Figure S1. Effect of expressing Cacna2d1 on α2δ-1 expression levels in the spinal cord and DRG tissues in naive rats, related to Figure 1.

(A,B) Original gel images and mean changes in the protein level of α2δ-1 (~140 kDa) in the dorsal spinal cord (A) and DRG (B) tissues of naive rats 5 weeks after intrathecal injection of the wild-type Cacna2d1 vector or control vector (n = 6 rats in each group). GAPDH was used as an internal control. Data are means ± s.e.m. **P < 0.01 (versus control vector-treated rats). Two-tailed Student’s t-test.
Figure S2. Intrathecal injection of Cacna2d1-specific siRNA reduces the α2δ-1 expression level in the spinal cord and DRG and pain hypersensitivity induced by nerve injury, related to Figure 2.

(A) Differential effects of Cacna2d1-specific siRNA (4 μg/day for 4 days) on the mRNA level of α2δ-1 and α2δ-2 in the DRG and dorsal spinal cord (n = 6 rats in each group). Data are presented as means ± s.e.m. *P < 0.05, **P < 0.01 (versus control siRNA-treated rats). Two-tailed Student’s t-test.

(B) Western blot analysis and quantification of the α2δ-1 protein level in the DRG and dorsal spinal cord (n = 6 rats in each group). GAPDH was used as an internal control. Data are means ± s.e.m. *P < 0.05, **P < 0.01 (versus control siRNA-treated rats). Two-tailed Student’s t-test.

(C) Effects of intrathecal treatment with control siRNA or Cacna2d1-specific siRNA (4 μg/day for 4 days) on the tactile, pressure and heat withdrawal thresholds of SNL rats (n = 8 rats in each group). Data are means ± s.e.m. *P < 0.05, **P < 0.01 (versus baseline, BL). One-way ANOVA analysis followed by Dunnett’s post hoc test.

(D) No significant effects were observed for intrathecal treatment with control siRNA or Cacna2d1-specific siRNA (4 μg/day for 4 days) on the tactile, pressure and heat withdrawal thresholds of naive control rats (n = 8 rats in each group).
Figure S3. Co-IP analysis using membrane extracts of HEK293 cells show that α2δ-1 does not interact with GluN1, GluN2A or GluN2B when these subunits are expressed individually with α2δ-1, related to Figure 3.

HEK293 cells were cotransfected with α2δ-1 and individual CFP-tagged NMDAR subunit constructs as indicated above the gel images. Co-IP was performed using an anti-GFP antibody, which cross-reacts with CFP. Immunoblotting (IB) was performed using an anti-α2δ-1 antibody. Data were from three independent experiments.
Figure S4. Gabapentin treatment normalizes nerve injury-induced increases in synaptic NMDAR activity of spinal dorsal horn neurons, related to Figures 4A-4C.

(A) Representative recordings and mean changes of the amplitude of evoked NMDAR-EPSCs, AMPAR-EPSCs and ratio of NMDAR-EPSCs to AMPAR-EPSCs of spinal dorsal horn neurons in sham (n = 10 neurons in the vehicle group, n = 11 neurons in the gabapentin [GBP] group) and SNL (n = 10 neurons in vehicle group, n = 12 neurons in the gabapentin group) rat spinal cord slices treated with vehicle or 100 μM gabapentin for 30 min. Data are means ± s.e.m. *P < 0.05 (versus corresponding value in the sham+vehicle group). One-way ANOVA analysis followed by Tukey’s post hoc test.

(B) Original traces and mean data show the AP5 effect on the amplitude of EPSCs of spinal dorsal horn neurons monosynaptically evoked by dorsal root stimulation in sham (n = 12 neurons) and SNL (n = 12 neurons in the vehicle group, n = 11 neurons in the gabapentin group) rat spinal cord slices treated with vehicle or gabapentin. Data are means ± s.e.m. *P < 0.05 (versus respective baseline). #P < 0.05 (versus baseline in the sham group). One-way ANOVA analysis followed by Tukey’s post hoc test.
Figure S5. Biophysical properties of NMDARs are not altered by α2δ-1 coexpression, related to Figures 4D and 4E.

(A) Response from an outside-out patch transfected with GluN1/GluN2A alone (black) or GluN1/GluN2A plus α2δ-1 (red) to a 1-ms 1-mM glutamate jump (deactivation) in the continual presence of 100 μM glycine.

(B) Summary of deactivation time constants from all patches transfected with either GluN1/GluN2A alone (black, n = 13 cells) or GluN1/GluN2A plus α2δ-1 (red, n = 12).

(C) Responses from the same patch as panel a to either a 6-s 1 mM glutamate jump (desensitization) or 4 s of glutamate followed by 2 s of glutamate plus MK-801 (300 nM, green trace) in the continual presence of 100 μM glycine.

(D) Summary of weighted desensitization from all patches transfected with either GluN1/GluN2A alone (black, n = 14) or GluN1/GluN2A plus α2δ-1 (red, n = 10).

(E) Summary of MK-801 time constants from all patches transfected with either GluN1/GluN2A alone (black, n = 12) or GluN1/GluN2A plus α2δ-1 (red, n = 10). Data are means ± s.e.m. (B, D and E, two-tailed Student’s t-test).
Figure S6. LRET nanopositioning system-based model of α2δ-1–NMDAR interaction, related to Figures 6A and 6B.

The LRET-determined distances to the acceptor fluorophore [59 Å for site 30 on GluN2A (cyan) and 57 Å for site 20 on GluN1 (magenta)] were used to generate the spheres: blue for GluN2A and green for GluN1. The extracellular domains of the α2δ-1 (orange) and NMDAR structures were moved to place the YFP fluorophore (yellow *) tagged to α2δ-1 at the plane of intersection of the LRET radii spheres with the additional constraint of having Cys1071 of the extracellular domain of α2δ-1 (red) near the membrane. There is currently no structural information for the transmembrane C-terminus (residues beyond 1071) of α2δ-1.
Figure S7. Treatment with gabapentin or α2δ-1Tat peptide does not affect VACC currents or the α2δ-1–Cav2.2 interaction, related to Figure 6.

