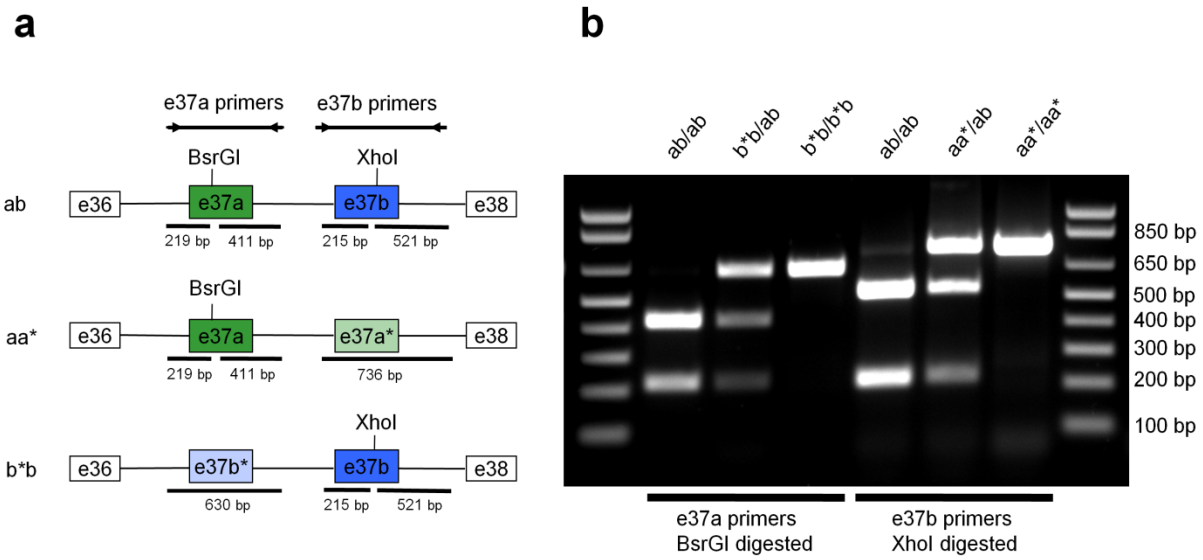
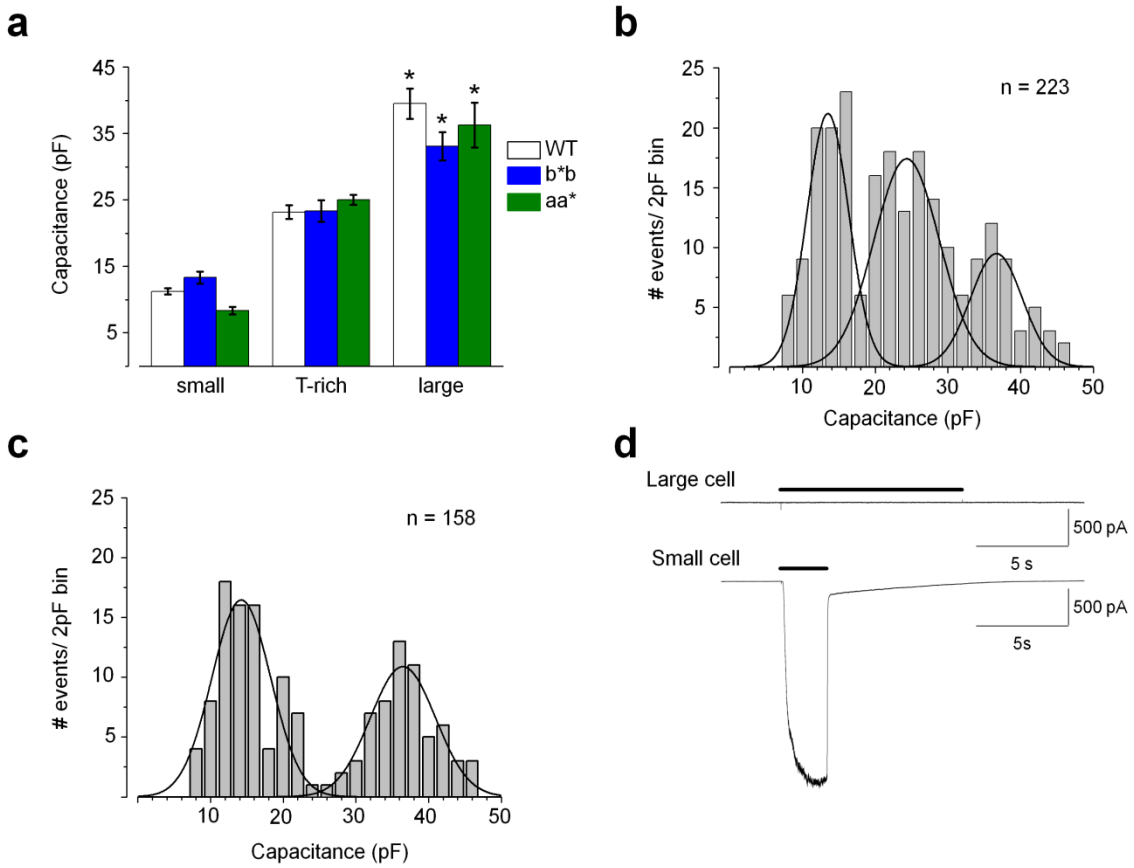
Supplementary figures



