Supporting Information

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SI Materials and Methods

Cell Culture and Heterologous Expression. HEK-293T cells were grown in a DMEM (DMEM/F-12; Invitrogen) containing 10% (vol/vol) FBS and maintained under standard conditions at 37 °C in a humidified atmosphere containing 5% CO_2. Cells were transfected using the jetPEI Transfection Reagent (Polyplus-Transfection Inc.) with either WT or mutant Cav3.2 channel. All of the constructs carried an HA epitope on the extracellular SS1-SS2 loop of domain I (1), an epitope useful for their detection and immunoprecipitation. Cells for electrophysiology experiments were cotransfected with EGFP-expressing vector to allow the identification of expressing cells. Cells were used 48–72 h after transfection.

Immunoprecipitation of Cav3.2 Channels. Rat brain tissue (four brains per immunoprecipitation excluding the cerebellum) was homogenized in 10% (wt/vol) lysis buffer (125 mM NaCl, 1% Nonidet P-40, 0.1% SDS, 50 mM Tris, pH 7.5 including a protease inhibitor mixture; Roche) and phosphatase inhibitors (50 mM sodium fluoride, 1 mM sodium orthovanadate, 5 mM sodium pyrophosphate, 25 mM β-glycerophosphate, 2 mM EDTA, 2 mM EGTA). The homogenate was centrifuged at 1,000 × *g* for 10 min, and the supernatant was collected and centrifuged again at 13,000 × *g* for 30 min at 4 °C. All of the resulting supernatant was further incubated with an antibody directed against a peptide in the C-terminal part of the protein Cav3.2 (100 μg per immunoprecipitation; sc-16263; Santa Cruz) for 3 h at 4 °C under gentle agitation. Protein G magnetic beads (Dynabeads Protein G; Life Technologies) were then added (1/100 v/v) followed by gentle agitation at 4 °C. After 1 h, the beads were recovered and washed three times with 1 mL cold lysis buffer. Elution was done by the addition of 140 μL NuPAGE LDS Sample Buffer (Life Technologies) supplemented with 6% (vol/vol) SDS and 10 mM DTT.

For immunoprecipition of the Cav3.2 channels from transfected HEK293T cells, the cells (five 100-mm confluent cell culture plates per immunoprecipitation) were lysed using the lysis buffer described above. After 30–60 min incubation on a rotation wheel, the lysate was centrifuged for 20 min at 16,000 × *g* at 4 °C. All of the recovered supernatant was further incubated with 100 μL anti-HA agarose beads (reference A2095; Sigma). After 3 h at 4 °C on a rotation wheel, the beads were packed in a column, washed with 10 mL cold lysis buffer, and eluted as above.

In-Gel Digestion. After alkylation for 30 min with 50 mM iodoacetamide, all of the eluted proteins obtained from the step described above were separated by SDS/PAGE and visualized by staining with Protein Staining Solution (Euromedex). The band corresponding to Cav3.2 was excised, diced into small pieces, and destained with 50% (vol/vol) acetonitrile in 50 mM ammonium bicarbonate. The gel band was further dehydrated by the addition of 100% acetonitrile and dried in a speed vacuum concentrator. Dried gel pieces were swollen with 25 mM ammonium bicarbonate containing 1 μg trypsin or chymotrypsin (ThermoScientific) and 20% (vol/vol) acetonitrile and incubated at 30 °C for 16 h. Digested peptide mixtures were extracted with 50% acetonitrile and then, two times with a solution containing 50% acetonitrile and 2.5% (vol/vol) formic acid. The extracted peptides were dried in a speed vacuum concentrator and stored at −20 °C until liquid chromatography–mass spectrometry (MS)/MS.

MS. The stored peptides were resuspended in 0.1% formic acid and analyzed using an Ultimate 3000 HPLC (ThermoScientific) directly coupled to an LTQ Orbitrap (XL, VELOS, or ELITE Pro; ThermoScientific). Desalting and preconcentration of samples were performed online on a Pepmap Precolumn (0.3 × 10 mm; ThermoScientific). A gradient consisting of 2–40% buffer B (3–33 min), 40–80% buffer B (33–34 min), and 80–0% buffer B (49–50 min) equilibrated for 20 min in 0% B (50–70 min) was used to elute peptides at 300 nL/min from a Acclaim Pepmap100 C18 Capillary (0.075 × 150-mm) Reverse-Phase Column (ThermoScientific). Buffer A consisted of 0.1% formic acid in water, and buffer B consisted of 100% acetonitrile with 0.1% formic acid. Mass spectra were acquired using a top 10 collision-induced dissociation data-dependent acquisition method. The LTQ-Orbitrap was programmed to perform a Fourier transform (FT) full scan (60,000 resolution) on a 400- to 1,400-Th mass range with the top 10 ions from each scan selected for LTQ-MS/MS with multistage activation on the neutral losses of 24.49, 32.66, and 48.99 Th. FT spectra were internally calibrated using a single lock mass (445.120024 Th). Target ion numbers were 500,000 for FT full scan on the Orbitrap and 10,000 MSn on the LTQ. Top six per 30-Da windows peak lists were extracted using MSconvert 3.0 and searched with Mascot 2.4 against the Swissprot Database for rat or Complete Proteome Set Database for human (68,511 entries for the human database and 27,815 entries for the rat database) with 7-ppm precursor mass tolerance, 0.6-Da fragment mass tolerance, and semitrypsin or chymotrypsin digestion. Carbamidomethylation of cysteines was set as static modification, and oxidation of methionine and phosphorylation of Ser, Thr, and Tyr were set as variable modifications. MS/MS spectra matching phosphorylated peptides with ion score over 15 were inspected manually and/or by using Prophossi software (2).