(A) Original current traces show the I_{Ba} of N-type Ca^{2+} channels reconstituted in HEK293 cells treated with vehicle, gabapentin (GBP, 100 μM) or α2δ-1Tat peptide (1 μM) for 60 min. Whole-cell I_{Ba} was elicited by a depolarizing pulse to 0 mV for 200 ms from a holding potential of –90 mV.

(B) Mean data show no effect of GBP or α2δ-1Tat peptide on N-type Ca^{2+} channel currents in HEK293 cells. Data are means ± s.e.m. The numbers of cells recorded in each group are indicated in parentheses (cells from 4 separate experiments).

(C,D) Original gel images (C) and quantification data (D) show the lack of an effect of α2δ-1Tat peptide on the interaction between α2δ-1 and Cav2.2 in the spinal cord of SNL and sham rats (n = 6 rats in each group). The dorsal spinal cord at the L5 and L6 level was removed 60 min after intrathecal injection of α2δ-1Tat peptide (1 μg) or control peptide (1 μg). Membrane proteins were immunoprecipitated first with a rabbit anti–α2δ-1 or anti-IgG antibody. Western immunoblotting was performed by using an anti-Cav2.2 antibody. S, sham; L, SNL; C, control peptide; P, α2δ-1Tat peptide. Data are means ± s.e.m.
Figure S8. Treatment with gabapentin or α2δ-1Tat peptide attenuates pain hypersensitivity induced by overexpression of Cacna2d1 in rats, related to Figure 6.

(A,B) Effects of a single intrathecal injection of α2δ-1Tat peptide (1 µg) or gabapentin (GBP, 30 µg) on the tactile (A) and pressure (B) withdrawal thresholds in rats treated with the Cacna2d1 vector or control vector (n = 7 rats in each group). Data are means ± s.e.m. *P < 0.05, **P < 0.01 (versus baseline before drug injection). One-way ANOVA analysis followed by Dunnett’s post hoc test.
Figure S9. Disruption of α2δ-1–NMDAR association attenuates nerve injury-induced increases in NMDAR activity of spinal dorsal horn neurons, related to Figure 7.

(A) Mean effects of α2δ-1Tat peptide (0.01, 0.1 and 1 μM) or scrambled control peptide (1 μM) on currents elicited by puff application of 100 μM NMDA to spinal dorsal horn neurons of SNL rats. Data are means ± s.e.m. *P < 0.05 (versus control peptide group). One-way ANOVA analysis followed by Dunnett’s post hoc test.

(B) Representative recordings and mean data show the AP5 effect on the amplitude of EPSCs of spinal dorsal horn neurons monosynaptically evoked by dorsal root stimulation in SNL rat spinal cord slices treated with 0.01, 0.1 and 1 μM α2δ-1Tat peptide or control peptide. Data are means ± s.e.m. *P < 0.05 (versus respective baseline). The numbers of cells recorded in each group are indicated in parentheses. One-way ANOVA analysis followed by Dunnett’s post hoc test.
Table S1. Lifetimes of terbium-labeled NMDAR subunits and YFP-tagged α2δ-1 constructs transfected in HEK293 cells, related to Figures 5A and 5B.

|                        | Donor-only lifetime (μs) | Donor-acceptor lifetime (μs) | Distance (Å) |
|------------------------|--------------------------|------------------------------|--------------|
| **GluN1*+GluN2A+YFP–α2δ-1** |                          |                              |              |
| No gabapentin          | 1,776 ± 1                | 1,312 ± 24                   | 57.1 ± 0.5   |
| With gabapentin        | 1,779 ± 1                | no LRET                      | > 95 #        |
| **GluN1*+GluN2A+YFP–α2δ-1 mutant** |                      |                              |              |
| No gabapentin          | 1,776 ± 1                | 1,319 ± 52                   | 57.3 ± 1.1   |
| With gabapentin        | 1,779 ± 1                | 1,344 ± 38                   | 57.9 ± 0.9   |
| **GluN1+GluN2A*+YFP–α2δ-1** |                          |                              |              |
| No gabapentin          | 1,755 ± 1                | 1,378 ± 15                   | 59.2 ± 0.4   |
| With gabapentin        | 1,770 ± 1                | no LRET                      | > 95 #        |
| **GluN1+GluN2A*+YFP–α2δ-1 mutant** |                      |                              |              |
| No gabapentin          | 1,755 ± 1                | 1,307 ± 21                   | 57.1 ± 0.5   |
| With gabapentin        | 1,770 ± 1                | 1,409 ± 16                   | 60.4 ± 0.7   |

n = 4 independent experiments in each condition. YFP-tagged α2δ-1 was used as the acceptor. *, terbium-labeled (donor) NMDAR subunits. #, $P < 0.05$ (versus the group without gabapentin). Mann-Whitney test.
Supplementary Experimental Procedures

Animal Models of Neuropathic Pain

All surgical preparations and experimental protocols were approved by the Animal Care and Use Committee of the University of Texas MD Anderson Cancer Center and conformed to the National Institutes of Health guidelines for the ethical use of animals. All animals were housed (2–3 rats per cage) in a standard 12:12 light–dark cycle with normal illumination. Male Sprague-Dawley rats (8–10 weeks of age) were purchased from Harlan Laboratories (Indianapolis, IN). We used L5 and L6 spinal nerve ligation (SNL) as an experimental model of neuropathic pain (Kim and Chung, 1992). In brief, we induced anesthesia with 2–3% isoflurane, isolated the left L5-L6 spinal nerves and ligated them tightly with 6–0 silk sutures. The rats in the sham control group underwent the same surgical procedures except for the nerve ligation.

