Supporting Information

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SI Materials and Methods

Molecular Biology. The rabbit voltage-gated calcium (Ca_V) channel $Ca_V2.2$ (D14157), rat β1b (1), rat $\alpha_2\delta$-1 (M86621.3), VAMP-mCherry (from T.A. Ryan, Weill-Cornell Medical College, New York), and free mCherry were used in these studies. $\alpha_2\delta$-1^{R241A}-HA was generated by standard techniques, with arginine 241 mutated to alanine. This construct was previously termed $\alpha_2\delta$-1^{R217A} (2), numbering residues after cleavage of the N-terminal signal sequence. pMT2 $Ca_V2.2$-mono-HA was generated using primer pairs 1 and 2 (Table S2) to generate fragments that were cut with BglII and SalI (fragment 1) or SalI and EcoRI (fragment 2), which were used for a three-part ligation with pMT2 $Ca_V2.2$ treated with BglII and EcoRI. To generate pMT2 $Ca_V2.2$-HAgHA, primer pair 3 was used to generate a fragment that was cut with BglII and SalI, which was ligated into pMT2 $Ca_V2.2$-HA treated with BglII and SalI (thus replacing fragment 1). pMT2 $Ca_V2.2$-mono-bungarotoxin binding site (BBS) was generated by replacing fragment 1 with a fragment generated from primer pair 4 (fragment and vector treated with BglII and SalI). pMT2 $Ca_V2.2$-BBS-BBS was generated by allowing primers in pair 5 to anneal to each other and ligating this short double-stranded sequence to pMT2 $Ca_V2.2$-BBS treated with SalI. Constructs that contained two BBS tags both in the correct orientation were chosen. pMT2 $Ca_V2.2$-HAgHA (termed $Ca_V2.2$-HA in this publication) and pMT2 $Ca_V2.2$-BBS-BBS (termed $Ca_V2.2$-BBS in this publication) were subcloned into pcDNA3 by treating the insert and the vector with EcoRI (removing the $Ca_V2.2$ fragment from pcDNA3 $Ca_V2.2$-EcoRI).

Cell Culture. Neuro2A mouse neuroblastoma cells (3) were maintained in DMEM/Opti-MEM (Invitrogen), 5% FBS (vol/vol), 1% GlutaMAX, 50 U/mL penicillin, 50 μg/mL streptomycin. Coverslips and glass-bottom dishes were coated in poly–L-lysine. The transfection reagent used was PolyJet (SignaGen), according to the manufacturer's protocol, in a ratio of 3:1 to DNA. Cells were transfected 48 h before immunocytochemistry. The medium was replaced ∼12 h after transfection with serum-free medium, containing gabapentin (Sigma) when stated. The DNA mixes consisted of pcDNA3 $Ca_V2.2$ to pcDNA3 $\alpha_2\delta$-1 or $\alpha_2\delta$-1-HA (4) to pRK5 β1b to mCherry (where used) in a ratio of 3:2:2:0.4, with 2 μL of DNA mix used per 35-mm dish.

Neuronal Cell Culture. Cell cultures were obtained following enzymatic and mechanical dispersal of rat dorsal root ganglia (DRG) as described previously (5). Briefly, DRG were removed from adult Sprague–Dawley rats and incubated in Hank's basal salt solution containing 1,000 U/mL DNase (Invitrogen), 5 mg/mL dispase (Invitrogen), and 2 mg/mL collagenase type 1A (Sigma) for 40 min at 37 °C. The partially digested ganglia were then washed and triturated in growth medium (DMEM/F12 with 10% FBS, 1% GlutaMAX, 50 U/mL penicillin, 50 μg/mL streptomycin, 6 g/L glucose). DRG neurons were transfected with $Ca_V2.2$-HA, $\alpha_2\delta$-1, β1b, and VAMP-mCherry (3:2:2:0.4) using an Amaxa Nucleofector (Lonza) according to the manufacturer's protocol. Cells were plated on laminin-coated coverslips and the medium was changed every other day. Immunostaining was performed on separate dishes from the same transfection for either cell-surface HA epitope or cell-surface $\alpha_2\delta$-1 epitope.

