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The α2δ-1-NMDA Receptor Complex Is Critically Involved in Neuropathic Pain Development and Gabapentin Therapeutic Actions

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SUMMARY

α2δ-1, commonly known as a voltage-activated Ca^{2+} channel subunit, is a binding site of gabapentinoids used to treat neuropathic pain and epilepsy. However, it is unclear how α2δ-1 contributes to neuropathic pain and gabapentinoid actions. Here, we show that *Cacna2d1* overexpression potentiates presynaptic and postsynaptic NMDAR activity of spinal dorsal horn neurons to cause pain hypersensitivity. Conversely, *Cacna2d1* knockdown or ablation normalizes synaptic NMDAR activity increased by nerve injury. α2δ-1 forms a heteromeric complex with NMDARs in rodent and human spinal cords. The α2δ-1-NMDAR interaction predominantly occurs through the C terminus of α2δ-1 and promotes surface trafficking and synaptic targeting of NMDARs. Gabapentin or an α2δ-1 C terminus-interfering peptide normalizes NMDAR synaptic

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SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, nine figures, and one table and can be found with this article online at https://doi.org/10.1016/j.celrep.2018.02.021.

AUTHOR CONTRIBUTIONS

J.C. performed chimera construction, HEK293 cell recordings, and *in vitro* coimmunoprecipitation. L.L. performed tissue coimmunoprecipitation, *Cacna2d1* overexpression, and behavioral experiments. S.-R.C. conducted spinal cord slice recordings, animal surgeries, and *Cacna2d1* KO mouse breeding. H.C. prepared spinal cord slices and performed slice recordings. J.-D.X. performed intrathecal injections and behavioral testing. R.E.S. performed LRET experiments. D.M.M. performed outside-out patch recordings. Y.Z. performed siRNA injection and behavioral testing. M.-H.Z. conducted VACC recordings. J.C., L.L., S.-R.C., R.E.S., D.M.M., V.J., and H.-L.P. analyzed the data. H.-L.P. conceived the project and wrote the manuscript, with input from the other authors.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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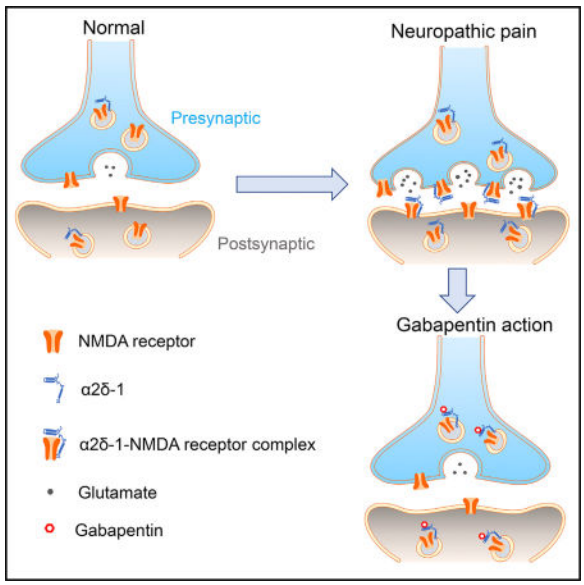
targeting and activity increased by nerve injury. Thus, α2δ-1 is an NMDAR-interacting protein that increases NMDAR synaptic delivery in neuropathic pain. Gabapentinoids reduce neuropathic pain by inhibiting forward trafficking of α2δ-1-NMDAR complexes.

In Brief



Chen et al. show that α2δ-1, through its C terminus, physically interacts with NMDA receptors and promotes synaptic expression of α2δ-1-NMDA receptor complexes in neuropathic pain. Gabapentin reduces neuropathic pain primarily by targeting α2δ-1-bound NMDA receptors.

INTRODUCTION

Chronic neuropathic pain is a major medical problem that remains difficult to treat. α2δ-1 (encoded by *Cacna2d1*) is expressed in the dorsal root ganglion (DRG) and spinal superficial dorsal horn neurons (Cole et al., 2005), and nerve injury increases α2δ-1 expression levels in both locations (Luo et al., 2001). Although α2δ-1 plays a key role in neuropathic pain (Li et al., 2004; Patel et al., 2013), it is unclear exactly how α2δ-1 causes neuropathic pain. α2δ-1 is commonly known to be a subunit of voltage-activated Ca^{2+} channels (VACCs) (Dolphin, 2012), but quantitative proteomic analysis indicates that α2δ-1 has a weak interaction with VACCs in the brain tissue (Müller et al., 2010). Gabapentinoids, including gabapentin and pregabalin, bind to α2δ-1 (Fuller-Bicer et al., 2009; Gee et al., 1996; Marais et al., 2001) and are widely used to treat neuropathic pain and epilepsy (Anhut et al., 1994; Dworkin et al., 2010; Rowbotham et al., 1998). However, gabapentinoids have little effect on VACC activity (Rock et al., 1993; Schumacher et al., 1998) or VACC-mediated neurotransmitter release at presynaptic terminals (Brown and Randall, 2005; Hoppa et al., 2012). Alternatively, gabapentin may reduce α2δ-1 interaction with thrombospondin, an astrocyte-secreted protein, and inhibit new synapse formation (but not already formed synapses) (Eroglu et al., 2009). Nevertheless, this action cannot fully account for the relatively rapid onset of gabapentinoid effects on pain hypersensitivity. A

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recent study suggests that the association between α2δ-1 and thrombospondin is rather weak and that there is no α2δ-1-thrombospondin interaction on the cell surface (Lana et al., 2016). Thus, the molecular mechanisms responsible for the therapeutic effects of gabapentinoids remain a long-standing puzzle.

Because α2δ-1 does not affect neuronal function independently, it is critically important to identify the relevant interacting proteins that involve it in neuropathic pain and gabapentinoid actions. The NMDA-type glutamate receptor (NMDAR) plays an essential role in learning, memory, synaptic plasticity, and neuropathic pain. Nerve injury potentiates NMDAR activity, at both pre- and postsynaptic sites, in the spinal dorsal horn (Chen et al., 2014b; Li et al., 2016). However, the molecular mechanisms underlying increased NMDAR activity and excitatory synaptic transmission in neuropathic pain remain unclear. Here we present our striking findings that α2δ-1, through its C-terminal domain, forms a heteromeric complex with NMDARs to potentiate their synaptic trafficking and excitatory synaptic transmission and that interrupting the α2δ-1-NMDAR interaction reverses the synaptic NMDAR hyperactivity associated with neuropathic pain. This new information redefines our understanding of the α2δ-1 function and the role of α2δ-1 in neuropathic pain development. Our findings also challenge the conventional perception about the molecular action of gabapentinoids, showing that α2δ-1-bound NMDARs, rather than VACCs, are the relevant target of gabapentinoids.

RESULTS

Cacna2d1 Overexpression Causes NMDAR-Mediated Pain Hypersensitivity

To study the relationship between α2δ-1 and NMDARs, we first determined whether *Cacna2d1* overexpression at the spinal cord level increased NMDAR activity in spinal dorsal horn neurons. We used intrathecal injection of lentiviral vectors, which effectively induce transgene expression in both spinal cord and DRG neurons (Li et al., 2016). Transfection with lentiviral vectors expressing GFP-*Cacna2d1* significantly increased α2δ-1 protein levels in the DRG and dorsal spinal cord in rats (Figures S1A and S1B). *Cacna2d1* overexpression caused long-lasting tactile allodynia and mechanical and thermal hyperalgesia, whereas injection of lentiviral vectors expressing GFP alone had no effect (Figure 1A). The pain hypersensitivity induced by *Cacna2d1* overexpression was readily reversed by intrathecal injection of (2R)-amino-5-phosphonopentanoate (AP5), a specific NMDAR antagonist, or systemic injection of memantine, a clinically used NMDAR antagonist (Figures 1B and 1C).

The first sensory synapse formed by central terminals of primary afferent neurons and spinal dorsal horn neurons is critically involved in nociceptive transmission and regulation. Electrophysiological recordings in spinal cord slices showed that *Cacna2d1* overexpression significantly increased postsynaptic NMDAR currents elicited by puff application of NMDA to the recorded neuron (Figure 1D). *Cacna2d1* overexpression also significantly potentiated presynaptic NMDAR activity, as reflected by the increase in the AP5-sensitive frequency of miniature excitatory postsynaptic currents (mEPSCs) of dorsal horn neurons (Chen et al., 2014a; Li et al., 2016) (Figures 1E and 1F). The increase in the mEPSC frequency induced by *Cacna2d1* overexpression was normalized by AP5 application within 5 min; therefore,

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the excitatory synaptic transmission potentiated by α2δ-1 is fully maintained by NMDARs. These data indicate that increased α2δ-1 expression at the spinal cord level augments pre- and postsynaptic NMDAR activity and leads to NMDAR-mediated pain hypersensitivity.