Molecular Biology. Mutagenesis of hCav3.2-pcDNA3.1 was performed using the QuikChange II XL Site-Directed Mutagenesis Kit (Agilent). The construction of the N-ter Ala mutant bearing five mutated serine residues (S29A, S32A, S49A, S51A, and S53A) was done in two steps: (*i*) generation of the triple mutant S49A, S51A, and S53A using the following primers: HS49AS51AS53A forward: 5′-ccgagctcggcgtggcacccgccgaggccccggcggcc-3′; hs49as51as53a reverse: 5′-cggccgccggggcctcggcgggtgccacgccgagctcgg-3′; and (*ii*) mutation of the construct generated in the first step on S29A and S32A using primers HS29AS32A forward: 5′-gcgttggtgggggcggccccggaggcccccggggcgc-3′ and HS29AS32A reverse: 5′-gcgccccgggggcctccggggccgcccccaccaacgc-3′. The LI-IIA Ala mutant, bearing seven mutated serine and threonine residues (S442A, S4445A, T446A, S532A, S535A, S558A, and S561A) was constructed in three steps: (*i*) mutation of S442A, S4445A, T446A using primers HS442AS445AT446A forward: 5′-gcagcgggcacgcgccacctggccaacgacgccgcgctggccagcttc-3′ and HS442AS445AT446A reverse: 5′-gaagctggccagcgcggcgtcgttggccaggtggcgtgcccgctgc-3′; (*ii*) mutation of the construct generated in *i* on S558A and S561A using primers S558S561 forward: ggcgcgcccccgcgccacctgccccaggccgcggacc and S558S561 reverse: ggtccgcggcctggggcaggtggcgcgggggcgcgcc; and (*iii*) mutation of the construct generated in *ii* on S532A and S535A using primers HS532AS535A forward: 5′-ccactaccatttcgcccatggcgccccccgcaggccc-3′ and HS532A-S535A reverse: 5′-gggcctgcggggggcgccatgggcgaaatggtagtgg-3′. For the LI-IIB Ala mutant (having eight phosphosites mutated: S650A, S653A, S687A, S715A, S717A, S749A, T751A, and S758A), a synthetic cDNA (660 bp) was generated (Eurofins) corresponding to the regions coding for part B of the loop I-II with the mutated phosphosites. The sequence was as follows: 5′-tccgacacggacgaggacaaggcggcggtccacttcgaggaggacttccacaagctcagagaactccagaccacagagctgaagatgtgttccctggccgtggcccccaacgggcacctggagggacgaggcgccctggcccctcccctcatcatgtgcacagctgccacgcccatgcctacccccaaggccgcaccattcctggat-

gcagcccccgccctcccagacgctcggcgtggcgccagcagctccggggacccgccactg-ggagaccagaagcctccggccagcctccgaagtgctccctgtgccccctggggccccagtgg-cgcctggagcgcccggcgctccagctggagcagcctgggccgtgcccccagcctcaagcgcc-gcggccagtgtggggaacgtgagtccctgctgtctggcgagggcaagggcgccgccgacgac-gaagctgaggacggcagggccgcgcccgggccccgtgccaccccactgcggcgggccgagg-ccctggacccacggcccctgcggccggccgccctcccgcctaccaagtgccgcgatcgcga-cgggcaggtggtggccctgcccagcgacttcttcctgcgcatcgacagccaccgtgaggatgc-agccgagcttgacgacgacgcg-3′. This sequence was further amplified by PCR, purified on an agarose gel, and then, used as a primer for a mutagenesis reaction.

The same procedure was used to construct the LII-III Ala mutant (having 16 phosphosites mutated: T1034A, S1035A, T1061A, S1071A, S1073A, S1091A, S1090A, S1099A, S1103A, S1107A, S1127A, S1144A, S1174A, S1175A, S1198A, and S1246A). The synthetic cDNA used (Eurofins) was 5′-agccactgctgctgccagcctcaggctggccacagggctgg-gcaccatgaactaccccacgatcctgccctcaggggtgggcagcggcaaaggcagcaccagcc-ccggacccaaggggaagtgggccggtggaccgccaggcaccgggggcacggcccgttggcc-ttgaacgcccctgatccctacgagaagatcccgcatgtggccggggagcatggactgggccaag-cccctggccatctgtcgggcctcagtgtgccctgccccctgcccgcccccccagcgggcacactg-acctgtgagctgaagagctgcccgtactgcacccgtgccctggaggacccggagggtgagctcg-ccggcgcggaaagtggagactcagatggccgtggcgtctatgaattcacgcaggacgtccggc-acggtgaccgctgggaccccacgcgaccaccccgtgcggcggacgcaccaggcccaggccca-ggcgcc-3′.

For the LIII-IV mutant (having three mutations S1587A, T1588A, S1591A), a mutagenesis reaction using the following primers was done: HS1587A forward: 5′-gaggaggcgcagggccgctttccccgccccagagg-cccagc-3′ and HS1587 reverse: 5′-gctgggcctctggggcggggaaagcggccc-tgcgcctcctc-3′. The C-terΔ mutant was constructed by introducing a stop codon at Q1886 using as primers Q1886Stop forward: 5′-gct-ggagatggcgtagggccccgggagtgc-3′ and Q1886Stop reverse: 5′-gcact-cccggggccctacgccatctccagc-3′.