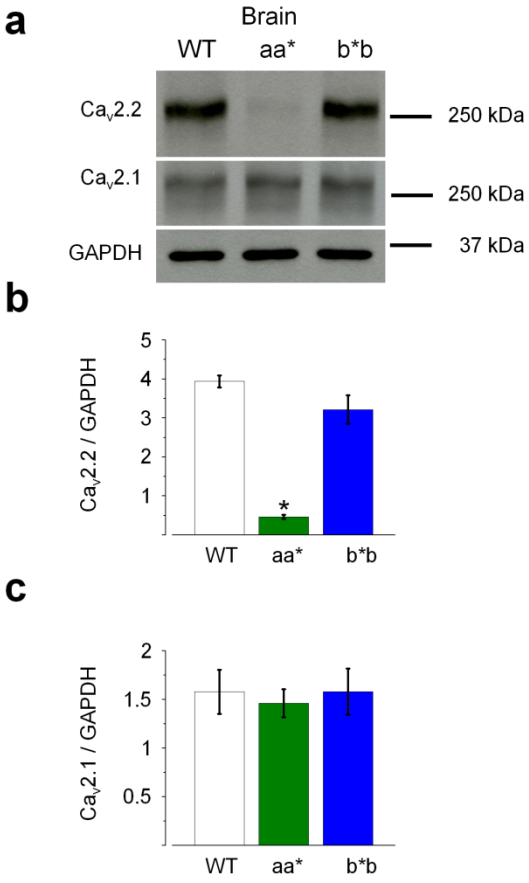
Supplementary Figure 1. Genotyping mouse strains. (**a**) Schematic representation of genotyping data obtained from genomic DNA of *Cacna1b* WT, aa* and b*b heterozygous and homozygous mice. To identify which exon (e37a or e37b*) was present in the position normally occupied by e37a, a 630 bp product was PCR amplified with e37a intronic primers that anneal upstream and downstream of the normal e37a position. Digestion of the product with BsrGI identified the exon present in the "a" position: e37a digests into two bands (219 and 411 bp), and e37b* does not digest (630 bp). Possible genotyping results for the "a" region (BsrGI digested) are: WT (ab/ab) = 219, 411 bp; heterozygous (b*b/ab) = 219, 411, 630 bp; homozygous (b*b/b*b) = 630 bp. To identify the exon (e37b or e37a*) present in the position normally occupied by e37b, a 736 bp product was PCR amplified with e37b intronic primers that anneal upstream and downstream of the normal e37b position, resulting in a 736 bp product. Digestion with XhoI identified the exon present in the "b" position: e37b digests into two bands (215 and 521 bp) and e37a* does not digest (736 bp). Possible genotyping results for the "b" region (XhoI digested) are: WT (ab/ab) = 215, 521 bp; heterozygous (aa*/ab) = 215, 521, 736 bp; homozygous (aa*/aa*) = 736 bp. (b) Representative genotyping of *Cacna1b* WT, aa* and b*b heterozygous and homozygous mice. To genotype the e37a region, mouse genomic DNA was PCR amplified using Taq Polymerase (New England Biolabs), 35 cycles, 60°C annealing, 1 min extension at 72°C with primers SD62for 5'-ATGGACGGGGTGCAACATGG (anneals to the intron upstream of the normal e37a position) and SD130rev 5'- GCATCCAGGATGCTGGAACG (anneals to the intron downstream of the normal e37a position). The resulting 630 bp product was digested with BsrGI, which only cuts e37a (219 and 411 bp). To genotype the e37b region,