α2δ-1 Is Essential for Increased Pre- and Postsynaptic NMDAR Activity in Neuropathic Pain

We next used intrathecally injected small interfering RNA (siRNA) (Laumet et al., 2015) to assess the contribution of α2δ-1 to the increased spinal NMDAR activity in rats that have undergone spinal nerve ligation (SNL), a commonly used animal model of neuropathic pain. Intrathecal injection of *Cacna2d1*-specific siRNA reduced the expression level of α2δ-1, but not α2δ-2, in the DRG and dorsal spinal cord and reduced pain hypersensitivity in SNL rats (Figures S2A–S2C). Treatment with *Cacna2d1*-specific siRNA had no effect on the withdrawal thresholds in naive control rats (Figure S2D). Remarkably, treatment with *Cacna2d1*-specific siRNA completely normalized SNL-induced increases in puff NMDA currents and the baseline frequency of the mEPSCs of dorsal horn neurons (Figures 2A and 2B).

To confirm that α2δ-1 plays a role in the increased NMDAR activity observed in neuropathic pain, we used *Cacna2d1* knockout (KO; $Cacna2d1^{-/-}$) mice, which show reduced pain hypersensitivity after nerve injury (Patel et al., 2013). Peripheral nerve injury markedly increased the amplitude of puff NMDA currents and the NMDAR-mediated frequency of mEPSCs of spinal dorsal horn neurons in wild-type ($Cacna2d1^{+/+}$) mice. Also, nerve injury significantly increased the amplitude of monosynaptic EPSCs evoked by dorsal root stimulation in spinal dorsal horn neurons in $Cacna2d1^{+/+}$ mice, which was readily normalized after bath application of AP5 (Figures 2C–2F). These data suggest that nerve injury causes tonic activation of presynaptic NMDARs expressed at primary afferent terminals, as shown previously (Li et al., 2016). These increases in pre- and postsynaptic NMDAR activity were abrogated in $Cacna2d1^{-/-}$ mice (Figures 2C–2F). Collectively, these findings strongly indicate that α2δ-1 is essential for the nerve injury-induced increase in synaptic NMDAR activity at the spinal cord level.

α2δ-1 Physically Interacts with NMDARs *In Vitro* and *In Vivo*

The functional link between α2δ-1 and NMDAR activity in neuropathic pain prompted us to examine whether α2δ-1 and NMDARs physically interact. To determine the interaction between α2δ-1 and NMDARs *in vivo*, we conducted coimmunoprecipitation (co-IP) analyses using membrane extracts of dorsal spinal cords obtained from SNL and sham control rats. An anti-α2δ-1 antibody coprecipitated the NMDAR subunits GluN1, GluN2A, and GluN2B in spinal membrane fractions (Figure 3A). The α2δ-1-GluN1 protein complex in the dorsal spinal cord was much higher (97.3 ± 8.3%) in SNL rats than in sham controls. Similarly, reciprocal coimmunoprecipitation showed that α2δ-1 was precipitated by an anti-GluN1 antibody but not by an irrelevant rabbit anti-immunoglobulin G (anti-IgG) antibody (Figure 3B). SNL caused a large increase in the GluN1-α2δ-1 complex in the spinal cord (by 116.0 ± 10.3%). Although α2δ-2 and α2δ-3 proteins were also present in rat spinal cords, they did not coimmunoprecipitate with GluN1 (Figure 3B). In addition, using membrane extracts from spinal cord tissues of human donors, we demonstrated that anti-

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α2δ-1, but not anti-IgG, antibody coimmunoprecipitated with GluN1 and vice versa (Figures 3C and 3D).

We next performed coimmunoprecipitation using membrane extracts of HEK293 cells expressing yellow fluorescent protein (YFP)-tagged α2δ-1 together with either GluN1 plus GluN2A or GluN1 plus GluN2B. GluN1, GluN2A, and GluN2B, when coexpressed with YFP-α2δ-1, were all precipitated by an anti-GFP antibody (Figure 3E). Conversely, precipitation with an anti-FLAG antibody identified a protein band corresponding to α2δ-1 in cells coexpressing either FLAG-GluN1 and GluN2A or FLAG-GluN1 and GluN2B (Figure 3F). This physical interaction is specific to α2δ-1, because neither α2δ-2 nor α2δ-3 coprecipitated (Figure 3F). However, α2δ-1 did not precipitate GluN1, GluN2A, or GluN2B on the membrane surface when these NMDAR subunits were expressed individually with α2δ-1 in HEK293 cells (Figure S3). These results demonstrate that α2δ-1 and NMDAR subunits form a heteromeric complex on the plasma membrane and that physical interactions between α2δ-1 and NMDARs occur both *in vitro* and *in vivo*.

α2δ-1 Interacts with NMDARs Primarily via Its C-Terminal Domain

We next attempted to identify the molecular determinants of the α2δ-1-NMDAR interaction. The large α2 protein is entirely extracellular, whereas the δ part has a C-terminal domain that anchors α2δ-1 to the membrane (Davies et al., 2007; Dolphin, 2013). Although the C terminus of α2δ-1 is predicted to be a transmembrane region, its structure has not been resolved by cryoelectron microscopy (Wu et al., 2016). Furthermore, one study suggests that α2δ-1 is a GPI-anchored protein (Davies et al., 2007). However, a subsequent study has disputed the presence of any glycophosphatidylinositol (GPI) motifs in α2δ-1 (Robinson et al., 2011). The highly conserved von Willebrand factor type-A (VWA) domain in α2δ-1 is required for its interaction with VACCs or thrombospondin (Eroglu et al., 2009; Hoppa et al., 2012), and the region just upstream of the VWA domain on the N terminus in α_2 is required for gabapentin binding (Field et al., 2006; Wang et al., 1999). We first deleted the VWA domain and expressed the truncated α2δ-1 (α2δ-$1_{\Delta VWA}$) with FLAG-GluN1 and GluN2A or with FLAG-GluN1 and GluN2B in HEK293 cells. Surprisingly, deleting the VWA domain did not affect coprecipitation of α2δ-1 and GluN1 in membrane extracts (Figures 3G and 3H). Because α2δ-2 and α2δ-3 do not interact with NMDARs, we next created chimeras by swapping the N terminus of α2δ-1 with that of α2δ-2 or α2δ-3 (termed α2δ-$1_{NT(α2δ-2)}$ and α2δ-$1_{NT(α2δ-3)}$, respectively) (Figure 3I). We then coexpressed YFP-α2δ-$1_{NT(α2δ-2)}$ or YFP-α2δ-$1_{NT(α2δ-3)}$ with FLAG-GluN1/GluN2A or FLAG-GluN1/GluN2B in HEK293 cells. Coimmunoprecipitation analysis showed that the anti-FLAG antibody still precipitated α2δ-$1_{NT(α2δ-2)}$ or α2δ-$1_{NT(α2δ-3)}$ similarly to wild-type α2δ-1 (Figures 3G and 3H).

Having found that neither the VWA domain nor the N terminus of α2δ-1 is essential for the interaction of α2δ-1 with NMDARs, we investigated whether the δ sequence and C terminus domain are required for α2δ-1-NMDAR interaction. We generated chimeras by replacing the δ sequence or C terminus of α2δ-1 with those of α2δ-2 and α2δ-3 (α2δ-$1_{δ(α2δ-2)}$ and α2δ-$1_{δ(α2δ-3)}$ or α2δ-$1_{CT(α2δ-2)}$ and α2δ-$1_{CT(α2δ-3)}$, respectively) (Figure 3I). We expressed these chimeras individually with GluN1/GluN2A or GluN1/GluN2B in HEK293 cells.

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Coimmunoprecipitation using cell-membrane extracts showed that substituting the δ sequence or C terminus of α2δ-1 with that of α2δ-2 markedly reduced coprecipitation of α2δ-1 and NMDARs, whereas replacing the δ sequence or C terminus of α2δ-1 with that of α2δ-3 nearly abolished α2δ-1-NMDAR coupling (Figures 3G and 3H). These findings demonstrate that α2δ-1 interaction with NMDARs predominantly requires the C-terminal, but not VWA, domain of α2δ-1.

Gabapentin Selectively Targets α2δ-1-Bound NMDARs and Their Surface Expression

Gabapentin binds to α2δ-1 (and to α2δ-2 at a lower affinity) (Fuller-Bicer et al., 2009; Gee et al., 1996; Marais et al., 2001; Wang et al., 1999). Because α2δ-1 directly interacts with NMDARs, we investigated whether gabapentin might block the increase in dorsal horn neuron NMDAR activity that results from nerve injury. Gabapentin treatment (100 μM for 30 min) normalized the amplitude of puff NMDA currents and the NMDAR-mediated mEPSC frequency that had been potentiated by SNL (Figures 4A–4C). Also, gabapentin reversed SNL-induced increases in the amplitude of NMDAR-mediated EPSCs and the ratio of NMDAR EPSCs to α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPAR) EPSCs monosynaptically evoked by dorsal root stimulation (Chen et al., 2014a; Li et al., 2016) (Figure S4).