Electrophysiology. Macroscopic currents were recorded by the whole-cell patch-clamp technique using a MultiClamp 700B Microelectrode Amplifier (Molecular Devices) at room temperature. Extracellular solutions contained 135 mM NaCl, 20 mM Tetraethylammonium Cl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, and 10 mM Hepes pH-adjusted to 7.44 with KOH. Patch pipettes were filed with an internal solution (140 mM CsCl, 10 mM EGTA, 3 mM $CaCl_2$, 10 mM Hepes, 3 mM Mg-ATP, 0.6 mM GTP pH-adjusted to 7.25 with KOH) and had a typical resistance of 2–3 MΩ. For the AP experiments, the cells were treated by adding AP (Roche) into the patch pipette (100 U/mL) and letting it dialyze into the cell for 30 min. This time point was picked in agreement with previous AP experiments reported for the Kv channel (3, 4). For data acquisition and analysis, the pClamp9 software was used (Molecular Devices). Additional analysis was done using GraphPad Prism software. Current voltage curves (I-V curves) were fitted using a combined Boltzmann and linear ohmic relationship: $I/I_{max} = G_{max} (V_m - E_{rev})/(1 + \exp^{(V1/2 - Vm)/k})$ for each individual cell. Normalized conductance–voltage curves (activation curves) were fitted with a Boltzmann equation $G/G_{max} = 1/(1 + \exp^{(V1/2 - Vm)/k})$, where G was calculated as follows: $G = I/(V_m - E_{rev})$. Similarly, the steady-state inactivation curves were fitted with $I/I_{max} = 1/(1 + \exp^{-(V1/2 - Vm)/k})$. In all of the equations, $V_{1/2}$ represents the half-activation and half-inactivation potentials, V_m is the membrane potential, E_{rev} is the inversion potential, k is the slope factor, G is the conductance, and I is the current at a given V_m; G_{max} and I_{max} are the maximum conductance and current, respectively.

NEURON Modeling. Action potential clamp experiments and neuronal firing simulations were done using the NEURON simulation environment downloaded from the database at Yale University (senselab.med.yale.edu/modeldb) (5). Action potential clamp studies were conducted using a modeled firing activity of thalamic reticular neurons originally described in the work by Destexhe et al. (6) and produced by a −180-pA current injected over 300 ms from a holding potential of −70 mV. The signals/values obtained were then converted into a pClamp stimulation file and further applied to transfected HEK293T cells as previously reported (7). For simulation experiments, the electrophysiological properties of the WT, LI-IIA1 mutant, and AP-treated WT channels were modeled using Hodgkin–Huxley equations as described by Huguenard and McCormick (8) and introduced back in the thalamic reticular neuron model (6).

Statistical Analysis. Data are presented as means ± SEMs. Statistical analyses were performed using Student's t test or one-way ANOVA combined with Tukey's posthoc analysis for multiple comparisons. $P < 0.05$ was considered statistically significant.

1. Dubel SJ, et al. (2004) Plasma membrane expression of T-type calcium channel alpha(1) subunits is modulated by high voltage-activated auxiliary subunits. *J Biol Chem* 279(28):29263–29269.
2. Martin DM, et al. (2010) Prophossi: Automating expert validation of phosphopeptide-spectrum matches from tandem mass spectrometry. *Bioinformatics* 26(17):2153–2159.
3. Murakoshi H, Shi G, Scannevin RH, Trimmer JS (1997) Phosphorylation of the Kv2.1 K+ channel alters voltage-dependent activation. *Mol Pharmacol* 52(5):821–828.
4. Park KS, Mohapatra DP, Misonou H, Trimmer JS (2006) Graded regulation of the Kv2.1 potassium channel by variable phosphorylation. *Science* 313(5789):976–979.
5. Hines ML, Carnevale NT (1997) The NEURON simulation environment. *Neural Comput* 9(6):1179–1209.
6. Destexhe A, Contreras D, Steriade M, Sejnowski TJ, Huguenard JR (1996) In vivo, in vitro, and computational analysis of dendritic calcium currents in thalamic reticular neurons. *J Neurosci* 16(1):169–185.
7. Chemin J, et al. (2002) Specific contribution of human T-type calcium channel isotypes (alpha(1G), alpha(1H) and alpha(1I)) to neuronal excitability. *J Physiol* 540(Pt 1):3–14.
8. Huguenard JR, McCormick DA (1992) Simulation of the currents involved in rhythmic oscillations in thalamic relay neurons. *J Neurophysiol* 68(4):1373–1383.