We implanted intrathecal catheters in some SNL group rats during isoflurane-induced anesthesia 2–3 weeks after SNL surgery. Briefly, the anesthetized animal was placed in a stereotaxic frame, and a small incision was made at the back of its neck. Then, a small puncture was made in the atlanto-occipital membrane of the cisterna magna, and a PE-10 catheter (~8 cm) was inserted with the caudal tip reaching the lumbar enlargement of the spinal cord (Chen et al., 2000; Chen et al., 2014a). We then exteriorized the rostral end of the catheter and closed the wound with sutures. We allowed the animals to recover for at least 4 days before we performed the intrathecal injections. Animals displaying signs of motor or neurological dysfunction were immediately killed with an intraperitoneal injection of phenobarbital (200 mg/kg) or by inhalation of CO2.

The doses of memantine (Tocris Bioscience, Ellisville, MO) and (2R)-amino-5-phosphonopentanoate (AP5) (Abcam, Cambridge, MA) were selected based on previous studies (Chen et al., 2014a; Zhou et al., 2012). α2δ-1–specific siRNA (CAAGCAACGAAGUUGCUA) or universal negative-control siRNA (#SIC001, Sigma-Aldrich, St. Louis, MO) was mixed with i-Fect (Neuromics, Edina, MN) to a final concentration of 400 mg/L for the intrathecal injections. α2δ-1–specific siRNA or negative control siRNA (4 μg/day for 4 days) was administered intrathecally in the SNL rats 3 weeks after surgery (Laumet et al., 2015).

Cacna2d1 KO Mice

Conventional Cacna2d1 KO mice (C57BL/6 genetic background) were generated as described previously (Fuller-Bicer et al., 2009). Two breeding pairs of Cacna2d1+/− mice were purchased from Medical Research Council (Harwell Didcot, Oxfordshire, UK), and Cacna2d1−/- mice and Cacna2d1+/+ (wild-type) littermates were obtained by breeding the heterozygous mice. The spared nerve injury (SNI) procedure (Laedermann et al., 2014) was performed on both male and female mice (10–11 weeks of age). Under a surgical microscope, we ligated and sectioned the left common peroneal and tibial nerves, leaving the sural nerve intact. The sham procedure consisted of the same surgery without nerve ligation and sectioning.

Behavioral Assessments of Nociception

To detect tactile allodynia, we applied von Frey filaments to the animals’ left hindpaws (ipsilateral to the nerve injury). We placed the rats or mice individually in suspended chambers on a mesh floor. After an acclimation period of 30 min, we applied a series of calibrated von Frey filaments (Stoelting, Wood Dale, IL) perpendicularly to the plantar surface of the hindpaw with sufficient force to bend the filament for 6 s. Brisk withdrawal or paw flinching was considered a positive response. After a response, the filament of the next lower force was applied. In the absence of a response, the filament of the next greater force was applied. Using the up-down method, we calculated the tactile stimulus that produced a 50% likelihood of a withdrawal response (Chaplan et al., 1994).

To quantify the mechanical nociceptive threshold in the rats and mice (Chen et al., 2014a; Laumet et al., 2015), we conducted the paw pressure test on the hindpaw with an analgesiometer (Ugo Basile, Varese, Italy). The device was activated by pressing a foot pedal, which triggered a motor that applied a constantly increasing force on a linear scale. When the animal displayed pain by either withdrawing its paw or vocalizing, the pedal was immediately released, and the animal’s nociceptive threshold was read on the scale (Chen et al., 2014a; Chen and Pan, 2005).

We tested the thermal sensitivity in rats by placing them on the glass surface (maintained at 30°C) of a
testing apparatus (IITC Life Sciences, Woodland Hills, CA). A mobile radiant heat source located under the glass was focused onto the hindpaw of animals. The paw withdrawal latency was recorded with a timer, and the hindpaw was tested twice to obtain the average. To measure the heat withdrawal threshold in mice, we used the increasing-temperature hot-plate test (Hot Plate Analgesia Meter, IITC Life Sciences). The investigators conducting the behavioral experiments were blinded to the treatment.

**Lentiviral Vector Constructs and Preparation**

The full-length coding sequence of rat \( \alpha_2\delta-1 \) tagged with enhanced green fluorescent protein (GFP) at the N-terminus or GFP alone was cloned into the lentiviral vector pLenti6/V5-DEST with a cytomegalovirus promoter (Invitrogen, Carlsbad, CA). The viral vector was produced using the ViraPower system (Invitrogen) according to the manufacturer’s instructions. Briefly, vectors were transfected into HEK293FT cells using Lipofectamine 3000 (#L3000015, Invitrogen). The virus-containing supernatant was collected 72 h after transfection and filtered through Millex-HV 0.45-μm filters (Millipore, Bedford, MA). The viruses were purified and concentrated about 1,000-fold by centrifugation at 90,000 × g. The viral titer, measured by infecting HEK293FT cells with 10 × gradient-diluted virus, was about 10^8 infectious units/mL. Viral vectors (2 × 10^6 viral particles in 20 μl) expressing GFP-Cacna2d1 (Cacna2d1 vector) or GFP only (control vector) were slowly injected into the rats through an intrathecal catheter, and the catheter was then removed. After vector injection, all rats were placed in a restricted biohazardous housing area for 2 weeks before undergoing behavioral tests. We have previously demonstrated that intrathecal injection of the same lentiviral vector can transfect ~90% of spinal dorsal horn neurons in laminae I-III (Li et al., 2016).