We then determined whether α2δ-1 and gabapentin affect NMDAR currents reconstituted in a heterologous expression system. In HEK293 cells expressing GluN1/GluN2A or GluN1/GluN2B diheteromers, cotransfection with α2δ-1 markedly increased the NMDAR current density (Figure 4D) but had no effect on the deactivation, desensitization, or apparent open probability of NMDARs (Figure S5). Remarkably, gabapentin (100 μM for 30 min) completely reversed the α2δ-1 coexpression-induced increase in the NMDAR current density but had no effect on NMDAR currents in cells expressing GluN1/GluN2A or GluN1/GluN2B alone (Figure 4D). α2δ-1 coexpression also reduced the voltage-dependent Mg^{2+} block of NMDARs reconstituted with GluN1/GluN2A at −40 and −60 mV, and gabapentin treatment reversed this effect (Figure 4E). However, α2δ-1 coexpression did not alter the voltage-dependent Mg^{2+} block of NMDARs containing GluN1/GluN2B. These data indicate that the interaction of α2δ-1 and NMDARs is a prerequisite for the effect of gabapentin on NMDAR activity.

We then used luminescence resonance energy transfer (LRET) (Sirrieh et al., 2013) to assess α2δ-1-NMDAR interaction on the plasma membrane and the gabapentin effect in live HEK293 cells cotransfected with GluN1, terbium-labeled GluN2A, and YFP-α2δ-1. LRET between terbium-labeled GluN2A and YFP-α2δ-1 yielded a single-exponential decay, with a corresponding distance of 59.2 ± 0.4 Å between YFP on the N terminus of α2δ-1 and terbium on the N terminus of GluN2A. Gabapentin treatment completely abrogated the LRET signal in cells transfected with wild-type α2δ-1 (Figure 5A; Table S1). When the wild-type α2δ-1 was replaced with the R217A mutant, which diminishes the therapeutic effect of gabapentinoids (Field et al., 2006; Lotarski et al., 2014), gabapentin failed to alter the LRET signal (Figure 5A; Table S1). Similar LRET findings were obtained for terbium-labeled GluN1 and YFP-α2δ-1 (Figure 5B; Table S1). The LRET nanopositioning system-based model illustrates a close interaction between α2δ-1 and GluN1/GluN2A proteins

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(Figure S6). These results demonstrate that α2δ-1 is in the proximity of NMDARs and that gabapentin diminishes the expression of $\alpha_2\delta$-1-bound NMDARs on the plasma membrane.

To determine the gabapentin effect on α2δ-1-bound NMDARs on the plasma membrane, we transfected HEK293 cells with YFP-α2δ-1 and GluN1/GluN2A or GluN1/GluN2B. Coimmunoprecipitation with membrane extracts revealed that gabapentin abolished the GluN1 band precipitated with anti-GFP antibody (Figure 5C). When the YFP-R217A mutant was cotransfected, the anti-GFP antibody still effectively precipitated the GluN1 protein, but gabapentin failed to produce an effect (Figure 5C). We then used biotinylation to label and isolate the membrane surface proteins of HEK293 cells (Li et al., 2014) expressing GluN1/GluN2A or GluN1/GluN2B heterodimers with or without α2δ-1. Coexpression with α2δ-1 markedly increased the amount of GluN1, GluN2A, and GluN2B proteins on the membrane surface (Figures 5D and 5E). Gabapentin (100 μM for 60 min) normalized the surface protein levels of GluN1, GluN2A, and GluN2B that had been increased by $\alpha_2\delta$-1 coexpression (Figures 5D and 5E), indicating that gabapentin diminishes α2δ-1-bound NMDARs on the membrane surface. These convergent data support the notion that gabapentin inhibits surface trafficking of α2δ-1-bound NMDARs.

The C Terminus of α2δ-1 Is Crucial for Increased Synaptic NMDAR Activity in Neuropathic Pain

Because the C terminus of α2δ-1 is essential for its interaction with NMDARs, we designed a 30-amino-acid peptide (VSGLNPSLWSIFGLQFILLWLVSGSRHYLW) mimicking the C-terminal domain of α2δ-1 to determine whether it could uncouple the α2δ-1-NMDAR interaction. Because α2δ-1 primarily promotes forward trafficking of intracellular α2δ-1-bound NMDARs (Figures 5D and 5E), we fused the C terminus-mimicking peptide to the cell-penetrating peptide Tat (YGRKKRRQRRR) (Schwarze et al., 1999), generating α2δ-1Tat to target intracellular α2δ-1-NMDAR complexes. Gabapentin or α2δ-1Tat peptide had no significant effect on VACC currents in HEK293 cells cotransfected with Cav2.2, Cavβ1, and α2δ-1 (Figures S7A and S7B). Also, intrathecal administration of α2δ-1Tat peptide had no effect on the interaction between α2δ-1 and Cav2.2 proteins in the spinal cord tissue (Figures S7C and S7D).

Application of the α2δ-1Tat peptide (1 μM for 60 min) disrupted the direct interaction between α2δ-1 and GluN1 in a concentration-dependent manner (Figures 6A and 6B). Intrathecal or systemic injection of the α2δ-1Tat peptide, but not a Tat-fused scrambled control peptide (FGLGWQPWSLSFYLVWSGLILSVLHLIRSN), produced a large reduction in the pain hypersensitivity of SNL rats (Figures 6C and 6D). Also, intrathecal injection of α2δ-1Tat peptide (1 μg) or gabapentin (30 μg) similarly reversed pain hypersensitivity caused by *Cacna2d1* overexpression in rats but had no effect on the withdrawal thresholds in rats treated with the control vector (Figures S8). The α2δ-1Tat peptide had no effect on nerve injury-induced pain hypersensitivity in *Cacna2d1* KO mice or on withdrawal thresholds in sham control rats. These data indicate that uncoupling the α2δ-1-NMDAR interaction via the C terminus of α2δ-1 alleviates neuropathic pain.

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α2δ-1 Promotes, whereas Gabapentin Inhibits, Synaptic Trafficking of NMDARs in Neuropathic Pain

In addition, we determined whether gabapentin affects nerve injury-induced synaptic targeting of α2δ-1 and NMDARs *in vivo*. Spinal cord slices from sham and SNL rats were treated with gabapentin or vehicle for 30 min, and then synaptosomes were isolated from the tissue slices. Immunoblotting showed that SNL increased the protein levels of α2δ-1 and GluN1 in spinal cord synaptosomes by about 124% and 25%, respectively. Gabapentin reversed the increased protein levels of GluN1, GluN2A, GluN2B, and α2δ-1 in the spinal synaptosomes of SNL, but not sham, rats (Figures 7A and 7B). These results indicate that gabapentin inhibits synaptic targeting and trafficking of α2δ-1-bound NMDARs. Similarly, treatment with the α2δ-1Tat peptide largely normalized the increased protein levels of GluN1, GluN2A, GluN2B, and α2δ-1 in the spinal cord synaptosomes of SNL, but not sham, rats (Figures 7A and 7B). However, the scrambled control peptide had no such effects in SNL or sham control rats. As expected, treatment with the α2δ-1Tat peptide (1 μM for 60 min), but not the control peptide (1 μM for 60 min), normalized the increased pre- and postsynaptic NMDAR activity of spinal dorsal horn neurons in SNL rats (Figures 7C–7E and S9). These findings support the concept that increased synaptic expression of α2δ-1-bound NMDARs is essential for the increased synaptic NMDAR activity observed in neuropathic pain.

DISCUSSION

Our findings reveal that α2δ-1 is a powerful regulator of NMDARs and that it contributes to neuropathic pain by potentiating the synaptic expression and targeting of NMDARs. Our study provides an eloquent example showing that an auxiliary subunit of VACCs is, in fact, a critical interacting protein regulating the ionotropic glutamate receptor. An interesting analogy is γ2 (stargazin), which was, like α2δ-1, initially identified as a VACC subunit but is now recognized as an auxiliary subunit of the AMPA subtype of glutamate receptors (Chen et al., 2000; Tomita et al., 2005). Although the coimmunoprecipitation assay showed that there is an association of NMDARs with α2δ-1 under the control condition, the level of α2δ-1-bound NMDAR complexes at the synaptic site is likely to be very low. Accordingly, presynaptic NMDARs are not tonically active in control animals but become activated under neuropathic pain conditions (Li et al., 2016; Xie et al., 2016). Because nerve injury induces a profound increase in α2δ-1 protein levels in the DRG and spinal cord, α2δ-1 can form more protein complexes with NMDARs and promote their trafficking to the synaptic sites. Our identification of α2δ-1 as an NMDAR-interacting protein provides new insight into the molecular composition, heterogeneity, and function of native NMDARs, as well as synaptic plasticity under pathophysiological conditions.