DNA was PCR amplified (as above) with primers SD132for 5'- CTGCTGCTGTGGTTCTCAGC (anneals to the intron upstream of the normal e37b position) and SD48rev 5'- CTGGGATGAGAGCAAAGGGT (anneals to the intron downstream of the normal e37b position). The resulting 736 bp product was digested with XhoI, which only cuts e37b (215 and 521 bp). Mouse genotypes are shown above the lanes. PCR primers and diagnostic restriction enzyme digestion shown below the lanes. Flanking lanes, 1 Kb Plus DNA ladder (Invitrogen).



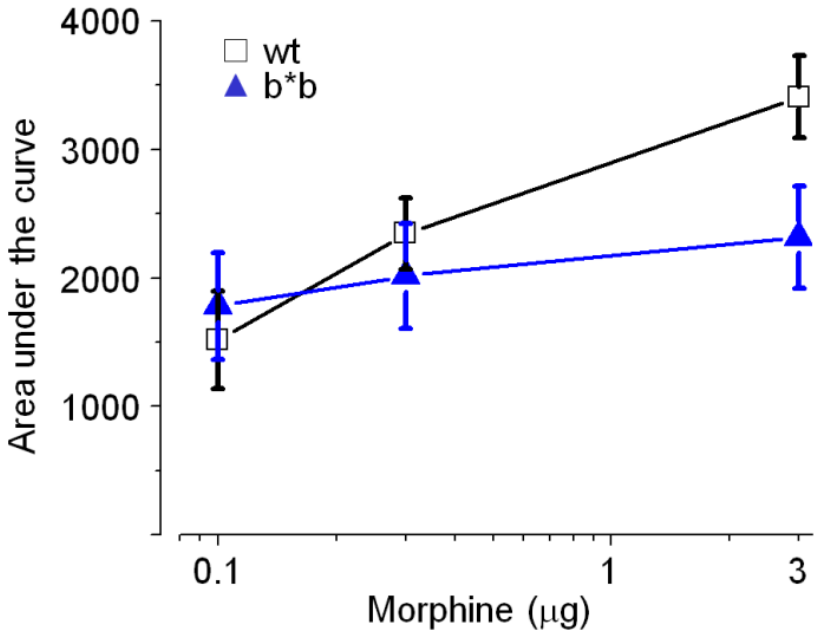
Supplementary Figure 2. Classification of DRG neurons from *Cacna1b*$^{wt/wt}$, *Cacna1b*$^{aa*/aa*}$, and *Cacna1b*$^{b*b/b*b}$ mice. Neurons isolated from the dorsal root ganglia of WT, b*b and aa* mice were classified in terms of their membrane capacitance, presence of T-type currents and capsaicin response. Three groups were found: small, T-type/medium and large cells. (**a**) Average of membrane capacitance ± s.e.m. of the three different groups of cells from the WT, b*b and aa* mice. Small cells: WT, 11.25 ± 0.46 (n = 25); b*b, 13.34 ± 0.89 (n = 83); aa*, 8.35 ± 0.59 (n = 23). T-type/medium cells: WT, 23.17 ± 1.04 (n = 17); b*b, 23.35 ± 1.6 (n = 83); aa*, 25.04 ± 0.77 (n = 24). Large cells: WT, 39.49 ± 1.04 (n = 24); b*b, 33.08 ± 2.14 (n = 65); aa*,

36.24 ± 3.37 (*n* = 16). *, *P* = 0.000246 (**b**) Exemplar of frequency distribution of membrane capacitance for genotype b*b fit with a triple gaussian function (x1 = 13.48, x2 = 24.3, x3 = 36.67, r^2 = 0.8829). (**c**) Same as in (**b**) but without including the cells that showed T-type currents. Two separate populations were found which correspond to small and large cells. (**d**) Capsaicin challenge. Small and large cells were exposed to a 2.5 s pulse of 2 μM capsaicin in whole cell configuration. 90% of small cells exhibited a noticeable inward current upon application of capsaicin. Large cells did not respond to capsaicin. To minimize false negative results we applied a 10 s pulse when an obvious response to capsaicin was not obtained.