**Electrophysiological Recordings in Spinal Cord Slices**

We induced anesthesia in rats or mice with isoflurane and removed the lumbar spinal cords via laminectomy. The spinal cords at the L5–L6 level were placed in ice-cold sucrose artificial cerebrospinal fluid containing (in mM) 234 sucrose, 3.6 KCl, 1.2 MgCl₂, 2.5 CaCl₂, 1.2 NaH₂PO₄, 12.0 glucose and 25.0 NaHCO₃, presaturated with 95% O₂ and 5% CO₂. The spinal cord tissue was glued onto the stage of a vibratome, and transverse slices (400 μm) of spinal cords were cut in ice-cold sucrose artificial cerebrospinal fluid and then preincubated in Krebs solution oxygenated with 95% O₂ and 5% CO₂ at 34°C for at least 1 h before being transferred to the recording chamber. The Krebs solution contained (in mM) 117.0 NaCl, 3.6 KCl, 1.2 MgCl₂, 2.5 CaCl₂, 1.2 NaH₂PO₄, 11.0 glucose and 25.0 NaHCO₃. The spinal cord slices were placed in a glass-bottom chamber and continuously perfused with Krebs solution at 5.0 ml/min at 34°C maintained by an inline solution heater and a temperature controller.

We filled a glass pipette (5–10 MΩ) with internal solution containing (in mM) 135.0 potassium gluconate, 5.0 TEA, 2.0 MgCl₂, 0.5 CaCl₂, 5.0 HEPES, 5.0 EGTA, 5.0 Mg-ATP, 0.5 Na-GTP and 10 lidocaine (lidocaine) N-ethyl bromide (adjusted to pH 7.2–7.4 with 1 M KOH; 290–300 mOsmol/L). The lamina II outer neurons were identified for recording because they receive nociceptive input from primary afferents and show increased synaptic N-methyl-D-aspartate receptor (NMDAR) activity after nerve injury (Chen et al., 2014b; Pan and Pan, 2004). It becomes impossible to visualize fluorescence-labeled projection neurons in other laminae in adult (>10-week-old) rodent spinal cords because of heavy laminar myelination. It has been shown that >85% neurons in lamina II are glutamate-releasing excitatory interneurons (Santos et al., 2007). Also, α2δ-1 is expressed in neurons and nerve terminals in the spinal lamina II (Cole et al., 2005; Taylor and Garrido, 2008). There is a large increase in synaptic NMDAR activity 2–3 weeks after SNL, although a small increase in evoked NMDAR activity of dorsal horn neurons occurs at days 3 and 7 after SNL (Chen et al., 2014b). In contrast, the NMDAR current in lamina I (putative ascending projection) neurons is very small and not affected by SNL (Chen et al., 2014b). Postsynaptic NMDAR currents were elicited by puff application of 100 μM NMDA to the recorded neuron using a positive pressure system (4 p.s.i., 15 ms; Toohey Company, Fairfield, NJ), and puff application of the vehicle produced no currents. The tip of the puff pipette was placed 150 μm away from the recorded neuron. To minimize the Mg²⁺ block of NMDARs, the puff NMDA currents were recorded in an extracellular solution containing no Mg²⁺, 10 μM glycine, and 1 μM tetrodotoxin at a holding potential of −60 mV (Chen et al., 2014a; Chen et al., 2014b). The pipette internal solution contained (in mM) 110.0 Cs₂SO₄, 2.0 MgCl₂, 0.1 CaCl₂, 1.1 EGTA, 10.0 HEPES, 2.0 Mg-ATP and 0.3 Na₂GTP (pH was adjusted to 7.25 with 1.0 M CsOH; 280–300
mOsmol/L).

In some experiments, the attached dorsal root was electrically stimulated with a stimulating electrode (0.5 ms, 0.6 mA and 0.1 Hz) to evoke monosynaptic excitatory postsynaptic currents (EPSCs). The α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPAR)-mediated EPSCs were recorded at a holding potential of −60 mV in the presence of 10 μM bicuculline and 1 μM strychnine, whereas NMDAR-mediated EPSCs were recorded at +40 mV in the presence of 10 μM 6-cyano-7-nitroquinoxaline-2,3-dione, 10 μM bicuculline, and 1 μM strychnine. Because the NMDAR channel is typically blocked by Mg2+ at a negative holding potential, the NMDAR-mediated EPSCs were recorded at a holding potential of +40 mV to remove the Mg2+ block of NMDAR channels (Chen et al., 2014a; Chen et al., 2014b).

Miniature EPSCs (mEPSCs) were recorded at a holding potential of −60 mV in the presence of 1 μM strychnine, 10 μM bicuculline and 1 μM tetrodotoxin, and presynaptic NMDAR-mediated glutamate release was tested by bath application of 50 μM AP5 (Chen et al., 2014a; Chen et al., 2014b). mEPSCs are predominantly mediated by AMPA receptors, and the postsynaptic NMDARs are minimally open under our recording conditions for mEPSCs (due to magnesium block at −60 mV). The input resistance was monitored, and the recording was abandoned if the input resistance changed more than 15%. All signals were recorded using an amplifier (MultiClamp700B; Axon Instruments Inc., Union City, CA), filtered at 1–2 kHz, digitized at 10 kHz, and stored for off-line analysis. Final spinal cord slice recordings were performed 3–4 weeks after the nerve injury or sham surgery. In all electrophysiological experiments, 3–5 animals were used for each recording protocol, and only 1 neuron was recorded in each spinal cord slice.

Gabapentin was obtained from Tocris Bioscience. The α2δ-1Tat peptide and scrambled control peptide were synthesized by Bio Basic Inc. (Marham, Ontario, Canada) and validated by using liquid chromatography and mass spectrometry.