Although gabapentinoids have been used clinically for treating epilepsy and neuropathic pain for >30 years, the definitive molecular mechanism responsible for their therapeutic actions remains uncertain. Our study challenges the conventional view that α2δ-1 potentiates, whereas gabapentinoids attenuates, neuropathic pain through modulation of VACCs. We provided substantial evidence showing that the interaction between α2δ-1 and NMDARs is independent of VACCs. Specifically, we showed that the α2δ-1-NMDAR

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interaction is recapitulated in HEK293 cells that are devoid of expression of VACC α1 subunits. Also, the α2δ-1-NMDAR interaction predominantly involves the C-terminal domain of α2δ-1. In contrast, the interaction between α2δ-1 and VACC α1 subunits is mediated through the VWA domain of α2δ-1 (Cantí et al., 2005; Hoppa et al., 2012), and the α2δ-1 without its C terminus can still increase VACC currents (Kadurin et al., 2012). Furthermore, gabapentin normalizes NMDAR-mediated mEPSCs and puff NMDAR currents of spinal dorsal horn neurons in SNL rats independent of the VACC activity. In addition, we showed that α2δ-1Tat peptide disrupts the α2δ-1 interaction with NMDARs but does not affect the VACC activity or α2δ-1-Cav2.2 interaction. The present study elucidates a new mechanism of action of a highly clinically relevant family of drugs, potentially opening fresh avenues for further development in this direction.

The differential effects of gabapentin and α2δ-1Tat peptide on NMDAR activity in sham and SNL rats indicate that α2δ-1-bound NMDARs are normally latent (not present at the synaptic sites) and become active when sensitized, as in neuropathic pain. This finding could explain why gabapentinoids inhibit neurotransmitter release only when persistent pain is present (Fehrenbacher et al., 2003; Kumar et al., 2010). Both α2δ-1 and NMDARs are expressed in DRG neurons and primary afferent nerve terminals in the spinal cord (Bauer et al., 2009; Liu et al., 1994; Taylor and Garrido, 2008). Our data suggest that presynaptic NMDARs that are tonically activated after nerve injury are present on primary afferent nerve terminals. Under neuropathic pain conditions, the ambient glutamate for tonic activation of presynaptic NMDARs probably originates from the same primary afferent terminals on which the NMDARs are expressed or from excitatory interneurons in the dorsal horn, owing to the diminished synaptic inhibition (Zhou et al., 2012), resulting in direct synaptic excitation of presynaptic boutons. In addition, tonic activation of presynaptic NMDARs in the spinal cord of SNL rats may result from glutamate (and glycine) diffusion from an adjacent active terminal, activity-dependent release of glutamate from the dendrites of a postsynaptic cell and/or reduced glutamate reuptake due to reduced glutamate transporter activity (Napier et al., 2012). The ability of gabapentin, α2δ-1Tat peptide, and α2δ-1 ablation to completely restore NMDAR activity raises the intriguing possibility that most, if not all, increased pre- and postsynaptic NMDAR activity after nerve injury is mediated by α2δ-1-bound NMDARs. We showed that the α2δ-1-mediated increase in pre- and postsynaptic NMDAR activity at the spinal cord level is primarily associated with a pathological condition (i.e., nerve-injury-induced neuropathic pain). This finding is important, because it points to a key strategy of targeting enhanced synaptic NMDAR activity in pathological pain conditions without affecting normal signaling. A similar strategy has been used to selectively target increased NMDAR function in chronic pain by uncoupling the Src kinase from the NMDAR complex (Liu et al., 2008). Thus, specifically targeting α2δ-1-bound NMDARs without affecting basal α2δ-1-free NMDARs represents an optimal strategy for alleviating neuropathic pain without impairing the other physiological functions of NMDARs.

Some studies suggest that α2δ-1 interacts with thrombospondins, particularly thrombospondin-4, to promote synaptogenesis in neuropathic pain. For example, thrombospondin-4 induces pain and promotes excitatory synaptic transmission in the spinal cord; this effect can be blocked by gabapentin and is absent in *Cacna2d1* KO mice (Kim et

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al., 2012; Park et al., 2016). It is unclear how α2δ-1's interactions with thrombospondin and NMDARs are related. NMDARs have a fundamental role in synaptogenesis (Cline and Haas, 2008; Sobczyk et al., 2005), but this role has been overlooked in studies testing the effects of thrombospondin and gabapentin on synaptogenesis. α2δ-1 may act as a scaffold protein by interacting with both thrombospondin (via its VWA domain) and NMDARs (via its C-terminal domain). In contrast to the α2δ-1 interaction with both VACCs and thrombospondins, which is mediated via the VWA domain of α2δ-1 (Cantí et al., 2005; Eroglu et al., 2009), we identified in this study that the C-terminal domain of α2δ-1 is critically involved in its interaction with NMDARs. We showed that blocking NMDARs rapidly reversed the *Cacna2d1* overexpression- and SNL-induced increases in the frequency of mEPSCs, suggesting that augmented glutamatergic input is likely maintained by potentiated presynaptic NMDAR activity, not by increased synaptic connections (synaptogenesis), in the spinal dorsal horn. Our findings strongly suggest that it is the interaction with NMDARs, but not with VACCs and thrombospondins, that accounts for the crucial role of α2δ-1 in increased excitatory synaptic transmission and chronic pain development.

We showed that gabapentin impairs the ability of α2δ-1 to facilitate synaptic delivery and cell-surface trafficking of functional NMDARs, suggesting that α2δ-1 promotes synaptic/plasma membrane trafficking of α2δ-1-bound NMDAR complexes. However, α2δ-1 may also stabilize NMDARs at the synaptic site. Similarly, although gabapentin inhibits surface trafficking of α2δ-1-bound NMDARs, it is possible that gabapentin may also promote internalization of α2δ-1-NMDAR complexes already present at the synapses. Because there is an ongoing dynamic exchange between surface and intracellular NMDARs, we cannot clearly distinguish between these two possibilities. It is uncertain why gabapentin impairs α2δ-1 surface expression but has no evident effect on VACC currents. There are conflicting reports about the effect of gabapentinoids on VACCs. For example, one study showed that chronic (48-hr), but not acute, treatment with gabapentin reduces the VACC activity and cell-surface expression in a cell line (Hendrich et al., 2008). Other studies reported that treatment with gabapentin for >72 hr has no effect on VACC-mediated neurotransmitter release or VACC trafficking in cultured neurons (Hoppa et al., 2012) and that gabapentin has no effect on the α2δ-1 interaction with VACC α1 subunits (Cassidy et al., 2014). The lack of a consistent effect of gabapentin on VACCs could be due to the weak interaction between α2δ-1 and VACC α1 subunits (Müller et al., 2010) or a minor role of α2δ-1 in regulating VACC activity (Wu et al., 2009).

Gabapentin and pregabalin bind to both α2δ-1 and α2δ-2 (Marais et al., 2001). Unlike α2δ-1, which is mainly expressed in excitatory neurons, α2δ-2 is predominantly expressed in inhibitory neurons (Cole et al., 2005; Taylor and Garrido, 2008) and may mediate some of the adverse effects of gabapentinoids, such as dizziness and sedation. We showed that the designed peptide targeting the C-terminal domain of α2δ-1 effectively disrupted the α2δ-1-NMDAR association and normalized the synaptic NMDAR activity and pain hypersensitivity induced by nerve injury. Because this sequence is unique to α2δ-1, it is conceivable that drugs that target this domain may have fewer adverse effects than gabapentinoids. Both α2δ-1 and NMDARs are coexpressed in many important brain regions. Because we demonstrated that α2δ-1 can physically interact with NMDARs and

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increase their activity even in a cell line, such interaction is unlikely to be limited to the spinal cord. Thus, targeting α2δ-1-bound NMDARs may be a promising strategy for treating neuropathic pain and other NMDAR-dependent neurological and psychiatric disorders such as epilepsy, neurodegenerative disease, anxiety, and alcohol and drug dependence.

EXPERIMENTAL PROCEDURES

Animal Models

All of the procedures were approved by the Animal Care and Use Committee of The University of Texas MD Anderson Cancer Center. We used L5 and L6 SNL in male rats (8–10 weeks of age) as an experimental model of neuropathic pain. Conventional *Cacna2d1* KO mice were generated as described previously (Fuller-Bicer et al., 2009). The spared nerve injury procedure was performed on mice (male and female, 10–11 weeks of age). The surgical preparation is described in detail in the Supplemental Information.