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   1 MTEGTLAADE VRVPLGASPP APAAPVRASP ASPGAPGREE QGGSGSGVLA PESPGTECGA DLGADEEQPV PYPALAATVF FCLGQTTRPR SWCLRLVCNP
 101 WFEHISMLVI MLNCVTLGMF RPCEDVECRS ERCSILEAFD DFIFAFFAVE MVIKMVALGL FGQKCYLGDT WNRLDFFIVM AGMMEYSLDG HNVSLSAIRT
 201 VRVLRPLRAI NRVPSMRILV TLLLDTLPML GNVLLLCFFV FFIFGIVGVQ LWAGLLRNRC FLDSAFVRNN NLTFLRPYYQ TEEGEENPFI CSSRRDNGMQ
 301 KCSHIPSRRE LRVQCTLGWE AYGQPQAEDG GAGRNACINW NQYYNVCRSG EFNPHNGAIN FDNIGYAWIA IFQVITLEGW VDIMYYVMDA HSFYNFIYFI
 401 LLIIVGSFFM INLCLVVIAT QFSETKQREN QLMREQRARY LSNDSTLASF SEPGSCYEEL LKYVGHIFRK VKRRSLRLYA RWQSRWRKKV DPSSTVHGQG
 501 PGRRPRRAGR RTASVHHLVY HHHHHHHHHY HFSHGGPRRP SPEPGAGDNR LVRACAPPSP PSPGHGPPDS ESVHSIYHAD CHVEGPQERA RVAHSIATAA
 601 SLKLASGLGT MNYPTILPSG TVNSKGGTSS RPKGLRGAGA PGAAVHSPLS LGSPRPYEKI QHVVGEQGLG RASSHLSGLS VPCPLPSPQA GTLTCELKSC
 701 PYCASALEDP EFEFSGSESG DSDAHGVYEF TQDVRHGDCR DPVQQPHEVG TPGHSNERRR TPLRKASQPG GIGHLWASFS GKLRRIVDSK YFNRGIMAAI
 801 LVNTLSMGVE YHEQPEELTN ALEISNIVFT SMFALEMLLK LLACGPLGYI RNPYNIFDGI VVVISVWEIV GQADGGLSVL RTFRLLRVLK LVRFLPALRR
 901 QLVVLMRTMD NVATFCMLLM LFIFIFSILG MHLFGCKFSL KTDSGDTVPD RKNFDSLLWA IVTVFQILTQ EDWNVVLYNG MASTSSWAAL YFVALMTFGN
1001 YVLFNLLVAI LVEGFQAEGD ATRSDTDEDK TSTQLEGDFD KLRDLRATEM KMYSLAVTPN GHLEGRGSLP PPLITHTAAT PMPTPKSSPN LDVAHALLDS
1101 RRSSSGSVDP QLGDQKSLAS LRSSPCTPWG PNSAGSSRRS SWNSLGRAPS LKRRSQCGER ESLLSGEGKG STDDEAEDSR PSTGTHPGAS PGPRATPLRR
1201 AESLDHRSTL DLCPPRPAAL LPTKFHDCNG QMVALPSEFF LRIDSHKEDA AEFDDDIEDS CCFRLHKVLE PYAPQWCRSR ESWALYLFPP QNRLRVSCQK
1301 VIAHKMFDHV VLVFIFLNCI TIALERPDID PGSTERAFLS VSNYIFTAIF VVEMMVKVVA LGLLWGEHAY LQSSWNVLDG LLVLVSLVDI IVAMASAGGA
1401 KILGVLRVLR LLRTLRPLRV ISRAPGLKLV VETLISSLRP IGNIVLICCA FFIIFGILGV QLFKGKFYYC EGTDTRNITT KAECHAAHYR WVRRKYNFDN
1501 LGQALMSLFV LSSKDGWVNI MYDGLDAVGI DQQPVQNHNP WMLLYFISFL LIVSFFVLNM FVGVVVENFH KCRQHQEAEE ARRREEKRLR RLERRRRKAQ
1601 RRPYYADYSH TRRSIHSLCT SHYLDLFITF IICLNVITMS MEHYNQPKSL DEALKYCNYV FTIVFVFEAA LKLVAFGFRR FFKDRWNQLD LAIVLLSIMG
1701 IALEEIEMNA ALPINPTIIR IMRVLRIARV LKLLKMATGM RALLDTVVQA LPQVGNLGLL FMLLFFIYAA LGVELFGRLE CSEDNPCEGL SRHATFTNFG
1801 MAFLTLFRVS TGDNWNGIMK DTLRECTRED KHCLSYLPAL SPVYFVTFVL VAQFVLVNVV VAVLMKHLEE SNKEAREDAE MDAEIELEMA QGSTAQPPPT
1901 AQESQGTQPD TPNLLVVRKV SVSRMLSLPN DSYMFRPVAP AAAPHSHPLQ EVEMETYTGP VTSAHSPPLE PRASFQVPSA ASSPARVSDP LCALSPRGTP
2001 RSLSLSRILC RQEAMHSESL EGKVDDVGGD SIPDYTEPAE NMSTSQASTG APRSPPCSPR PASVRTRKHT FGQRCISSRP PTLGGDEAEA ADPADEEVSH
2101 ITSSAHPWPA TEPHSPEASP TASPVKGTMG SGRDPRRFCS VDAQSFLDKP GRPDAQRWSS VELDNGESHL ESGEVRGRAS ELEPALGSRR KKKMSPPCIS
2201 IEPPTEDEGS SRPPAAEGGN TTLRRRTPSC EAALHRDCPE PTEGPGTGGD PVAKGERWGQ ASCRAEHLTV PNFAFEPLDM GGPGGDCFLD SDQSVTPEPR
2301 VSSLGAIVPL ILETELSMPS GDCPEKEQGL YLTVPQTPLK KPGSTPATPA
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   1 MTEGARAADE VRVPLGAPPP GPAALVGASP ESPGAPGREA ERGSELGVSP SESPAAERGA ELGADEEQRV PYPALAATVF FCLGQTTRPR SWCLRLVCNP
 101 WFEHVSMLVI MLNCVTLGMF RPCEDVECGS ERCNILEAFD AFIFAFFAVE MVIKMVALGL FGQKCYLGDT WNRLDFFIVV AGMMEYSLDG HNVSLSAIRT