Supplementary Figure 3. Western blot analysis of brain $Ca_V2.2$ and $Ca_V2.1$ channel levels in *Cacna1b*$^{wt/wt}$, *Cacna1b*$^{aa*/aa*}$, and *Cacna1b*$^{b*b/b*b}$ mice. Three 9-10 week mouse brains of each genotype were homogenized and tested for levels of $Ca_V2.2$, $Ca_V2.1$ and GAPDH (as a control). (**a**) One representative set of western blot results (of three) is shown. The un-cropped Western blot is shown in supplementary Fig. 5 below. (**b**) Protein levels were quantified, and

results are shown as an average of the three $Ca_V2.2$ bands for each genotype after normalization to the average of three GAPDH bands for each genotype. We found that *Cacna1b*$^{aa*/aa*}$ and *Cacna1b*$^{b*b/b*b}$ mice express $Ca_V2.2$ protein at roughly 12% and 82% that of *Cacna1b*$^{wt/wt}$ mice respectively. The results (in arbitrary units) are as follows: 3.9 ± 0.15, 0.47 ± 0.048, and 3.21 ± 0.37 for *Cacna1b*$^{wt/wt}$, *Cacna1b*$^{aa*/aa*}$ and *Cacna1b*$^{b*b/b*b}$ respectively. The errors were calculated as standard errors. The *P* values are 0.000014 for *CACNA1B*$^{aa*/aa*}$ and 0.07 for *CACNA1B*$^{b*b/b*b}$, using a one-tailed, unpaired *t* test. (**c**) The $Ca_V2.2$ blots described above were stripped and re-probed for $Ca_V2.1$. Channel levels were determined for each transgenic mouse line using the same methods as for $Ca_V2.2$. We found that all lines express $Ca_V2.1$ channel at similar levels. The results are: 1.58 ± 0.23, 1.46 ± 0.14, and 1.58 ± 0.24 for *Cacna1b*$^{wt/wt}$, *Cacna1b*$^{a*a/a*a}$ and *Cacna1b*$^{b*b/b*b}$, respectively. *P* values were 0.34 for *CACNA1B*$^{aa*/aa*}$ and 0.50 for *CACNA1B*$^{b*b/b*b}$.