Behavioral Assessments of Nociception

Tactile allodynia was tested using von Frey filaments and the ‘‘up-down’’ method. The mechanical nociceptive threshold was tested using a noxious pressure stimulus. See the Supplemental Information for details.

Spinal Cord Slice Recordings

Lumbar spinal cord slices from rats and mice were prepared as described previously (Chen et al., 2014a, 2014b). Spinal lamina II neurons were visualized and selected for whole-cell recording of NMDAR activity. In all electrophysiological experiments, 3–5 animals were used for each recording protocol, and only 1 neuron was recorded in each spinal cord slice. Details are provided in the Supplemental Information.

Recording of NMDAR Currents in HEK293 Cells

HEK293 cells were transfected using PolyJet reagents at a GluN1/GluN2/α2δ-1 ratio of 1:3:1. Whole-cell currents were recorded at a holding potential of −60 mV. Glycine (10 μM) and NMDA (300 μM) were used to elicit NMDAR currents. See the Supplemental Information for details.

DNA Constructs and Lentiviral Vector Preparation

The rat cDNAs of the NMDAR subunits GluN1-1a, GluN2A, and GluN2B and α2δ-1 were expressed in the pcDNA 3.1 vector. Various α2δ-1 constructs were made using the QuickChange Site-Directed Mutagenesis Kit or In-Fusion HD Cloning Plus. All cDNA clones and constructs were confirmed by DNA sequencing.

The full-length coding sequence of rat α2δ-1 tagged with enhanced GFP at the N terminus or GFP alone was cloned into the lentiviral vector pLenti6/V5-DEST with a cytomegalovirus promoter. The viral vector was produced using the ViraPower system, which is described in detail in the Supplemental Information.

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Western Blotting and Immunoprecipitation

Western blotting and immunoprecipitation using spinal cord tissues and HEK293 cells were performed using standard methods described in the Supplemental Information.

LRET Measurements

Cysteines at the GluN1 and GluN2A sites labeled with thiol-reactive terbium chelate served as donor fluorophore sites, and YFP fused to α2δ-1 served as the acceptor fluorophore. HEK293 cells transfected with GluN1, GluN2A, and YFP–α2δ-1 were harvested and labeled with 200 nM terbium chelate (Sirrieh et al., 2013). The sample was excited at 337 nm, and emission was detected at 527 nm in a QuantaMaster QM3-SS system. Details are provided in the Supplemental Information.

Statistical Analysis

Results are expressed as means ± SEM. We used a two-tailed Student's t test to compare two groups and one-way or two-way ANOVA to compare more than two groups. We used the appropriate nonparametric analysis when electrophysiological and behavioral data were not normally distributed. $p < 0.05$ was considered statistically significant. See the Supplemental Information for details.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

- α2δ-1 forms a heteromeric complex with NMDARs, mainly through its C terminus domain
- α2δ-1 is essential for nerve injury-induced pre- and postsynaptic NMDAR hyperactivity
- α2δ-1 promotes synaptic and surface expression of α2δ-1-NMDAR complexes
- α2δ-1-bound NMDARs are critical for neuropathic pain development and gabapentin actions

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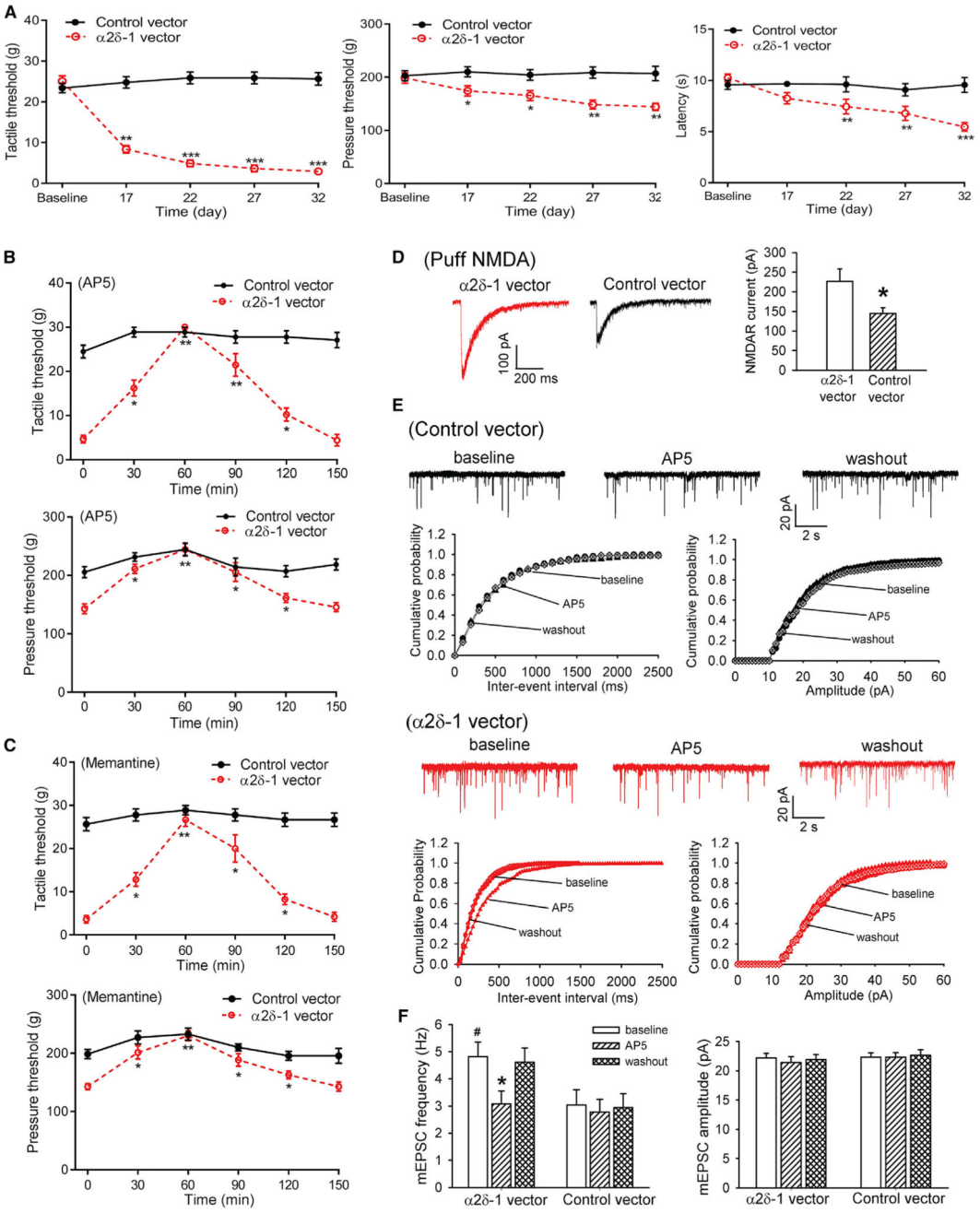

Figure 1. α2δ-1 Overexpression Induces Pain Hypersensitivity and Increases Pre- and Postsynaptic NMDAR Activity of Spinal Dorsal Horn Neurons

(A) Time course of changes in the tactile and pressure withdrawal thresholds and heat withdrawal latency after a single intrathecal injection of the *Cacna2d1* vector or control vector (n = 7 rats in each group). Data are expressed as means ± SEM. *p < 0.05; **p < 0.01; ***p < 0.001 (versus respective baseline), one-way ANOVA followed by Dunnett's post hoc test.

(B and C) Effects of a single intrathecal injection of 5 μg AP5 (B) or intraperitoneal injection of 10 mg/kg memantine (C) on the tactile and pressure withdrawal thresholds in rats treated with the *Cacna2d1* vector or control vector (n = 8 rats in each group). Data are

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expressed as means ± SEM. *p < 0.05; **p < 0.01 (versus baseline before drug injection, time 0), one-way ANOVA followed by Dunnett's post hoc test.
(D) Original traces and mean changes of NMDAR currents elicited by puff application of 100 μM NMDA to spinal dorsal horn neurons in rats 5 weeks after treatment with the *Cacna2d1* vector or control vector (n = 12 neurons in each group). Data are expressed as means ± SEM. *p < 0.05 (versus control vector-treated rats), two-tailed Student's t test.
(E and F) Representative traces and cumulative plots (E) and mean changes (F) of miniature excitatory postsynaptic currents (mEPSCs) of spinal dorsal horn neurons before (baseline), with (AP5), and after (washout) bath application of 50 μM AP5. Slice recordings were performed using rat spinal cords 5 weeks after treatment with the control vector (n = 10 neurons) or *Cacna2d1* vector (n = 11 neurons). Data are expressed as means ± SEM. *p < 0.05 (versus baseline). $^{\#}$p < 0.05, compared with the baseline value in the control vector-treated group, one-way ANOVA followed by Tukey's post hoc test.