Immunocytochemistry, Imaging, and Analysis. For fixed Neuro2A cells, coverslips were washed twice at room temperature in PBS before fixation with 4% paraformaldehyde (PFA) for 5 min. For live labeling experiments, cells in glass-bottom dishes (MatTek) were washed once with Krebs Ringer Hepes (KRH) buffer and then labeled with α-bungarotoxin (BTX)-AF 488 (Invitrogen; 1:100 in KRH buffer) at 17 °C for 30 min. Cells were then washed twice with KRH buffer before fixation. For internalization experiments, cells were incubated at either 17 °C or 37 °C for 10–30 min before fixation with 4% PFA for 5 min. Cells were then treated as for fixed samples. For DRG neurons, coverslips were washed with PBS and fixed with 4% PFA/sucrose in PBS for 5 min and then washed twice with PBS. When antigen retrieval was performed for the immunodetection of $\alpha_2\delta$-1, the cells were incubated for 10 min at 95 °C in 10 mM citrate buffer (pH 6.0). When permeabilization was performed, cells were incubated with 0.2% Triton X-100 in PBS for 5 min. Cells were then incubated with blocking buffer (20% goat serum, 4% BSA in PBS). Antibodies were applied in blocking buffer diluted with PBS, with four PBS washes between each antibody incubation step. The primary antibody was applied for 1 h at room temperature, except for DRG neurons, where primary antibody was applied overnight at 4° C; secondary antibody and streptavidin were applied for 1 h at room temperature. DAPI was applied for 5 min at room temperature. Coverslips were mounted using Vectashield mounting medium (Vector Laboratories). The primary antibodies used were rat anti-HA (Roche), rabbit anti-$Ca_V2.2$ directed against the intracellular II-III linker (6), and mouse anti–α_2-1 (mAb; Sigma). The secondary antibodies used as appropriate were goat anti-rabbit AF 488, goat anti-rabbit AF 594, goat anti-rat AF 488, goat anti-rat AF 594, goat anti-rat biotin, and streptavidin AF 633. All antibodies were used at 1:500.

Imaging was performed on a Zeiss LSM 780 confocal microscope with excitation wavelengths 405 nm, 488 nm, 543 nm, and 633 nm. For imaging Neuro2A cells, after choosing a region of interest containing transfected cells as determined by either mCherry or intracellular $Ca_V2.2$ or $\alpha_2\delta$-1 staining, the tile function was then used to acquire images around this region, composed of 3×3 tiles (one tile, $1{,}024 \times 1{,}024$ pixels), to reduce the impact of collection bias. Every cell identified as transfected by the presence of mCherry or intracellular staining was included in the measurements, to ensure that no transfected cells were missed. Images were analyzed using ImageJ (National Institutes of Health). Surface expression was measured using the freehand line tool (line width of 0.92 μm) and manually tracing the surface of the cell. After measuring surface expression, the background signal was measured in an area of the image lacking cells. Internal staining was measured using the freehand selection tool and drawing around the cell (omitting the nucleus), with the background measured in the same way as above. The background was subtracted from the measurements, and then the measurements were subjected to baseline subtraction by comparing measurements with those in conditions lacking the construct being measured. All data were then normalized to the appropriate positive control. Data were always normalized to the mean for each experiment before combining experiments. In Fig. 3*B*, analyzed cells were categorized as having surface staining for either $\alpha_2\delta$-1-HA (red triangles), $Ca_V2.2$-BBS (green circles), or both $\alpha_2\delta$-1-HA and $Ca_V2.2$-BBS (black squares). Parameters used were green if normalized $\alpha_2\delta$-1-HA <0.6, red if $\alpha_2\delta$-1-HA >0.6 and $Ca_V2.2$-BBS <0.7, and black if $\alpha_2\delta$-1-HA >0.6 and $Ca_V2.2$-BBS >0.7.