 201 VRVLRPLRAI NRVPSMRILV TLLLDTLPML GNVLLLCFFV FFIFGIVGVQ LWAGLLRNRC FLDSAFVRNN NLTFLRPYYQ TEEGEENPFI CSSRRDNGMQ
 301 KCSHIPGRRE LRMPCTLGWE AYTQPQAEGV GAARNACINW NQYYNVCRSG DSNPHNGAIN FDNIGYAWIA IFQVITLEGW VDIMYYVMDA HSFYNFIYFI
 401 LLIIVGSFFM INLCLVVIAT QFSETKQRES QLMREQRARH LSNDSTLASF SEPGSCYEEL LKYVGHIFRK VKRRSLRLYA RWQSRWRKKV DPSAVQGQGP
 501 GHRQRRAGRH TASVHHLVYH HHHHHHHHYH FSHGSPRRPG PEPGACDTRL VRAGAPPSPP SPGRGPPDAE SVHSIYHADC HIEGPQERAR VAHAAATAAA
 601 SLRLATGLGT MNYPTILPSG VGSGKGSTSP GPKGKWAGGP PGTGGHGPLS LNSPDPYEKI PHVVGEHGLG QAPGHLSGLS VPCPLPSPPA GTLTCELKSC
 701 PYCTRALEDP EGELSGSESG DSDGRGVYEF TQDVRHGDRW DPTRPPRATD TPGPGPGSPQ RRAQQRAAPG EPGWMGRLWV TFSGKLRRIV DSKYFSRGIM
 801 MAILVNTLSM GVEYHEQPEE LTNALEISNI VFTSMFALEM LLKLLACGPL GYIRNPYNIF DGIIVVISVW EIVGQADGGL SVLRTFRLLR VLKLVRFLPA
 901 LRRQLVVLVK TMDNVATFCT LLMLFIFIFS ILGMHLFGCK FSLKTDTGDT VPDRKNFDSL LWAIVTVFQI LTQEDWNVVL YNGMASTSSW AALYFVALMT
1001 FGNYVLFNLL VAILVEGFQA EGDANRSDTD EDKTSVHFEE DFHKLRELQT TELKMCSLAV TPNGHLEGRG SLSPPLIMCT AATPMPTPKS SPFLDAAPSL
1101 PDSRRGSSSS GDPPLGDQKP PASLRSSPCA PWGPSGAWSS RRSSWSSLGR APSLKRRGQC GERESLLSGE GKGSTDDEAE DGRAAPGPRA TPLRRAESLD
1201 PRPLRPAALP PTKCRDRDGQ VVALPSDFFL RIDSHREDAA ELDDDSEDSC CLRLHKVLEP YKPQWCRSRE AWALYLFSPQ NRFRVSCQKV ITHKMFDHVV
1301 LVFIFLNCVT IALERPDIDP GSTERVFLSV SNYIFTAIFV AEMMVKVVAL GLLSGEHAYL QSSWNLLDGL LVLVSLVDIV VAMASAGGAK ILGVLRVLRL
1401 LRTLRPLRVI SRAPGLKLVV ETLISSLRPI GNIVLICCAF FIIFGILGVQ LFKGKFYYCE GPDTRNISTK AQCRAAHYRW VRRKYNFDNL GQALMSLFVL
1501 SSKDGWVNIM YDGLDAVGVD QQPVQNHNPW MLLYFISFLL IVSFFVLNMF VGVVVENFHK CRQHQEAEEA RRREEKRLRR LERRRRSTFP SPEAQRRPYY
1601 ADYSPTRRSI HSLCTSHYLD LFITFIICVN VITMSMEHYN QPKSLDEALK YCNYVFTIVF VFEAALKLVA FGFRRFFKDR WNQLDLAIVL LSLMGITLEE
1701 IEMSAALPIN PTIIRIMRVL RIARVLKLLK MATGMRALLD TVVQALPQVG NLGLLFMLLF FIYAALGVEL FGRLECSEDN PCEGLSRHAT FSNFGMAFLT
1801 LFRVSTGDNW NGIMKDTLRE CSREDKHCLS YLPALSPVYF VTFVLVAQFV LVNVVVAVLM KHLEESNKEA REDAELDAEI ELEMAQGPGS ARRVDADRPP
1901 LPQESPGARD APNLVARKVS VSRMLSLPND SYMFRPVVPA SAPHPRPLQE VEMETYGAGT PLGSVASVHS PPAESCASLQ IPLAVSSPAR SGEPLHALSP
2001 RGTARSPSLS RLLCRQEAVH TDSLEGKIDS PRDTLDPAEP GEKTPVRPVT QGGSLQSPPR SPRPASVRTR KHTFGQRCVS SRPAAPGGEE AEASDPADEE
2101 VSHITSSACP WQPTAEPHGP EASPVAGGER DLRRLYSVDA QGFLDKPGRA DEQWRPSAEL GSGEPGEAKA WGPEAEPALG ARRKKMSPP CISVEPPAED
2201 EGSARPSAAE GGSTTLRRRT PSCEATPHRD SLEPTEGSGA GGDPAAKGER WGQASCRAEH LTVPSFAFEP LDLGVPSGDP FLDGSHSVTP ESRASSSGAI
2301 VPLEPPESEP PMPVGDPPEK RRGLYLTVPQ CPLEKPGSPS ATPAPGGGAD DPV
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Fig. S1. Liquid chromatography–MS/MS sequence coverage of the Cav3.2 protein. (*A*) The amino acid sequence of the canonical rat Cav3.2 isoform (Swiss-Prot G3V9C6) immunopurified from rat brain. (*B*) The amino acid sequence of the canonical human Cav3.2 isoform (Swiss-Prot O95180) immunopurified from HEK293T cells. Peptide coverage obtained is highlighted in yellow. Underlined sequences represent the proposed large intracellular loops.