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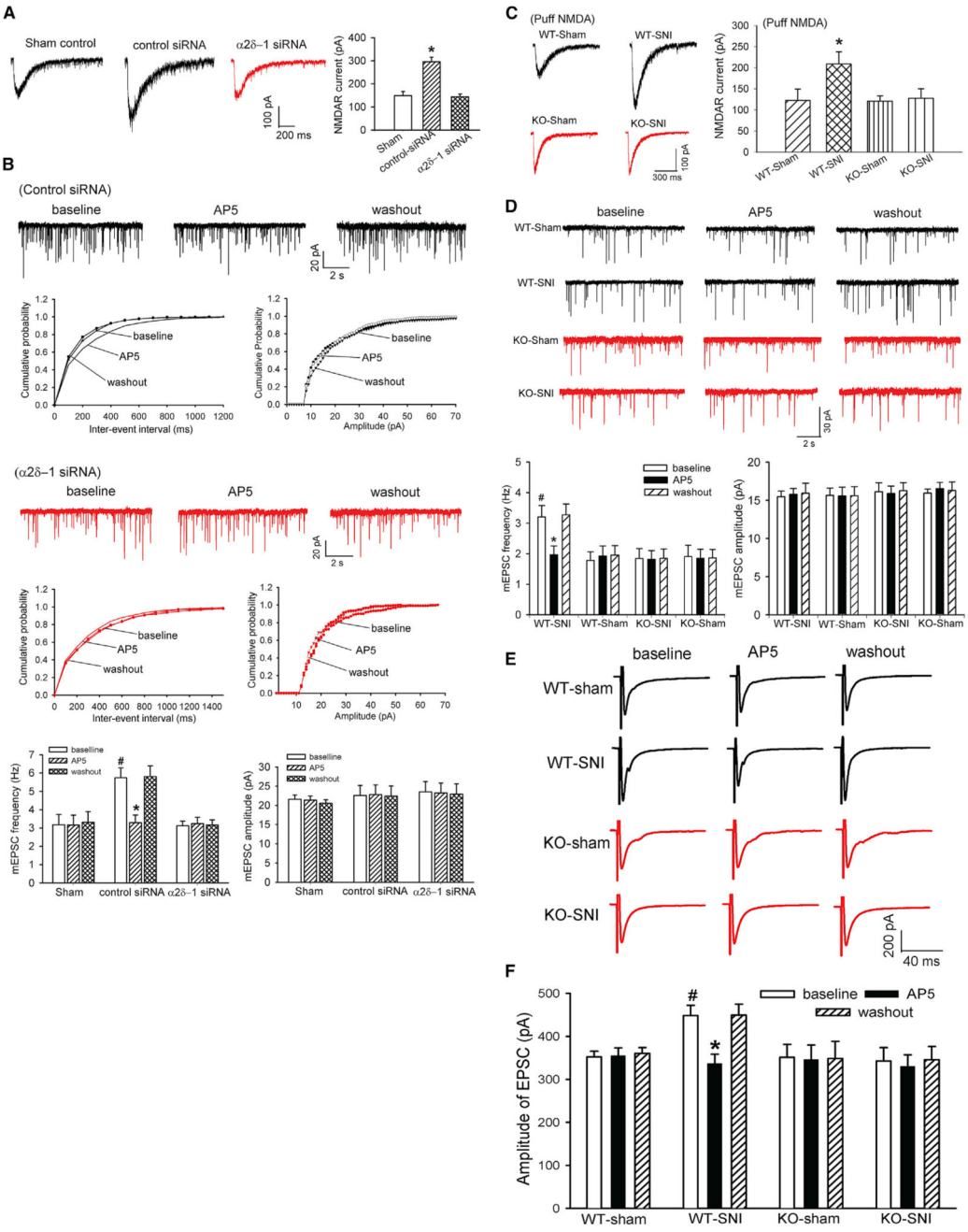

Figure 2. α2δ-1 Contributes to Increased Pre- and Postsynaptic NMDAR Activity of Spinal Dorsal Horn Neurons after Nerve Injury

(A) Representative recording traces and mean changes of NMDAR currents elicited by puff application of 100 μM NMDA to spinal dorsal horn neurons of sham control rats (n = 11 neurons) and SNL rats injected with control siRNA (n = 12 neurons) or *Cacna2d1*-specific siRNA (n = 12 neurons). Data are expressed as means ± SEM. *p < 0.05 (versus sham group), one-way ANOVA followed by Tukey's post hoc test.

(B) Original traces, cumulative plots, and mean changes in the baseline frequency and amplitude of mEPSCs and the AP5 effect in spinal dorsal horn neurons recorded from sham control rats (n = 14 neurons) and SNL rats receiving control siRNA (n = 13 neurons) or

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Cacna2d1-specific siRNA (n = 12 neurons) before (baseline), with (AP5), and after (washout) bath application of 50 μM AP5. Data are expressed as means ± SEM. *p < 0.05 (versus baseline); #p < 0.05 (versus baseline in sham group), one-way ANOVA followed by Tukey's post hoc test.

(C) Original current traces and mean changes in NMDAR currents elicited by puff application of 100 μM NMDA to spinal dorsal horn neurons in wild-type (WT; n = 12 neurons in each group) and *Cacna2d1* KO (n = 11 neurons in each group) mice 3 weeks after spared nerve injury (SNI) or sham surgery. Data are expressed as means ± SEM. *p < 0.05 (versus WT sham group), one-way ANOVA followed by Tukey's post hoc test.

(D) Representative traces and mean changes in baseline values and the AP5 effect on the frequency and amplitude of mEPSCs of spinal dorsal horn neurons in wild-type (WT; n = 11 neurons in each group) and *Cacna2d1* KO (n = 12 neurons in each group) mice subjected to spared nerve injury (SNI) or sham surgery. Data are expressed as means ± SEM. *p < 0.05 (versus baseline); #p < 0.05 (versus baseline in the WT sham group), one-way ANOVA followed by Tukey's post hoc test.

(E and F) Representative traces (E) and mean changes (F) in baseline values and the AP5 effect on the amplitude of EPSCs of spinal dorsal horn neurons monosynaptically evoked by dorsal root stimulation in wild-type (WT) and *Cacna2d1* KO mice subjected to spared nerve injury (SNI; n = 11 neurons in each group) or sham surgery (n = 12 neurons in each group). Data are expressed as means ± SEM. *p < 0.05 (versus baseline); #p < 0.05 (versus baseline in the WT sham group); one-way ANOVA followed by Tukey's post hoc test.

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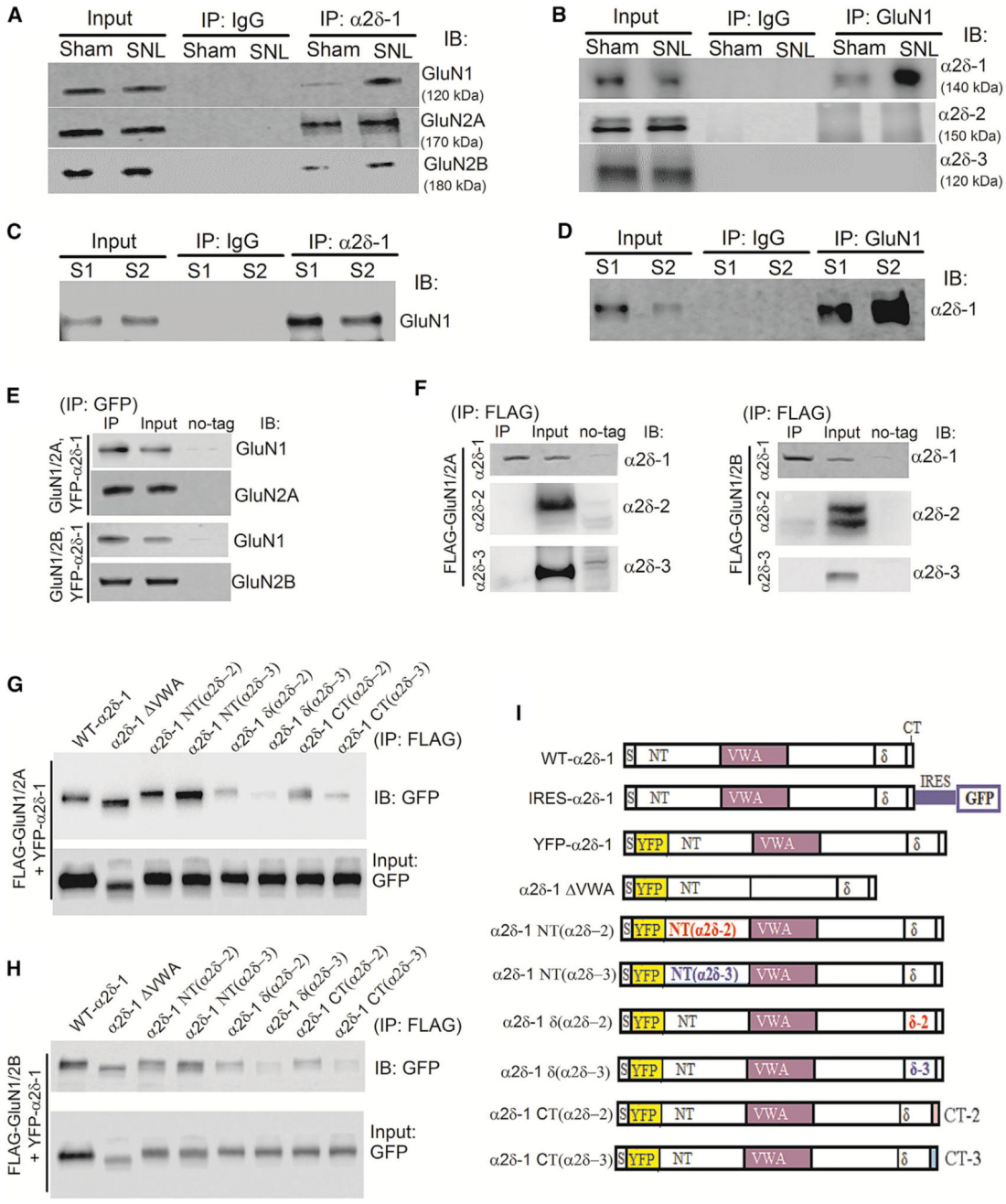