**Western Immunoblotting Analysis of α2δ-1**

Western blotting was used to quantify the α2δ-1 expression level in the DRG and dorsal spinal cord. Spinal cord and DRG tissues at the L5 and L6 levels were removed, dissected, and homogenized in 300 μl radioimmunoprecipitation assay buffer containing 50 mM Tris-HCl (pH 7.4), 1% Nonidet P-40, 0.25% sodium-deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM Na3VO4, and 1 mM NaF in the presence of a protease inhibitor cocktail (Sigma-Aldrich). Samples were then put on ice for 30 min with shaking. Lysates were centrifuged at 13,000 × g for 30 min at 4°C. The supernatant was carefully collected, and the protein concentration was measured using a DC Protein Assay Kit (Bio-Rad, Hercules, CA). Thirty μg of total proteins from each sample was loaded and separated by 4–15% Tris-HCl sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Bio-Rad). The resolved proteins were transferred to an Immobilon-P membrane (Millipore). The membrane was treated with 5% nonfat dry milk in Tris-buffered saline (TBS) at 25°C for 1 h and then incubated in TBS supplemented with 0.1% Triton X-100, 1% bovine serum albumin and rabbit anti-α2δ-1 (#C5105, 1:1,000, Sigma-Aldrich) overnight at 4°C. The membrane was washed 3 times and then incubated with horseradish peroxidase-conjugated anti-rabbit IgG (1:5,000; Jackson ImmunoResearch, West Grove, PA) for 1 h at room temperature. The protein band detected by using the α2δ-1 antibody was confirmed to be specific, because this band was absent in tissues obtained from Cacna2d1 KO mice. The protein bands were detected with an ECL kit (Thermo Fisher Scientific, Waltham, MA), and protein band intensity was visualized and quantified using the Odyssey Fc Imager (LI-COR Biosciences, Lincoln, NE). The amount of α2δ-1 protein in the DRG and spinal cord tissues was normalized to the amount of GAPDH in the same gels.

**Coimmunoprecipitation Using Spinal Cord Tissue Membrane Extracts**

The dorsal quadrants of rat spinal cords at the L5 and L6 levels or the frozen lumbar spinal cord tissues from 4 human donors (2 males and 2 females, age range 18–42 years, postmortem interval of 17–29 h; supplied by the University of Maryland Brain and Tissue Bank) were dissected and homogenized in ice-cold hypotonic buffer (20 mM Tris [pH 7.4], 1 mM CaCl2, 1 mM MgCl2, and protease inhibitors) for membrane preparation. The nuclei and unbroken cells were removed by centrifugation at 300 × g for 5 min. The supernatant was centrifuged for 20 min at 21,000 × g. The pellets were re-suspended and solubilized in immunoprecipitation buffer (50 mM
Tris [pH 7.4], 250 mM NaCl, 10% glycerol, 0.5% NP-40, 20 mM NaF, 1 mM Na3VO4, 10 mM N-ethylmaleimide, 1 mM phenylmethylsulfonyl fluoride, 2 mM benzamidine, and protease inhibitors), and the soluble fraction was incubated at 4°C overnight with Protein A/G beads (#16-266, Millipore) prebound to mouse anti-GluN1 antibody or rabbit anti-α2δ-1 antibody. Protein A/G beads prebound to mouse IgG or rabbit IgG were used as controls. Samples were washed 5 times with immunoprecipitation buffer and then immunoblotted. The pull-down efficiency with α2δ-1 and GluN1 antibodies was ~30% and ~45%, respectively. The following antibodies were selected for immunoblotting on the basis of previously published studies (Bourdin et al., 2015; Cheng et al., 2013; Cordeira et al., 2014; Fourgeaud et al., 2010; Li et al., 2014a): rabbit anti-α2δ-1 (#C5105, 1:1,000, Sigma-Aldrich; #ACC-015, Alomone Labs, Jerusalem, Israel), rabbit anti-α2δ-2 (#ACC-102, 1:500, Alomone Labs), rabbit anti-α2δ-3 (#ACC-103, 1:500, Alomone Labs), rabbit anti-Cav2.2 (#ACC-002, 1:500, Alomone Labs), mouse anti-GluN1 (#75-272, 1:1,000, NeuroMab, Davis, CA), mouse anti-GluN2A (#75-288, 1:1,000, NeuroMab), and mouse anti-GluN2B (#75-097, 1:1,000, NeuroMab). The amount of α2δ-1 and GluN1 proteins in the spinal cord tissues was normalized to the amount of their respective proteins in the input lanes in the same gels.

**Spinal Cord Synaptosome Preparation**

The spinal cord slices were pooled from 2 rats and were homogenized using glass-Teflon homogenizer in 10 volumes of ice-cold HEPES-buffered sucrose (0.32 M sucrose, 1 mM EGTA, and 4 mM HEPES at pH 7.4) containing a protease inhibitor cocktail (Sigma-Aldrich). The homogenate was centrifuged at 1,000 × g for 10 min at 4°C to remove the nuclei and large debris. The supernatant was centrifuged at 10,000 × g for 15 min to obtain the crude synaptosomal fraction. The synaptosomal pellet was lysed via hypo-osmotic shock in 9 volumes of ice-cold HEPES buffer with the protease inhibitor cocktail for 30 min. The lysate was centrifuged at 25,000 × g for 20 min at 4°C to obtain the synaptosomal membrane fraction, which was then dissolved in sodium dodecyl sulfate sample buffer at a final concentration of 0.25 μg/μl for immunoblotting. The amount of α2δ-1 and NMDAR proteins in the spinal cord synaptosomes was normalized to the amount of PSD-95 in the same gels.

**Quantitative PCR Analysis**

Total RNA was extracted from the DRG and spinal cord tissues at the L5 and L6 levels using TRIzol-chloroform and then treated with DNase I (Invitrogen). cDNA was prepared by using the SuperScript III First-Strand Synthesis Kit and then treated with RNase H (Invitrogen). Quantitative PCR was performed using the iQ5 Real-Time PCR Detection System (Bio-Rad, Hercules, CA) and SYBR Green (Bioline, Taunton, MA). The following primers were used: rat α2δ-1 forward, CGAGCATGATGAGACACCTG; rat α2δ-1 reverse, TTATGGCAGCAGGCTGAG; rat α2δ-2 forward, GGCCTCTGCCTACAGCTTCC; rat α2δ-2 reverse, CAGGTTCCGATTGTCCTTGT; rat Gapdh forward, TGCCACTCAGAAGACTGTGG; rat Gapdh reverse, TTCAGCTCTGGGATGACCTT; rat α2δ-1R217A forward, ACGCCAACTGTGTTAAATTC; mouse α2δ-1 reverse, CTTGCAAAATCTTCCCTCCA; mouse Gapdh forward, GGGTGTAACACCACGAAAT; and mouse Gapdh reverse, CTTCCCACATGCAAGAT. Relative mRNA levels were calculated using the 2-ΔΔCT method and normalized to the Gapdh level in the same sample.