For analysis of internalization experiments (Fig. 3*F*), data were normalized to the value for $Ca_V2.2$-BBS/β1b/$\alpha_2\delta$-1-HA at time 0 min, and fit by a single exponential ($y = y_0 + A \times e^{-x/\tau}$, where y_0

is initial amplitude, A is amplitude of decay, and τ is decay time constant).

Electrophysiology. The electrophysiological properties of the tagged $Ca_V2.2$ constructs were assessed by recording from tsA-201 cells 2 d after transfection. Cells were transfected with $Ca_V2.2$:$\alpha_2\delta$-1:β1b:CD8 in the ratio 3:2:2:0.4 using FuGENE 6 as transfection reagent. Transfected cells were identified by using anti-CD8 beads. Cells were bathed in an external solution of 160 mM tetraethylammonium bromide, 3 mM KCl, 1 mM $NaHCO_3$, 1 mM $MgCl_2$, 10 mM Hepes, 4 mM D-glucose, and 1 mM $BaCl_2$, which was adjusted to pH 7.4 using Tris base and to 310 mosM with sucrose. The intracellular solution contained 140 mM Cs·aspartate, 5 mM EGTA, 2 mM $MgCl_2$, 0.1 mM $CaCl_2$, 2 mM K_2ATP, and 20 mM Hepes, and was adjusted to pH 7.2 with Tris base and to 320 mosM with sucrose. ω-Conotoxin GVIA (Alomone) was applied by perfusion. Electrophysiology was performed using an Axopatch 1D or 200B amplifier with pClamp 9.2 (Molecular Devices). Pipettes of resistance 2–4 MΩ were used. Data were filtered at 1–2 kHz and digitized at 5–10 kHz. To record current–voltage (IV) relationships, cells were held at −90 mV, and a 50-ms step potential was applied to between −30 and +60 mV in 5-mV intervals every 10 s. I_{Ba} was measured at 10 ms after the start of the step. For steady-state inactivation, a 5-s depolarizing step from −110 mV to the stated prepulse potential was applied before a 50-ms step to + 10 mV every 10 s. Analysis of data was performed using pClamp 9.2 and Origin 7 or 9 (Originlab). Current records were subjected to leak and residual capacitance current subtraction (P/8 protocol in pClamp). IV relationships were fit by a modified Boltzmann equation as follows: $I = G_{max} \times (V - V_{rev})/(1 + \exp(-(V - V_{50,\ act})/k))$, where I is the current density (in picoamperes per picofarad), G_{max} is the maximum conductance (in nanosiemens per picofarad), V_{rev} is the apparent reversal potential, $V_{50,\ act}$ is the midpoint voltage for current activation, and k is the slope factor. Steady-state inactivation data were fitted with a single Boltzmann equation of the form $I/I_{max} = (A1 - A2)/[1 + \exp((V - V_{50,\ inact})/k)] + A2$, where I_{max} is the maximal current and $V_{50,\ inact}$ is the half-maximal voltage for current inactivation.

Immunoblotting. Western blotting was performed as described previously (7). The primary antibodies used were anti–α_2-1 (1:2,000; mouse monoclonal; Sigma) and rabbit anti-HA antibody (1:1,000; BD Biosciences). The secondary antibodies used were goat anti-mouse or anti-rabbit coupled to HRP (Bio-Rad).

1. Tomlinson WJ, et al. (1993) Functional properties of a neuronal class C L-type calcium channel. *Neuropharmacology* 32(11):1117–1126.
2. Field MJ, et al. (2006) Identification of the α_2-δ-1 subunit of voltage-dependent calcium channels as a molecular target for pain mediating the analgesic actions of pregabalin. *Proc Natl Acad Sci USA* 103(46):17537–17542.
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4. Kadurin I, et al. (2012) Calcium currents are enhanced by $\alpha_2\delta$-1 lacking its membrane anchor. *J Biol Chem* 287(40):33554–33566.
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6. Raghib A, et al. (2001) Dominant-negative synthesis suppression of voltage-gated calcium channel $Ca_v2.2$ induced by truncated constructs. *J Neurosci* 21(21):8495–8504.
7. Page KM, et al. (2004) Dominant-negative calcium channel suppression by truncated constructs involves a kinase implicated in the unfolded protein response. *J Neurosci* 24(23):5400–5409.