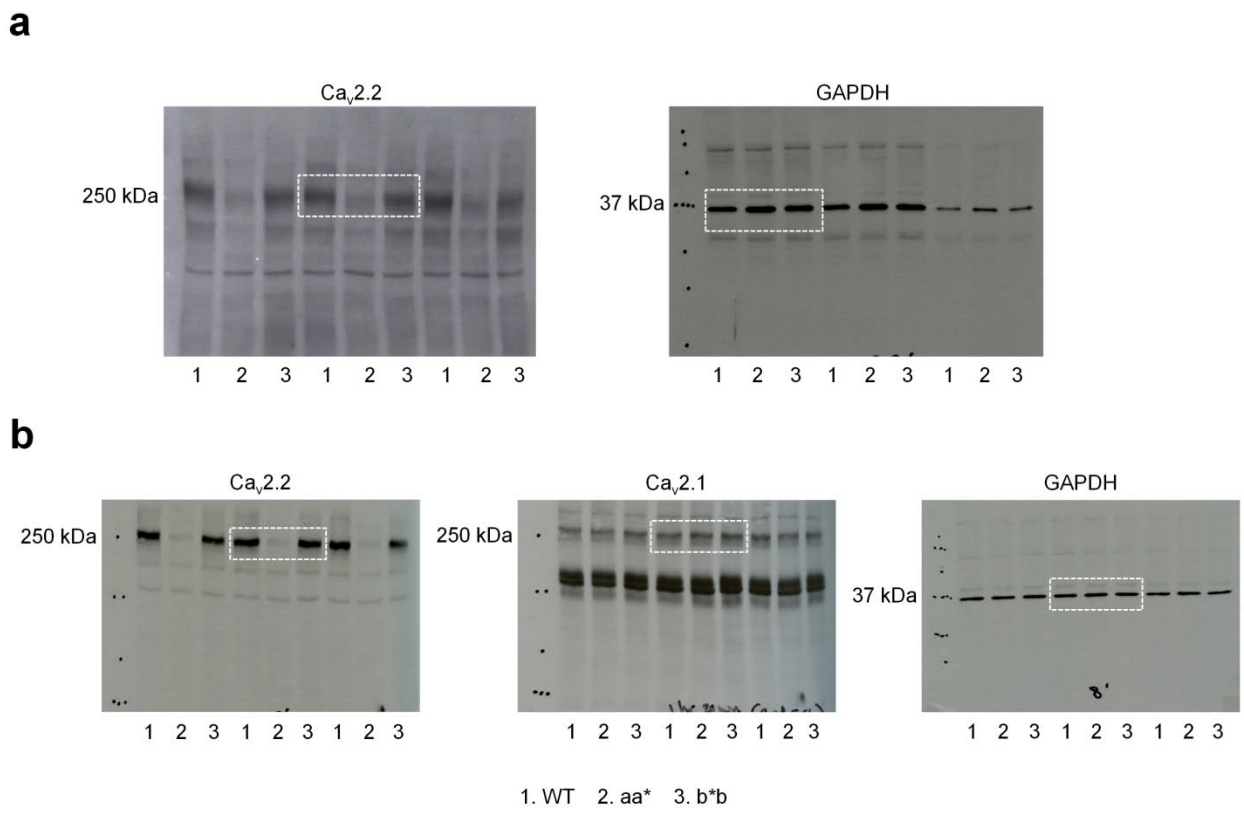


Supplementary Figure 4. Effect of intrathecal morphine in wild-type and *Cacna1b*$^{b*b/b*b}$ mice. The overall efficacy of three doses of intrathecal morphine, as an anti-nociceptive to a standard noxious thermal stimulus to the hind paw, are compared in wild-type (open squares) and *Cacna1b*$^{b*b/b*b}$ (solid blue, triangles) mice. The area under the morphine effect time course curve was calculated from the integral of the maximum possible effect (MPE) over a 1 hr time

period, following morphine injection. Values shown are averages ± standard errors ($n = 6$ for the 0.1 μg dose and $n = 10$ for the 1 and 3 μg doses, for each genotype). There is a significant reduction in the anti-nociceptive efficacy of intrathecal morphine at the highest dose (3 μg; $P = 0.046$, *t* test, $n = 10$ mice for each genotype). There is also a significant difference in the slope of the dose-response relationship between genotypes. Average slope values are 761 ± 172 for wild-type and 301 ± 45 for *Cacna1b*$^{b*b/b*b}$ mice, values are significantly different ($P = 0.040$, Student's *t* test, $n = 6$ for each genotype).

Supplementary Table 1. Survival of progeny from crosses of heterozygous *CACNA1B* mutant mice.

Cross	ab/aa* x ab/aa*	ab/b*b x ab/b*b
# Litters	53	60
# Progeny	367	381
Avg. Litter Size	6.9	6.4
Homozygous	54	95
Heterozygous	186	190
Wildtype	115	91
# Homozygotes dead @ weaning	12	5
Ratio of Progeny Observed* (mut/mut:wt/mut:wt/wt)	0.5 : 1.6 : 1.0	1.0 : 2.1 : 1.0
*Ratio of Progeny Expected (1 : 2 : 1)		



Supplementary Figure 5. Complete blots for Westerns shown in Fig. 2d and supplementary Fig. 3a. (a) Full-length blots for $Ca_V2.2$ and GAPDH on samples derived from DRG of five different mice of the three genotypes indicated by the numbers at the bottom of blots: 1: WT, 2: aa* and 3: b*b. The last three lanes of the GAPDH blot are a 1:2 dilution of the samples used in lanes 1-6. Cropped versions of these blots appear in **Fig. 2d**. (**b**) Images of full-length blots for $Ca_V2.2$, $Ca_V2.1$ and GAPDH included in the quantitative analysis shown in **Supplementary Fig. 3b** and 3**c** to determine the levels of each protein in the brain of three different mice for each genotype. In both panels, the dashed box indicates the cropped area shown in **Fig. 2d** and **Supplementary Fig. 3a**. Dots are drawn on the left hand side of each gel where molecular weight markers were; pertinent sizes are indicated. In the $Ca_V2.2$ blot in **a**, the molecular weight markings show as white dots, in all other gels they are black.