**DNA Constructs**

The rat cDNAs of the NMDAR subunits GluN1-1a, GluN2A, and GluN2B and α2δ-1 (encoded by Cacna2d1) were all expressed in the expression vector pcDNA 3.1. The α2δ-2 (encoded by Cacna2d2) and α2δ-3 (encoded by Cacna2d3) constructs were obtained from Addgene (Cambridge, MA). To generate N-terminal tagged fusion constructs (FLAG-GluN1, CFP-GluN1 and YFP–α2δ-1), the coding sequences of the tags FLAG, cyan fluorescent protein (CFP) and yellow fluorescent protein (YFP) were inserted after the predicted signal peptide cleavage site of GluN1 and α2δ-1 by using recombinant PCR techniques. The NMDAR currents reconstituted with these constructs were similar to those of wild-type GluN1 and α2δ-1 in HEK293 cells. The Cacna2d1 coding sequence was inserted between the Nhe I and EcoR I sites of the pIREs-AcGFP1 vector, which was used for electrophysiological recording. The mutant α2δ-1R217A was generated by mutating the gabapentin binding site on α2δ-1 (the R217A mutant was also termed R241A when amino acid numbering included the N-terminal signal sequence of α2δ-1). The von Willebrand factor type A (VWA) domain of α2δ-1 (residues
239–417) was deleted to generate α2δ-1AVWA.

The chimeras of α2δ-1 (which swapped the N-terminus or δ peptide of α2δ-1 for that of α2δ-2 or α2δ-3) were generated based on multiple sequence alignment using the ClustalW2 program. α2δ-1NTG(α2δ-2) and α2δ-1NTG(α2δ-3) were generated by replacing the α2δ-1 N-terminus (residues 25–237) with the N-terminal domain of α2δ-2 (residues 65–278) and α2δ-3 (residues 34–240), respectively. The δ peptide of α2δ-1 (residues 945–1091) was replaced with the δ part of α2δ-2 (residues 1001–1157) or α2δ-3 (residues 951–1085) to generate α2δ-1ΔG(α2δ-2) and α2δ-1ΔG(α2δ-3), respectively. α2δ-1C(α2δ-2) and α2δ-1C(α2δ-3) were generated by replacing the C-terminus of α2δ-1 (residues 1059–1091) with the C-terminus of α2δ-2 (residues 1111–1157) and α2δ-3 (residues 1061–1085), respectively. The amino acid numbering includes the N-terminal signal sequence of α2δ-1.

**HEK293 Cell Culture, Transfection and Whole-Cell Recording of NMDAR Currents**

HEK293 cells (ATCC, Manassas, VA) were maintained in Dulbecco's modified Eagle medium (DMEM) (#10-017-CV, Corning, Manassas, VA) supplemented with 10% fetal bovine serum (#F4135, Sigma-Aldrich) and penicillin/streptomycin (#30-002-CI, Corning). Cells were passaged after they reached a confluence of 80–90%, approximately every 2 days. HEK293 cells were transfected using PolyJet reagents (#SL100688, SignaGen Laboratories, Gaithersburg, MD) at a GluN1/GluN2/δ ratio of 1:1:1. The amino acid numbering includes the N-terminal signal sequence of α2δ-1. The constructs were made using the QuickChange Site-Directed Mutagenesis Kit (#200521, Agilent Technologies, Santa Clara, CA) or In-Fusion HD Cloning Plus (#638916, Clontech Laboratories, Inc., Mountain View, CA). All cDNA clones made using the QuickChange Site-Directed Mutagenesis Kit (#200521, Agilent Technologies, Santa Clara, CA) or In-Fusion HD Cloning Plus (#638916, Clontech Laboratories, Inc., Mountain View, CA). All cDNA clones and mutated constructs were confirmed by DNA sequencing.

Whole-cell recording was performed using a fire-polished pipette (3–5 MΩ) pulled from borosilicate glass and filled with an intracellular solution containing (in mM) 135 CsF, 2 MgCl2, 0.5 CaCl2, 5 EGTA, 2 Mg-ATP and 10 HEPES (pH, 7.25; 285 mOsmol/L). The extracellular solution contained (in mM) 160 NaCl, 2.5 KCl, 0.2 CaCl2, 10 HEPES, and 10 glucose (pH, 7.35; 300–310 mOsmol/L). Whole-cell currents were recorded with an EPC-10 amplifier (HEKA Instruments, Lambrecht, Germany) at a holding potential of −60 mV. After the whole-cell configuration was established, the cell membrane capacitance and series resistance were electronically compensated for. Glycine (10 μM) and NMDA (300 μM) were used to elicit NMDAR currents. For voltage-dependent Mg2+ block experiments, the conductance-voltage relation of NMDARs was assessed when 2 mM MgCl2 was added to the extracellular solution. The sensitivity of NMDARs to Mg2+ was estimated by fitting current-voltage relationships to a Boltzmann function (Chen and Huang, 1992). All HEK293 cell recording experiments were performed at 25°C.

**Cell Culture, Transfection and Recording of VACC Currents in HEK293 Cells**

HEK293 cells were cultured in DMEM supplemented with 10% fetal bovine serum (Sigma-Aldrich) in an incubator at 37°C in 5% CO2. For cell transfection, 1.2 × 10^4 cells were plated on poly-D-lysine–coated coverslips in each well of a 24-well plate. After 24 h, PolyJet reagents were used to transiently transfect the cells with Cav2.2, Cavβ1 and α2δ-1 subunits at a ratio of 1:1:1 (Zhou et al., 2015). Electrophysiological recordings were performed 48 h after transfection.