Fig. S2. Representative MS/MS spectra of the rat Cav3.2 peptide containing S442, S445, and T446. The spectrum of a doubly charged, monophosphorylated Cav3.2 peptide at *m/z* 1,345.590698 (Mascot score 55) was fragmented to produce a tandem mass spectrum with y- and b-ion series (red on the spectrum) that described the sequence YLS442NDS445T446LASFSEPGSCYEELLK (amino acids 440–462). The spectrum is hybrid, with b and y ions indicating the coelution of two monophosphorylated peptides: one phosphorylated at S442 and the other one phosphorylated at S445 or T446. The b5 (*m/z* 575.1998+) and b5-98 (*m/z* 673.1912+) ions (green) indicate phosphorylation at S442, and the y20 (*m/z* 1,163.4553++) and y20-98 (*m/z* 1,114.4895++; blue) ions indicate phosphorylation at S445 or T446. *Inset* shows a zoom on the low *m/z* values (from 500 to 1,200).

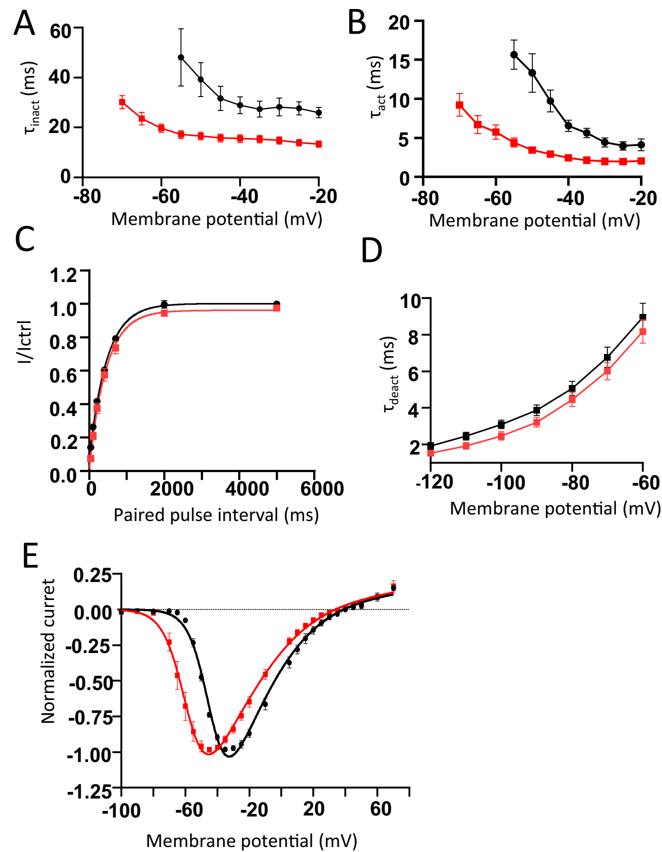

Fig. S3. AP effect on the biophysical properties of Cav3.2 channels. HEK293T cells expressing human Cav3.2 were treated with AP by adding AP into the patch pipette (100 U/mL) and letting it dialyze into the cell for 30 min. (*A*) Inactivation time constants from traces shown in Fig. 2*C*. (*B*) Activation time constants of activation from traces shown in Fig. 2*C*. (*C*) Recovery from inactivation: recordings were done using two 100-ms test pulses at −30 mV (holding potential was −110 mV) separated by increasing interpulse durations. (*D*) Deactivation kinetics: after a 7-ms test pulse at −30 mV (holding potential was −110 mV), tail currents were measured at different membrane potentials (−120, −110, −100, −90, −80, −70, and −60 mV) and fitted with a monoexponential function. (*E*) I-V curves: the currents were elicited from a holding potential of −100 mV to different test pulse potentials (−100, −90, −80, −70, −65, −60, −55, −50, −45, −40, −35, −30, −25, −20, −10, 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 60, and 70 mV). The reversal potential was 39.78 ± 2.73 mV for the non–AP-treated cells and 34.2 ± 1.91 mV for AP-treated cells (Student's *t* test; $P > 0.1$; nonsignificant). The non–AP-treated cells are represented with black circles (n = 3–5), and the AP-treated cells are represented with red squares (n = 4–11).

Fig. S4. Effects of alanine mutations of the phosphorylation loci S442, S445, and T446 on the Cav3.2 channel expressed in NG-108–15 cells. (*A*) Steady-state inactivation curve of the WT (black circles; $V_{1/2}$ = −69.77 ± 1.676 mV; n = 8) and LI-IIA1 mutant (red squares; $V_{1/2}$ = −81.75 ± 2.04 mV; n = 9; P < 0.001). (*B*) Steady-state activation curve of the WT (black circles; $V_{1/2}$ = −44.91 ± 1.15 mV; n = 7) and LI-IIA1 mutant (red squares; $V_{1/2}$ = −56.65 ± 1.93 mV; n = 9; P < 0.001). (*C* and *D*) Inactivation and activation time constants, respectively, for the WT (black circles) and the LI-IIA1 mutant (red squares).