Figure 3. α2δ-1 Physically Interacts with NMDARs *In Vivo* and *In Vitro*

(A and B) Reciprocal coimmunoprecipitation analysis shows the protein-protein interaction between α2δ-1 and NMDAR subunits in the membrane extracts of dorsal spinal cord tissues of rats from the sham control and SNL groups 3 weeks after surgery. (A) Proteins were immunoprecipitated first with a rabbit anti-α2δ-1 or anti-IgG antibody. Western immunoblotting (IB) was performed by using mouse anti-GluN1, anti-GluN2A, or anti-GluN2B antibodies. (B) Proteins were immunoprecipitated initially with a mouse anti-GluN1 or anti-IgG antibody. IB was performed by using a rabbit anti-α2δ-1, anti-α2δ-2, or anti-α2δ-3 antibody. IgG and input (tissue lysates only, without immunoprecipitation) were

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used as negative and positive controls, respectively. Similar data were obtained from 4 independent experiments.

(C and D) Reciprocal coimmunoprecipitation analysis shows the α2δ-1 and GluN1 interaction in the membrane extracts of two human lumbar spinal cord tissue samples (labeled as S1 and S2). (C) Proteins were immunoprecipitated first with a rabbit anti–α2δ-1 or anti-IgG antibody. Western immunoblotting (IB) was performed by using a mouse anti-GluN1 antibody. (D) Proteins were immunoprecipitated initially with a mouse anti-GluN1 or anti-IgG antibody. IB was performed by using a rabbit anti–α2δ-1 antibody. Similar data were obtained from spinal cord tissues of each of the 4 human donors.

(E and F) Coimmunoprecipitation analysis shows that α2δ-1 heterodimerized with NMDAR subunits in membrane extracts of HEK293 cells. HEK293 cells were cotransfected as indicated on the left side of the gel images. (E) GluN1/GluN2A/YFP-α2δ-1 (top images) or GluN1/GluN2B/YFP-α2δ-1 (bottom images). (F) FLAG-GluN1/ GluN2A (left) or FLAG-GluN1/GluN2B (right) with α2δ-1, α2δ-2, or α2δ-3. IRES-α2δ-1 (no-tag) was used as a negative control for YFP-α2δ-1 in (E), and untagged GluN1 (no-tag) was used as a negative control for FLAG-GluN1 in (F). Proteins were immunoprecipitated first with anti-GFP (E) or anti-FLAG antibody (F) using membrane fractions of HEK293 cells. IB was performed by using the antibodies indicated on the right side of the gel images. Data were from 5 independent experiments.

(G and H) α2δ-1 interacts with NMDARs predominantly through its C-terminal domain. Coimmunoprecipitation analysis of FLAG-GluN1/GluN2A (G) and FLAG-GluN1/GluN2B (H) interactions with various YFP-tagged α2δ-1 constructs, indicated above the gel images, coexpressed in HEK293 cells. Coimmunoprecipitation and IB were performed using the antibodies indicated on the right side of the gel images. Data were from 5 independent experiments.

(I) Schematic representation of α2δ-1 constructs and chimeras used for coexpression with NMDAR subunits in HEK293 cells. S, signal peptide; NT, putative N terminus; VWA, von Willebrand factor type-A domain; δ, δ peptide; CT, C-terminal domain. Pink boxes represent the domains of α2δ-2, and blue boxes represent the domains of α2δ-3. All α2δ-1 constructs and chimeras used for coimmunoprecipitation experiments were tagged with yellow fluorescent protein (YFP).

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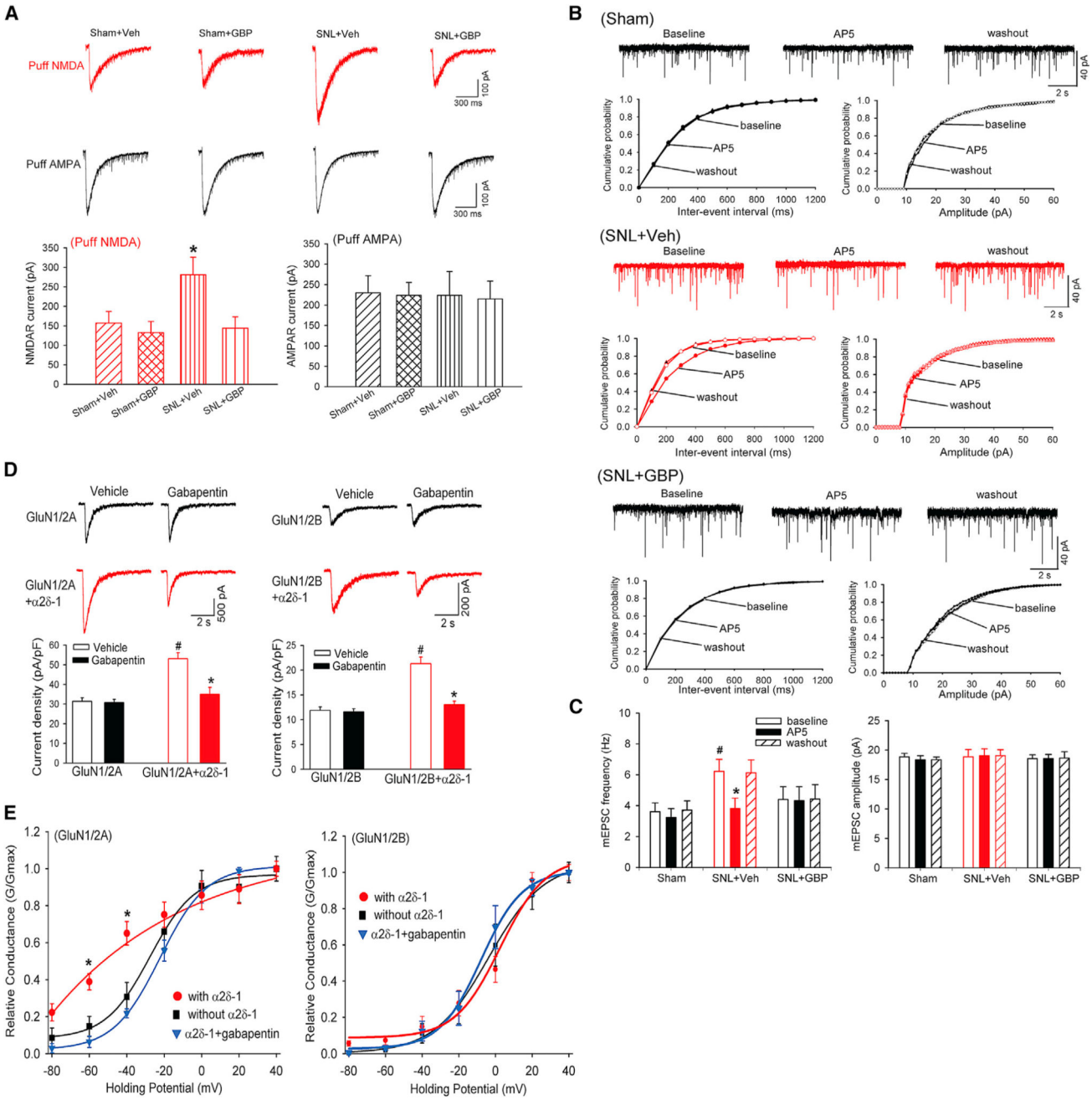