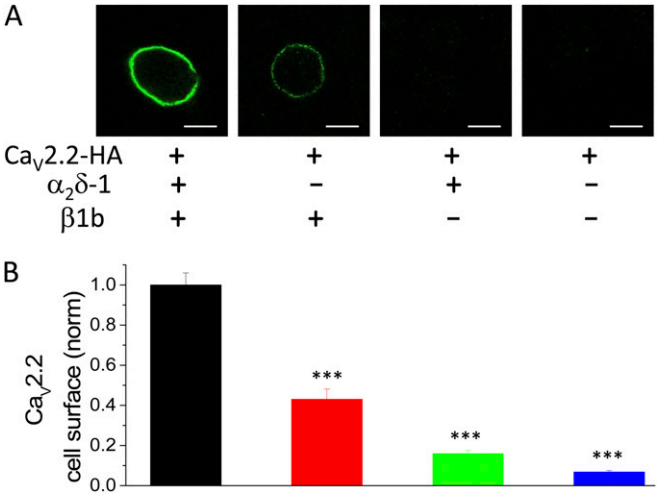

Fig. S1. Cell-surface expression of $Ca_V2.2$-HA: effect of $\alpha_2\delta$-1 and β1b. (*A*) Representative images showing cell-surface expression of $Ca_V2.2$-HA in Neuro2A cells. $Ca_V2.2$-HA expressed +$\alpha_2\delta$-1/β1b (panel 1), +β1b (panel 2), +$\alpha_2\delta$-1 (panel 3), and alone (panel 4). (Scale bars, 10 μm.) (*B*) Bar chart of mean (±SEM) cell-surface $Ca_V2.2$-HA density for $Ca_V2.2$-HA/$\alpha_2\delta$-1/β1b (black bar; $n = 369$), $Ca_V2.2$-HA/β1b (red bar; $n = 187$), $Ca_V2.2$-HA/$\alpha_2\delta$-1 (green bar; $n = 191$), and $Ca_V2.2$-HA alone (blue bar; $n = 233$). Data were obtained from two separate transfections. Statistical differences were determined by one-way ANOVA and Bonferroni post hoc tests. ***$P < 0.001$ compared with $Ca_V2.2$-HA/$\alpha_2\delta$-1/β1b. Cells were selected that were positive for internal $Ca_V2.2$.