Table S1. Summary and comparison of the phosphorylation sites identified in vivo (rat brain) and in vitro (human Cav3.2 expressed in HEK293T cells)

In vivo (brain tissue): Rat Isoform		In vitro (HEK293T): Human isoform		
Phosphosite(s)	Peptide sequence	Phosphosite(s)	Peptide sequence	Remark
S18-p*	VPLGAsPPAPAAPVR(41)	P18	VPLGAPPPGPAALVG	Not conserved in human
S29-p*	VPLGASPPAPAAPVRAsPASPGAPGR(23)	S29-p*	VGAsPESPGAPGR(22)	
S32-p*	VPLGASPPAPAAPVRASPAsPGAPGR(23)	S32-p*	VGASPEsPGAPGR(46)	
L49	GSGSGVLAPESPGTEC	S49-p*	GSELGVsPSESPAAER(62)	Not conserved in rat
P51	GSGSGVLAPESPGTEC	S51-p +S53-p (diP)*	GSELGVSPsEsPAAER(43)	Not conserved in rat
S53	GSGSGVLAPESPGTEC	S53-p*	GSELGVSPSEsPAAER(72)	Noncovered region in rat
S442-p*	YLsNDSTLASFSEPGSCYEELLK(55)	S442/S445/T446-p*	ARHLsNDstLASFSEPGSCY-EELLK(49)	
S445/T446-p*	YLSNDstLASFSEPGSCYEELLK(55)	S442/S445/T446-p*	ARHLsNDstLASFSEPGSCY-EELLK(49)	S445 (1); T446 (2)
S442-p and S445/T446-p (diP)*	YLsNDstLASFSEPGSCYEELLK(23)	S442/S445/T446-p*	ARHLsNDstLASFSEPGSCY-EELLK(49)	
S533-p*	sHGGPRRPSPEPGAGDNRL(22)	S532/S535-p*	sHGsPRRPGPEPGACDTRL(21)	
S541-p*	SHGGPRRPsPEPGAGDNRL(37)	E542	sHGsPRRPGPEPGACDTRL	Not conserved in human (3)
S559/S562-p*	ACAPPsPPsPGHGPPDSESVHSIYHADC-HVEGPQER(19)	S558-p +S561-p (diP)*	AGAPPsPPsPGR(21)	
S601-p*	VAHSIATAAsLK(28)	S601	VAHAAATAAASLR	
S647-p + S650-p + S653-p (triP)*	GAGAPGAAVHsPLsLGsPRPYEK(34)	S650/S653-p*	WAGGPPGTGGHGPLsLNsP-DPYEK(25)	
S687-p*	ASSHLSGLSVPCPLPsPQAGTLTCELK(57)	S687-p*	SVPCPLPsPPAGTL(20)	
S715	ALEDPEFEFSG	S715-p*	ALEDPEGELsG(43)	Noncovered region in rat
S715, S717	ALEDPEFEFSGSESGDSDAH	S715-p+S717-p (diP)*	ALEDPEGELsGsESGDSDGR(69)	Noncovered region in rat
T751-p*	HGDCRDPVQQPHEVGtPGHSNE(40)	T749/T751+S758 (diP)*	AtDtPGPGPGsPQR(33)	T749 (4)
S755-p*	HGDCRDPVQQPHEVGTPGHsNER(40)	T749/T751+S758 (diP)*	AtDtPGPGPGsPQR(33)	
T751-p+S755-p (diP)*	HGDCRDPVQQPHEVGtPGHsNER(19)	T749/T751+S758 (diP)*	AtDtPGPGPGsPQR(33)	
S767-p*	KAsQPGGIGHLWASFSGK(40)	G770	APGEPGWMGRLWVTFSGK	Not conserved in human
T1031/S1032/T1033-p*	SDTDEDKtstQLEGDFDKLR(65)	T1034/S1035-p*	SDTDEDKtsVHFEEDFHK(27)	
T1058	AVTPNGHLEGR	T1061-p*	AVtPNGHLEGR(16)	
S1068	GSLPPPLITHTAATPMPTPK	S1071-p*	GsLSPPLIMCTAATPMPTPK(35)	
P1070	GSLPPPLITHTAATPMPTPK	S1073-p*	GSLsPPLIMCTAATPMPTPK(56)	
S1087/S1088-p*	ssPNLDVAHALLDSR(34)	S1090/S1091-p*	ssPFLDAAPSLPDSR(42)	
A1096	SSPNLDVAHALLDSR	S1099-p*	SSPFLDAAPsLPDSR(38)	
S1100	LDVAHALLDSR	S1103-p*	LDAAPSLPDsR(56)	
S1104-p*	RSsSGSVDPQLGDQK(73)	S1107-p*	RGsSSSGDPPLGDQKPPASLR(66)	5
S1124	RSSPCTPW	S1127-p*	RSsPCAPW(21)	
S1144-p*	SSWNsLGR(34)	S1144-p*	RSsWSSLGR(44)	
S1171-p + T1172-p (diP)*	SQCGERESLLSGEGKGstDDEAEDSRPST-GTHPGASPGPR(36)	S1174-p+S1175-p (diP)*	ESLLSGEGKGstDDEAEDGR(21)	
S1203-p*	RRAEsLDHRSTL(23)	S1198-p*	RRAEsLDPRPL(34)	6
I1257	EDAAEFDDDIEDSCCFR	S1246-p*	EDAAELDDDsEDSCCLR(80)	
—	—	S1587-p*	RRsTFPSPEAQR(47)	
—	—	T1588-p*	RStFPSPEAQR(26)	
—	—	S1591-p*	STFPsPEAQR(37)	
—	—	S1905-p*	RVDADRPPLPQEsPGAR(41)	
S1927-p*	MLsLPNDSYMFR(45)	S1926-p*	MLsLPNDSYMFR(61)	
S1966-p*	TGPVTSAHsPPLEPR(43)	S1970-p*	GSVASVHsPPAESCASL(60)	
S1983-p*	ASFQVPSAASsPAR(53)	S1987-p*	AVSsPARSGEPL(42)	
S1995-p*	VSDPLCALsPR(66)	S1999-p*	SGEPLHALsPR(31)	
S2054-p*	sPPCSPRPASVR(17)	S2057-p*	TPVRPVTQGGSLQsPPR(62)	
S2119-p*	CISSRPPTLGGDEAEAADPADEEVSHITS-SAHPWPATEPHSPEAsPTASPVK(24)	S2123-p*	QPTAEPHGPEAsPVAGGE(36)	
S2140-p*	FCsVDAQSFLDKPGRPDAQR(64)	S2137	LYSVDAQGFLDKPGRADEQW	