Table 2S

a

Small cells										
	n	Total current			N-type current			Non-N-type current		
Parameters		$V_{1/2}$(mV)	k(mV)	E_r (mV)	$V_{1/2}$(mV)	k (mV)	E_r (mV)	$V_{1/2}$(mV)	k (mV)	E_r (mV)
wt	27	-7.6 ± 0.7	5.3 ± 0.2	55.1 ± 0.6	-6.7 ± 0.9	3.8 ± 0.2	61.7 ± 1.1	-7.3 ± 1.0	3.9 ± 0.2	50.0 ± 1.1
b*b	11	-7.4 ± 1.0	5.4 ± 0.2	56.9 ± 0.8	-6.6 ± 0.9	4.1 ± 0.2	61.8 ± 1.2	-9.6 ± 1.8	3.6 ± 0.2	50.5 ± 1.6
aa*	10	-10.2 ± 1.0	4.2 ± 0.1	53.0 ± 1.2	-2.1 ± 1.4	2.1 ± 0.1	58.1 ± 4.9	-11.4 ± 2.3	3.6 ± 0.1	47.5 ± 2.8

b

Large cells										
	n	Total current			N-type current			Non-N-type current		
Parameters		$V_{1/2}$(mV)	k(mV)	E_r (mV)	$V_{1/2}$(mV)	k (mV)	E_r (mV)	$V_{1/2}$(mV)	k (mV)	E_r (mV)
wt	10	-12.7 ± 0.1	5.1 ± 0.2	58.1 ± 1.6	-10.3 ± 1.6	3.8 ± 0.4	59.0 ± 1.5	-16.1 ± 1.6	4.1 ± 0.2	54.1 ± 2.2
b*b	16	-11.2 ± 0.4	5.9 ± 0.2	53.0 ± 0.5	-9.0 ± 0.4	4.2 ± 0.2	56.0 ± 0.9	-14.1 ± 0.4	4.1 ± 0.2	49.8 ± 0.7
aa*	9	-13.9 ± 0.8	5.2 ± 0.3	52.1 ± 0.4	-13.1 ± 1.3	1.8 ± 0.1	55.7 ± 1.2	-13.1 ± 1.3	2.0 ± 0.1	55.7 ± 1.2

c

T-rich cells												
	n	Total current				N-type current			Non-N-type current			
Parameters		$^1V_{1/2}$(mV)	^{1}k (mV)	$^2V_{1/2}$(mV)	E_r (mV)	$V_{1/2}$(mV)	^{1}k (mV)	E_r (mV)	$^1V_{1/2}$(mV)	^{1}k (mV)	$^2V_{1/2}$(mV)	E_r (mV)
wt	10	-41.9 ± 1.6	6.7 ± 0.4	-10.6 ± 1.1	59.8 ± 1.7	-8.9 ± 1.5	3.1 ± 0.2	54.8 ± 2.2	-43.8 ± 2.4	6.2 ± 0.8	-12.4 ± 1.7	56.6 ± 2.1
b*b	14	-41.6 ± 0.8	7.0 ± 0.3	-10.1 ± 0.1	56.8 ± 0.6	-10.1 ± 0.7	2.7 ± 0.2	64.6 ± 2.4	-45.3 ± 0.6	6.0 ± 0.2	-13.2 ± 1.2	53.4 ± 1.2
aa*	8	-45.1 ± 1.5	6.3 ± 0.3	-13.2 ± 1.4	55.2 ± 0.7	-14.5 ± 1.7	2.7 ± 0.6	60.2 ± 2.9	-46.8 ± 0.9	6.2 ± 0.5	-15.4 ± 1.0	53.9 ± 1.4

Supplementary Methods

Construction of genomic clones for mouse exon substitution.

Detailed cloning steps

We cloned the ~9.9 kb AscI fragment from MHPN59f07 spanning e36-e38 of *Cacna1b* into the BssHII site of *pBluescript II SK*+ (Stratagene). To facilitate mutagenesis, we subcloned the 2.8 kb BamHI-EagI fragment containing e37a and e37b into *pBluescript II SK*+. After several rounds of mutagenesis (QuickChange II XL Site-Directed Mutagenesis Kit, Stratagene) and we reinserted the mutated fragments into the BamHI-EagI sites to recreate the ~12 kb AscI final constructs for gene targeting.

