066919.2 (CACNA1I).

**Adrenal cortical gene expression.** Data on calcium channel expression in human adrenal cortex were extracted from a previously described data set.

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