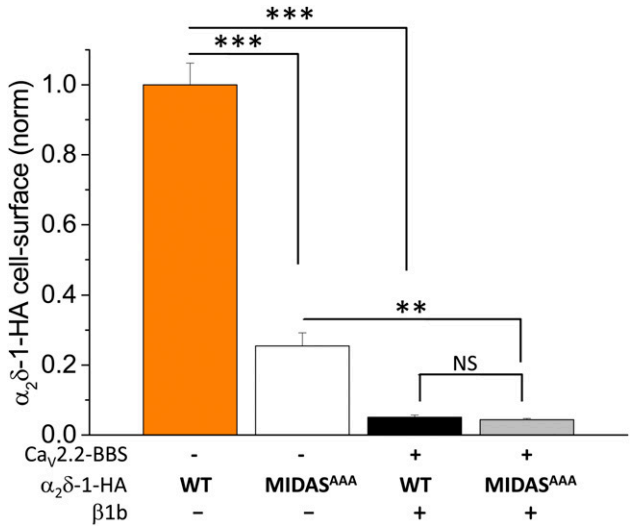

Fig. S2. Cell-surface expression of $\alpha_2\delta$-1-MIDAS^{AAA} in Neuro2A cells. Bar chart of mean (±SEM) cell-surface $\alpha_2\delta$-1-HA density for WT $\alpha_2\delta$-1-HA alone (orange bar; $n = 173$), $\alpha_2\delta$-1-MIDAS^{AAA}-HA alone (white bar; $n = 218$), $Ca_V2.2$-BBS/WT $\alpha_2\delta$-1-HA/β1b (black bar; $n = 148$), and $Ca_V2.2$-BBS/$\alpha_2\delta$-1-MIDAS^{AAA}-HA/β1b (gray bar; $n = 137$). Cells were selected for analysis that were positive for internal $\alpha_2\delta$-1, and also for internal $Ca_V2.2$ under the conditions in which this was transfected. Data were obtained from two separate transfections and normalized to the value for WT $\alpha_2\delta$-1-HA alone in each experiment. Statistical differences were determined by one-way ANOVA and Bonferroni post hoc tests. ***$P < 0.001$, **$P < 0.01$; not significant (NS), $P > 0.05$.

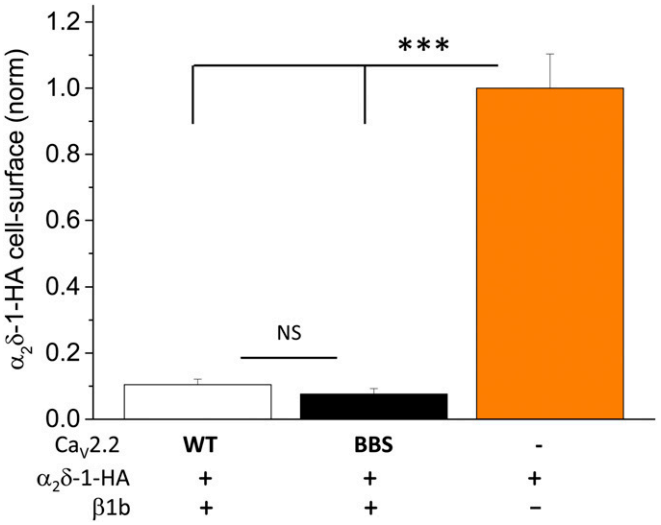

Fig. S3. $\alpha_2\delta$-1-HA epitope is masked on the cell surface by wild-type $Ca_V2.2$. Bar chart of mean (±SEM) cell-surface $\alpha_2\delta$-1-HA density for WT $Ca_V2.2$/$\alpha_2\delta$-1-HA/β1b (white bar; $n = 162$), $Ca_V2.2$-BBS/$\alpha_2\delta$-1-HA/β1b (black bar; $n = 74$), and $\alpha_2\delta$-1-HA alone (orange bar; $n = 79$). Cells were selected for analysis that were positive for internal $\alpha_2\delta$-1, and for internal $Ca_V2.2$ under the conditions in which this was transfected. Data were obtained from two separate transfections and normalized to the value for $\alpha_2\delta$-1-HA alone in each transfection. Statistical differences were determined by one-way ANOVA and Bonferroni post hoc tests. ***$P < 0.001$ compared with $\alpha_2\delta$-1-HA alone; NS, $P > 0.05$.

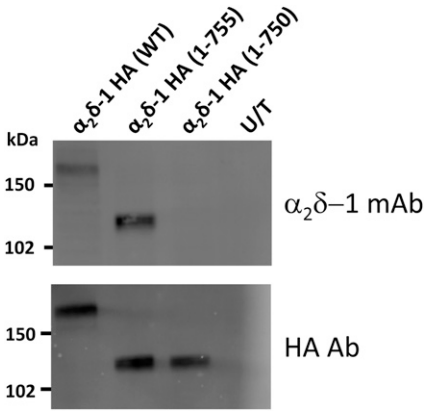

Fig. S4. Mapping the epitope of the monoclonal $\alpha_2\delta$-1 antibody used in this study. Sequential C-terminal truncation of $\alpha_2\delta$-1-HA identified the binding site for the mouse monoclonal antibody (Ab), which was downstream of the internal HA tag. Lane 1: full-length $\alpha_2\delta$-1-HA (WT); lane 2: $\alpha_2\delta$-1-HA (1–755); lane 3: $\alpha_2\delta$-1-HA (1–750); lane 4: untransfected tsA-201 cells (U/T). (*Upper*) Mouse monoclonal $\alpha_2\delta$-1 Ab immunoblot. (*Lower*) Anti-HA immunoblot, to identify all of the constructs used. The antigenic epitope therefore includes residues FYKRS.