To create the e37a-e37a* construct used to make the *Cacna1b*$^{aa*/aa}$ mice, we created an intermediate construct (e37a-Δe37b) with primers SD7for and SD8rev. We created another intermediate construct (*e37a*-e37b*) to delete the BsrGI site in e37a by introducing a silent single nucleotide mutation with primers SD19for and SD20rev. We PCR amplified a mutagenesis primer that contained e37a* flanked by the upstream and downstream intronic sequences of e37b from the *e37a*-e37b* construct with primers SD17for and SD18rev; we used the resulting primer to insert e37a* into the De37b position of the *e37a-De37b* construct, creating the *e37a-e37a** targeting construct. We used a similar strategy to create the *e37b*-e37b* construct used to make *Cacna1b*$^{b*b/b*b}$ mice. We created the *De37a-e37b* construct with primers SD5for and SD6rev and with primers SD21for and SD22rev, created the *e37a-e37b** intermediate construct to delete the XhoI site in e37b. We used the mutagenesis primer, amplified with primers SD15for and SD16rev, to insert e37b* into the De37a position of the *De37a-e37b* construct, creating the *e37b*-e37b* targeting construct.

Genomic DNA isolation

We incubated a 0.3-0.5 cm tail piece from 7-9 day old mouse overnight at 60°C with 300 ml lysis buffer (50 mM Tris-HCl pH7.5, 50 mM EDTA pH 8.0, 100 mM NaCl, 5 mM DTT, 0.5 mM spermidine, 1% SDS) and 20 ml 10 mg ml^{-1} fresh proteinase K, then extracted once with 300 ml phenol/choloform/isoamyl alcohol (25:24:1) by shaking vigorously for 30 sec. We separated phases by centrifugation for 15 min , 16k relative centrifugal force (r.c.f.), transferred the aqueous phase to a new tube and precipitate genomic DNA with 300 ml of isopropanol and centrifugation for 10 min (16k r.c.f.) at room temperature. After re-suspending in 200 ml TE (10 mM Tris-HCl pH 8.0, 1mM EDTA), DNA was precipitated by 20 ml of 3M sodium acetate pH5.2

and 500 ml ethanol and centrifugation (16k r.c.f.) for 10 min. We dissolved the DNA pellet in 300 ml 10mM Tris-HCl pH 8.0 and used 1 ml for genotyping PCR reaction.

RNA extraction and reverse transcription polymerase chain reaction (RT-PCR)

We extracted total DRG RNA from 10 P10 mice by homogenization with TriZol reagent (Invitrogen) following the manufacturer's recommendations. We reverse transcribed from 1 mg of total RNA using SuperScript First Strand system (Invitrogen) with oligo-$(dT)_{12\text{-}18}$ priming. We PCR amplified a 430 bp fragment spanning e35 and e38 from 1 ml of the first-strand RT reaction using Taq Polymerase (New England Biolabs) [35 cycles, 60°C annealing, 1 min extension at 72°C] and primers SD151 for and SD153rev. We used BsrGI (which cuts e37a) and XhoI (which cuts e37b) to distinguish WT from mutant mRNAs.

PCR Primers:

SD5for 5'- CCTGTAACATTTCCTTTCCAGTGTTTGCAACTTCAGAGCCTCC
SD6rev 5'- GGAGGCTCTGAAGTTGCAAACACTGGAAAGGAAATGTTACAGG
SD7for 5'- CCTCTGGAACGGGTTTCCAGTGTAGACCTTGACCCTGCACTG
SD8rev 5'- CAGTGCAGGGTCAAGGTCTACACTGGAAACCCGTTCCAGAGG
SD15for 5'- CCTGTAACATTTCCTTTCCAGTGGGCGCATCAGTTACAATGAC
SD16rev 5'- GGAGGCTCTGAAGTTGCAAACCTTGTATGCAACTCGAGCCG
SD17for 5'- CCTCTGGAACGGGTTTCCAGTTGCCGGATTCATTATAAGGATATG
SD18rev 5'- CAGTGCAGGGTCAAGGTCTACCTTGTAGGCCAACCTACGAGG
SD19for 5'- GCCGGATTCATTATAAGGATATGTATAGTTTGTTGCGTTGTATTG
SD20rev 5'- CAATACAACGCAACAAACTATACATATCCTTATAATGAATCCGGC
SD21for 5′- GAAGAAATGCCCGGCACGAGTTGCATACAAGGTTTG
SD22rev 5′- CAAACCTTGTATGCAACTCGTGCCGGGCATTTCTTC
SD29for 5'- CATGAGCGCTGAATTCCTGCAGCCCAATTCC
SD31rev 5'- CTAGAGCGCTCCCTCGAGGGACCTAATAACTTCG
SD47for 5'- GGGAAGACAATAGCAGGCATGC
SD48rev 5'- CTGGGATGAGAGCAAAGGGT
SD62for 5'- ATGGACGGGGTGCAACATGG
SD64rev 5'- CTACCCGGTAGAATTTCGACG
SD77for 5'- CCGTGGTGGCATTTGAGGC
SD79rev 5'- CCAAGGCTATGTGACTCACC
SD151for 5'- AGGCCTGGCATGAGATCATGC