Whole-cell recordings were performed using an EPC-10 amplifier. The whole-cell Ca2+ currents carried by barium (I_Ba) were recorded using an extracellular recording solution containing (in mM) 140 TEA, 2 MgCl2, 3 BaCl2, 10 glucose and 10 HEPES (pH, 7.4; 320 mOsm). Electrodes (resistance, 4-6 MΩ) were filled with pipette solution (in mM) 120 CsCl, 1 MgCl2, 10 HEPES, 10 EGTA, 4 MgATP and 0.3 NaGTP (pH, 7.2; osmolarity, 300 mOsm). To test the drug's effect on I_Ba, transfected cells were preincubated with 100 μM gabapentin or 1 μM α2δ-1Tat peptide for 60 min. The cell membrane capacitance and series resistance were electronically compensated. Signals were filtered at 1 kHz, digitized at 10 kHz and acquired using the Pulse program (HEKA Instruments).

**Outside-Out Patch Recording of NMDAR Currents in HEK293 Cells**

Outside-out patches were excised from transfected HEK293 cells with thick-walled borosilicate glass pipettes of 2-5 MΩ resistance coated with bees wax, fire polished and filled with a solution containing (in mM)
135 CsF, 33 CsOH, 11 EGTA, 10 HEPES, 2 MgCl₂ and 1 CaCl₂. The external solution was composed of (in mM) 150 NaCl, 10 HEPES, 10 tricine, 1 CaCl₂ and 100 μM added glycine (pH 7.4 with NaOH). All recordings were performed at 25°C and a holding potential of −80 mV (Axopatch 200B, Molecular Devices, Sunnyvale, CA). Data were acquired at 40 kHz using pCLAMP 10 software and filtered at 10 kHz. Series resistance was compensated for ~95% where the absolute amplitude exceeded 100 pA. Solution exchange was performed using a piezoelectric system (#LSS-3000, Burleigh Instruments) and in-house triple-barrel glass application pipettes as described previously (MacLean et al., 2014). Solution exchange times as measured from open tip potentials were between 100 and 300 μs (10–90% rise time). For MK-801 experiments, patches were first equilibrated in the control solution from the first barrel, then jumped to the second barrel perfusing glutamate (1 mM) and finally jumped to the third barrel perfusing glutamate plus MK-801 (300 nM). Mean responses from each patch were fit with either double-exponential (deactivation) or single-exponential (desensitization and MK-801 inhibition) equations (Borschel et al., 2015; Hansen et al., 2013). For deactivation experiments, the weighted time constant was reported.

Coimmunoprecipitation Using HEK293 Cell Membrane Extracts

HEK293 cells were transfected with FLAG–GluN1-1a, GluN2A/GluN2B, YFP–α2δ-1, α2δ-1 mutants, α2δ-2 or α2δ-3 when cells were grown to 80–90% confluence in 75-cm² culture flasks. HEK293 cells expressing NMDARs and α2δ-1 were suspended, washed in phosphate-buffered saline (PBS) twice and incubated at 25°C for 20 min with a rabbit anti-FLAG antibody (#F7425, Sigma-Aldrich) to immunoprecipitate FLAG-tagged GluN1. A mouse anti-GFP antibody (#75-132, NeuroMab, Davis, CA), which cross-reacts with YFP, was used to immunoprecipitate YFP-tagged wild-type α2δ-1 or α2δ-1 mutants. Cells were then washed 3 times in PBS and lysed in Pierce IP Lysis Buffer (#87787, Thermo Fisher Scientific) with a cocktail of protease and phosphatase inhibitors (#78440, Thermo Fisher Scientific). The lysates were incubated on ice for 30 min and then centrifuged at 13,000 × g for 10 min at 4°C. The supernatant was transferred to a tube with Dynabeads (#147187300, Thermo Fisher Scientific) and incubated with rotation at 25°C for 20 min. The Dynabeads-antibody-antigen complex was washed 3 times using 200 μl IP Lysis Buffer for each wash. The IP Lysis Buffer with 100 μg/ml immunogen peptides of FLAG (#F3290, Sigma-Aldrich) was added to elute the coimmunoprecipitation complex. The eluted proteins were subjected to Western blot analysis with the following antibodies: rabbit anti-GluN1 (#G8913, Sigma-Aldrich), rabbit anti–α2δ-1 (#C5105, Sigma-Aldrich), rabbit anti-GFP (#G1544, Sigma-Aldrich), rabbit anti-GluN2A (#1500-NR2A, PhosphoSolutions, Aurora, CO) and mouse anti-GluN2B (#5580S, Cell Signaling, Danvers, MA).

Isolation and Analysis of HEK293 Cell Membrane Surface Proteins.

HEK293 cells were transfected with GluN1-1a, GluN2A/GluN2B and/or α2δ-1 after they had grown to 80–90% confluence in 75-cm² culture flasks. Cell surface biotinylation was performed using the Pierce Cell Surface Protein Isolation Kit (#89881, Thermo Fisher Scientific) according to the manufacturer’s instructions. Briefly, cells were incubated with Sulfo-NHS-SS-biotin, which covalently binds to primary amino groups of extracellular proteins at 4°C with constant rotation for 30 min. Excess biotin was quenched with quenching solution (Li et al., 2014b). The cells were washed, harvested by gentle scraping and lysed using the provided lysis buffer in the cocktail of protease and phosphatase inhibitors (#78440, Thermo Fisher Scientific) for 30 min at 4°C. The lysates were then centrifuged for 2 min at 10,000 × g at 4°C, and the clear supernatants were added to NeutrAvidin agarose and incubated for 60 min at 25°C with end-over-end mixing. The unbound (unbiotinylated) proteins, representing the intracellular fraction, were separated from the captured surface proteins by centrifugation of the column. Finally, the captured surface proteins were eluted from the biotin-NeutrAvidin agarose by incubation with dithiothreitol in SDS-PAGE sample buffer and subjected to Western blot analysis with antibodies against GluN1, GluN2A, GluN2B and α2δ-1. Na⁺/K⁺ ATPase (#ab7671, Abcam), a specific plasma membrane marker, was used as an internal control. Protein bands were quantified using Image Studio software (version 3.1, LI-COR Biosciences).