Table S1. Cont.

In vivo (brain tissue): Rat Isoform		In vitro (HEK293T): Human isoform		
Phosphosite(s)	Peptide sequence	Phosphosite(s)	Peptide sequence	Remark
S2159/S2160-p*	WssVELDNGESHLESGEVR(82)	S2156	WRPSAELGSGEPGEAK	
S2195-p*	KMsPPCISIEPPTEDEGSSRPPAAEGGNTTLR(57)	S2188-p*	KMsPPCISVEPPAEDEGSARPSA-AEGGSTTLR(58)	1, 7
S2200-p*	KMSPPCIsIEPPTEDEGSSRPPAAEGGNTTLR(38)	S2193-p*	KMSPPCIsVEPPAEDEGSARPSA-AEGGSTTLR(27)	
T2227/S2229	TPSCEAALHRDCPEPTEGPGTGGDPVAK	T2220/S2222-p*	tPsCEATPHRDSLEPTEGSGAGG-DPAAK(31)	
C2238	RTPSCEAALHRDCPEPTEGPGTGGDPVAK	S2231-p*	RTPSCEATPHRDsLEPTEGSGAG-GDPAAK(26)	Not conserved in rat
S2354-p*	KPGSTPATPAPDDsGDEPV(55)	G2348	KPGSPSATPAPGGGADDPV	Not conserved in human (1, 3)

For the ambiguous sites, the serine or threonine is underlined. The peptide sequence corresponds to the peptide on which the serine/threonine residues were found phosphorylated by MS. The mascot identification score is given in parentheses after each peptide. Except for the peptides marked with diphosphorylated peptide (diP) or triphosphorylated peptide (triP), all of the peptides were monophosphorylated. When the phosphorylated serine/threonine residue in the rat isoform has no correspondence to a phosphorylated serine/threonine residue in the human isoform or vice versa, the corresponding amino acid and amino acid sequences are given.

*The identified phosphosites are indicated with -p in the Phosphosite columns and lowercase letters in the Peptide sequence columns.

1. Huttlin EL, et al. (2010) A tissue-specific atlas of mouse protein phosphorylation and expression. *Cell* 143(7):1174–1189.
2. (2007) *CST Curation Set: 3170; Year: 2007; Biosample/Treatment: Cell Line, Jurkat/Pervanadate & Calyculin; Disease: T cell Leukemia; SILAC: -; Specificity of Antibody Used to Purify Peptides Prior to MS2: Anti-pTyr Antibody Used to Purify Peptides Prior to MS2: Phospho-Tyrosine Mouse mAb (P-Tyr-100) Cat#: 9411, PTMScan(R) Phospho-Tyr Motif (Y*) Immunoaffinity Beads Cat#: 1991*. Available at www.phosphosite.org.
3. Trinidad JC, et al. (2012) Global identification and characterization of both O-GlcNAcylation and phosphorylation at the murine synapse. *Mol Cell Proteomics* 11(8):215–229.
4. (2008) *CST Curation Set: 5418; Year: 2008; Biosample/Treatment: Cell Line, EGI-1/Untreated; Disease: Bile-Duct Cancer; SILAC: -; Specificity of Antibody Used to Purify Peptides Prior to MS2: Anti-pTyr Antibody Used to Purify Peptides Prior to MS2: Phospho-Tyrosine Mouse mAb (P-Tyr-100) Cat#: 9411, PTMScan(R) Phospho-Tyr Motif (Y*) Immunoaffinity Beads Cat#: 1991*. Available at www.phosphosite.org.
5. Hu C, Depuy SD, Yao J, McIntire WE, Barrett PQ (2009) Protein kinase A activity controls the regulation of T-type CaV3.2 channels by Gbetagamma dimers. *J Biol Chem* 284(12):7465–7473.
6. Yao J, et al. (2006) Molecular basis for the modulation of native T-type Ca2+ channels in vivo by Ca2+/calmodulin-dependent protein kinase II. *J Clin Invest* 116(9):2403–2412.
7. (2007) *CST Curation Set: 2262; Year: 2007; Biosample/Treatment: Cell Line, M059K/UV; Disease: Glioblastoma; SILAC: -; Specificity of Antibody Used to Purify Peptides Prior to MS2: Anti-(s/t)Q(phosphorylation) Antibody Used to Purify Peptides Prior to MS2: Phospho-(Ser/Thr) ATM/ATR Substrate Antibody Cat#: 2851*. Available at www.phosphosite.org.

Other Supporting Information Files

Dataset S1 (PDF)