SD153rev 5'- TGAGGGCCATCAGTGTGGACG

Western blot analysis.

We homogenized tissue on ice in RIPA buffer (50 mM Tris-HCl pH 7.5, 150mM NaCl, 1% Igepal, 0.5% sodium deoxycholate, 0.1% SDS) containing protease inhibitor (Complete, mini; Roche Diagnostics), followed by 2-3 hr incubation on ice and centrifugation at 14.9k r.c.f. for 20 min at 4^{o}C to remove debris. We diluted a volume of supernatant equivalent to 20 µg of protein (determined by BCA Protein Assay) for each sample in 2X reducing buffer (100mM Tris-HCl pH 6.8, 4% SDS, 0.2% bromophenol blue, 20% glycerol, 200 mM dithiothreitol). We incubated samples at room temperature for 15 min before resolving on 6% ($Ca_V2.2$) or 15% (GAPDH) SDS-polyacrylamide gels (4% stacking gels were used in both cases). We discovered that boiling samples at this stage caused $Ca_V2.2$ protein to aggregate, impeding protein movement through the gel. Proteins were transferred using electrophoresis to 0.45 mm nitrocellulose membranes (Whatman) for 1hr on ice. After blocking with 5% non-fat dry milk, 0.1% Tween-20, PBS for 1 hr, membranes were probed with antibodies against $Ca_V2.2$ (Alomone) and $Ca_V2.1$ diluted 1:200 (Alomone) and Glyceraldehyde 3-Phosphate Dehydrogenase (GAPDH) diluted 1:1,000 (Cell Signaling, clone 14C10) for one hour at room temperature. We then washed membranes (3X; 0.1% Tween-20, PBS) and incubated with horseradish peroxidase labeled donkey anti-rabbit IgG secondary antibodies diluted 1:15,000 (Jackson) for 1 hr at room temp. All antibodies were diluted in 1% BSA, 0.1% Tween-20, 1X PBS unless otherwise recommended by vendor. Membrane stripping (before reprobing) was performed with stripping buffer: (2%SDS, 62.5 mM Tris-HCL pH 6.8, 100 mM 2-mecaptoethanol) and incubation for 30 minutes at 50^{o}C (with rocking), followed by two washing steps with excess PBS-T, and blocking as usual.

Settings for immune-fluorescence and details of the analysis software used

Immuno-fluorescence was visualized using a Zeiss Axiovert 200M fluorescence microscope equipped with EC Plan Neofluar 20x/0.50 Ph2 lenses. Images were taken by a Zeiss AxioCam MRc5 camera using the software Zeiss Axio Vision 4.4.1.0. The space and time resolution settings were as follows: scaling x, 0.5 µm/pixel; y, 0.5 µm/pixel; z, 1 pixel/pixel. The image size was 1344 x 1024 pixel. Excitation, emission wavelengths and filters for each phluorochrome were: i) Fluorescein isothiocyanate (FITC) [chroma set 41001, excitation 460 – 500nm, emission 510 – 560 nm]; ii) Alexa

Fluor® 555 [chroma set 41001, excitation 460-500 nm, emission 510-560 nm (bandpass filter)]. iii) Cy5 [chroma set 41024, excitation 590-650 nm, emission 665 nm and above (long pass filter)]. We used the software MetaMorph 7.6 (Molecular Devices) to further improve all our images we applied identical enhanced contrast, brightness and gamma correction settings to all images.