Luminescence Resonance Energy Transfer (LRET) Measurements

For constructs used for LRET measurements, the extracellular non–disulfide-bonded cysteines were
mutated to serines so that they were not labeled with the maleimide reactive fluorophore. For the GluN1 subunit, Cys22 and Cys459 were mutated; for the GluN2A subunit, Cys231, Cys399 and Cys461 were mutated. For measurement of LRET between GluN1 and \(\alpha_2\delta_1\), Cys22 was not mutated and was used as the site for attachment of the thiol-reactive fluorophore. Additionally, for quantitative analysis of the background, a thrombin recognition sequence (amino acids LVPRGS) was inserted immediately at the C-terminal to the cysteine (Sirrieh et al., 2015). For measurement of LRET between GluN2A and \(\alpha_2\delta_1\), a cysteine followed by the thrombin recognition sequence was inserted after Lys29 (Cys30). The thrombin cleavage site allowed subtraction of the background signal from free cysteines of \(\alpha_2\delta_1\) and other proteins on the surface of HEK293 cells (Sirrieh et al., 2013; Sirrieh et al., 2015), thus providing the specific measurement of the LRET signal between the NMDAR and \(\alpha_2\delta_1\).

Cysteines at the GluN1 and GluN2A sites labeled with thiol-reactive terbium chelate served as donor fluorophore sites, and YFP fused to \(\alpha_2\delta_1\) served as the acceptor fluorophore. HEK293 cells transfected with GluN1, GluN2A and YFP–\(\alpha_2\delta_1\) were harvested and labeled with 200 nM terbium chelate (Invitrogen) for 1 h at 25°C. After labeling, cells were washed twice in extracellular buffer composed of (in mM) 150 NaCl, 2.8 KCl, 1 CaCl2 and 5 HEPES (pH 7.3). The washed and labeled cells were then re-suspended in extracellular buffer and probed in a cuvette-based LRET analysis. The sample was excited at 337 nm, and emission was detected at 527 nm in a QuantaMaster QM3-SS system with Fluorescan software (Photon Technology International, Edison, NJ). The temperature was maintained at 20°C using a Peltier TE temperature controller. Data were analyzed with Origin 8.6 software (OriginLab Corp., Northampton, MA). Each sample was scanned 3 times for each ligated condition, and each scan was recorded as an average of 99 sweeps. To determine the gabapentin effect, cells were maintained in 100 \(\mu\)M gabapentin (Tocris Bioscience) for 30 min before terbium labeling. The distance between the donor and acceptor was calculated using the Förster equation (Sirrieh et al., 2015).

**LRET Nanopositioning System-based Model of \(\alpha_2\delta_1\)–NMDAR Interaction**

With the distances obtained from the LRET measurements, we used a method similar to previously reported nanopositioning methods (Muscieloik and Michaelis, 2011; Shaikh et al., 2016). Spheres were drawn around the positions of the donor fluorophore on the NMDAR (PDB ID: 4EP5) using the LRET-determined distances as radii. Using the extracellular domain of the \(\alpha_2\delta_1\) structure (PDB ID:5GJV) tagged with YFP at the N-terminus, a series of rigid-body translations and rotations were performed to superpose the acceptor fluorophore on the plane of intersection of the donor fluorophore spheres, with the additional constraint of maintaining the C-terminus of the extracellular domain of \(\alpha_2\delta_1\) near the membrane. The model that best fit the distance with minimal steric clashes positioned the extracellular domain of \(\alpha_2\delta_1\) next to the extracellular domains of the NMDAR. While the precise location of \(\alpha_2\delta_1\) is hard to predict based on the LRET distances, the LRET distance measurements indicated that it is in close proximity to and has extensive interactions with the extracellular domains of the NMDAR. The EM structure of \(\alpha_2\delta_1\) ends at Cys1071 (Wu et al., 2016), and there is currently no structural information for the C-terminus (residues beyond 1071) of \(\alpha_2\delta_1\).

**Data and Statistical Analysis**

Data are presented as means ± s.e.m. No statistical methods were used to predetermine sample sizes for biochemical studies, but our sample sizes were similar to those generally employed in the field. Data collection was randomized, and data distribution was determined using the Kolmogorov-Smirnov normality test. For proper exclusion of data points, the criteria were established before data collection. Animals in which motor function was impaired after intrathecal cannulation or treatment were excluded. In electrophysiological recording experiments, we monitored cell capacitance, input resistance, series resistance, resting membrane potential, and baseline holding current; we excluded cells if the recording indicated a rundown condition. The PulseFit software program (HEKA Instruments) was used to analyze the whole-cell current data obtained from transfected HEK293 cells. The amplitude of evoked EPSCs and NMDAR currents recorded from spinal dorsal horn neurons was analyzed with Clampfit 9.2 software (Axon Instruments). The amplitude and frequency of mEPSCs were analyzed off-line with a peak detection program (MiniAnalysis; Synaptosoft Inc., Decatur, GA). The cumulative probability of the amplitude and interevent interval was compared using the Kolmogorov-Smirnov test, which estimates the probability that two cumulative distributions are similar. We used a two-tailed Student’s t test to compare two groups and one-way or two-way analysis of variance (ANOVA, followed by Dunnett’s and Tukey’s post hoc tests).
to compare more than two groups. We used the nonparametric analysis (i.e., the Mann-Whitney or Kruskal-Wallis test) when electrophysiological and behavioral data were not normally distributed. Statistical analyses were performed using Prism software (version 7, GraphPad Software Inc., La Jolla, CA). The level of significance was set at $P < 0.05$.

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