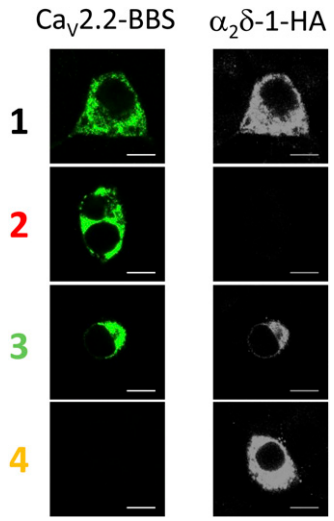

Fig. S5. Internal $Ca_V2.2$ and $\alpha_2\delta$-1 immunostaining in transfected Neuro2A cells. Representative images for the mean data shown in Fig. 3*E*, showing internal $Ca_V2.2$ (green, identified by II-III loop Ab; *Left*) and internal $\alpha_2\delta$-1-HA (white; *Right*) for the subunit combinations $Ca_V2.2$-BBS/$\alpha_2\delta$-1-HA/β1b (condition 1), $Ca_V2.2$-BBS/β1b (condition 2), $Ca_V2.2$-BBS/$\alpha_2\delta$-1-HA (condition 3), and $\alpha_2\delta$-1-HA alone (condition 4). Cells were selected that were positive for internal $\alpha_2\delta$-1 or $Ca_V2.2$. (Scale bars, 10 μm.)

Table S1. Mean parameters of current density–voltage relationships for the $Ca_V2.2$ constructs described

Constructs	$Ca_V2.2$	$Ca_V2.2$-BBS	$Ca_V2.2$-HA
G_{max}, nS/pF	4.10 ± 0.68	3.44 ± 0.68*	3.93 ± 1.02*
V_{rev}, mV	47.1 ± 1.0	47.0 ± 0.83*	48.8 ± 1.2*
V_{50}, mV	−4.27 ± 0.70	−6.09 ± 0.71*	−5.53 ± 1.36*
k	4.01 ± 0.09	4.32 ± 0.14*	4.40 ± 0.19*
n	30	17	13

None of the parameters show any statistical differences between the different constructs (one-way ANOVA and Tukey's post hoc test). G_{max}, maximum conductance; *k*, slope factor; $V_{50,\,act}$, potential for 50% activation; V_{rev}, reversal potential.
*NS, not significant.

Table S2. Primers used in molecular biology

Primer pair	Primer orientation	Sequences, 5′–3′
1	F	ACT AGT GTC GAC GTC AAG CCA GGG ACC TCC
	pMT2-R	GGT CGA ACC ATG ATG GCA GC
2	pMT2-F	AGC TTG AGG TGT GGC AGG CTT
	R	ACT AGT GTC GAC GCC GGC GTA GTC GGG GAC GTC GTA GGG GTA GCC AGC AGC CCA GAC CAC
3	F	pMT2-F
	R	ACT AGT GTC GAC GCC GGC GTA GTC CGG GAC GTC GTA CGG GTA GCC GGC GTA GTC GGG GAC GTC GTA GGG GTA AGC AGC CCA GAC CAC
4	F	pMT2-F
	R	ACT AGT GTC GAC GCC GTC AGG GTA GGG CTC CAG GGA GCT CTC GTA GTA TCT CCA GCC AGC AGC CCA GAC CAC
5	F	P_i TCG ACG GAT GGA GAT ACT ACG AGA GCT CCC TGG AGC CCT ACC CTG ACA GAT CTG
	R	P_i TCG ACA GAT CTG TCA GGG TAG GGC TCC AGG GAG CTC TCG TAG TAT CTC CAT CCG

The resultant tags and flanking sequences are Cav2.2-BBS, VVWAA-GWRYYESSLEPYPDGVDGWRYYESSLE-PYPDRSVD-VKPGT, and Cav2.2-HA, VVWAA-GYPYDVPDYAGYPYDVPDYAVD-VKPGT. F, forward; R, reverse.