Figure 4. Gabapentin Restores NMDAR Activity Increased by Nerve Injury or α2δ-1 Coexpression *In Vivo* and *In Vitro*

(A) Original traces and mean effects of gabapentin (GBP; 100 μM for 30 min) on currents elicited by puff application of 100 μM NMDA or AMPA to spinal dorsal horn neurons in rats that had undergone sham surgery (n = 12 neurons in the vehicle group; n = 13 neurons in the gabapentin group) or SNL (n = 11 neurons in the vehicle group; n = 12 neurons in gabapentin group) 3 weeks after surgery. Data are expressed as means ± SEM. *p < 0.05 (versus sham rats treated with vehicle), one-way ANOVA followed by Tukey's post hoc test. (B and C) Representative traces and cumulative probabilities (B) and mean changes (C) of baseline values and the AP5 effect on the frequency and amplitude of mEPSCs of spinal

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dorsal neurons recorded from rats subjected to sham surgery (n = 10 neurons) or SNL (n = 12 neurons in the vehicle group; n = 11 neurons in the gabapentin group). Data are expressed as means ± SEM. *p < 0.05 (versus respective baseline); #p < 0.05, compared with the baseline in sham group, one-way ANOVA followed by Tukey's post hoc test.

(D) Original traces and mean changes show the effect of α2δ-1 coexpression and gabapentin treatment (100 μM for 30 min) on whole-cell NMDAR currents in HEK293 cells expressing GluN1/GluN2A (n = 10 cells in each group) or GluN1/GluN2B (n = 12 cells in each group). Current responses were elicited by application of 300 μM NMDA plus 10 μM glycine. Data are expressed as means ± SEM. *p < 0.05 (versus respective vehicle control); #p < 0.05, compared with the current reconstituted with GluN1/GluN2A or GluN1/GluN2B alone, one-way ANOVA followed by Tukey's post hoc test.

(E) Effect of α2δ-1 coexpression and gabapentin treatment on the conductance-voltage relationship of NMDAR channels in HEK293 cells expressing GluN1/GluN2A (n = 12 cells for GluN1/GluN2A with α2δ-1; n = 10 cells for GluN1/GluN2A without α2δ-1; n = 13 cells for GluN1/GluN2A with α2δ-1 and gabapentin) or GluN1/GluN2B (n = 10 cells in each group) in the presence of Mg^{2+}. In cells expressing GluN1/GluN2A, coexpression of α2δ-1 increased NMDAR conductance at −40 and −60 mV in the presence of 2 mM Mg^{2+}, and the increase was reversed by treatment with gabapentin (100 μM for 30 min). Currents were normalized by values obtained at +40 mV. Data are expressed as means ± SEM. *p < 0.05 (versus cells expressing GluN1/GluN2A alone at the same voltage), two-way ANOVA followed by Tukey's post hoc test.

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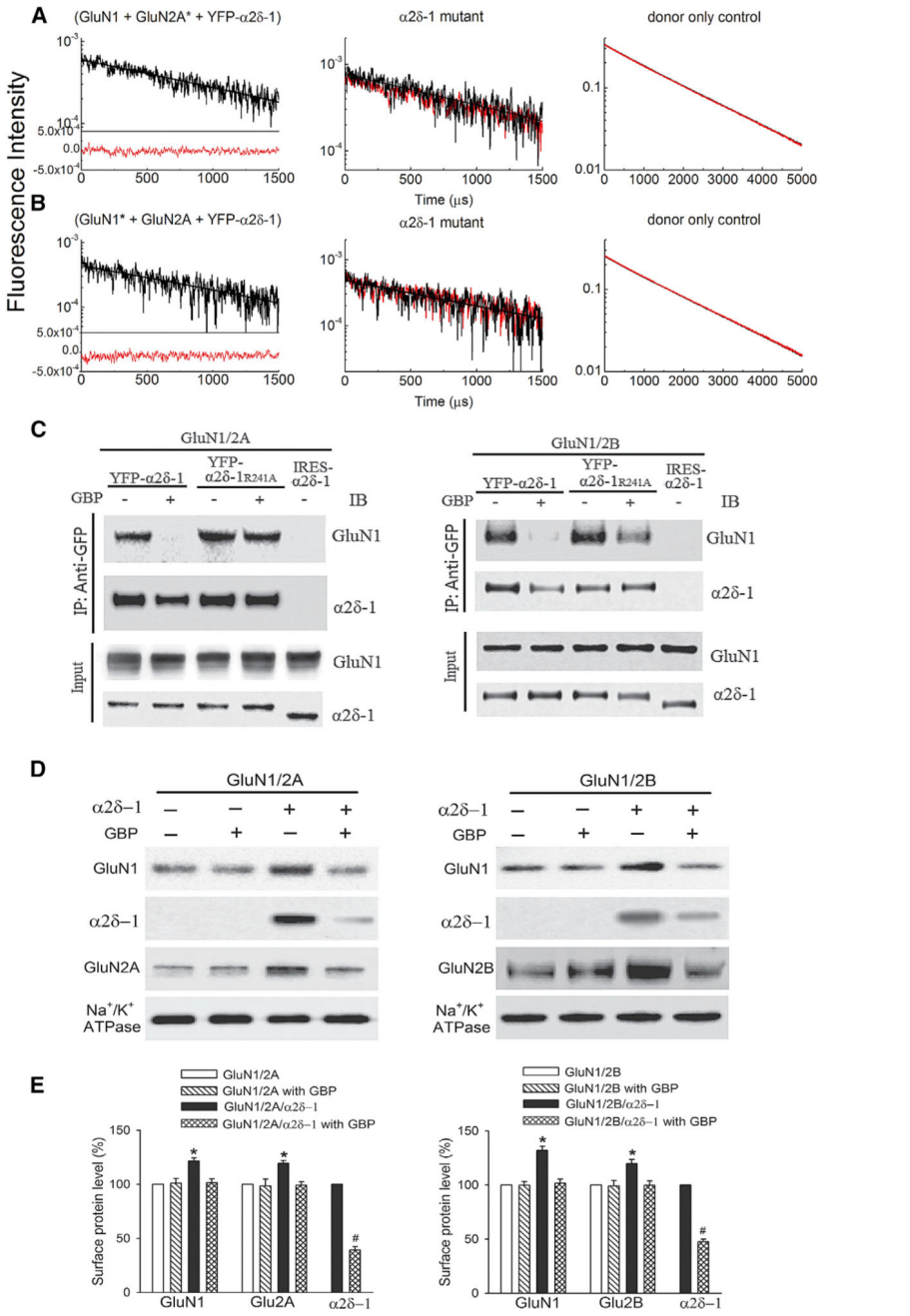

Figure 5. Gabapentin Diminishes Membrane Surface Expression of α2δ-1-Bound NMDARs

(A) Luminescence resonance energy transfer (LRET) between terbium-labeled GluN2A (GluN2A*), GluN1, and YFP-α2δ-1. Black curve: without gabapentin; red curve: with gabapentin. Left: LRET lifetime signals show that gabapentin resulted in loss of the LRET signal between terbium labeled-GluN2A* and YFP-α2δ-1 on themembrane. Middle: gabapentin had no effect on the LRET signal when YFP-α2δ-1 was replaced with its R217A mutant. Right: donor-only curves of terbium-labeled GluN2A* and unlabeled α2δ-1. Data were from 4 or 5 independent experiments (Table S1).

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(B) LRET between terbium-labeled GluN1 (GluN1*), GluN2A, and YFP-α2δ-1. Black curve: without gabapentin; red curve: with gabapentin. Left: LRET lifetime signals show that gabapentin diminished the interaction between terbium labeled-GluN1* and YFP-α2δ-1 on the membrane. Middle: gabapentin had no effect on the LRET signal when YFP-α2δ-1 was replaced with its R217A mutant. Right: donor-only curves of terbium-labeled GluN1* and unlabeled α2δ-1. Data were from 4 or 5 independent experiments (Table S1).
(C) Coimmunoprecipitation and immunoblotting (IB) analysis shows that gabapentin (GBP; 100 μM for 30 min) diminished the expression of α2δ-1-bound NMDARs in the membrane extract of HEK293 cells. Left: the GluN1/GluN2A heterodimer was cotransfected with YFP-α2δ-1, a YFP-α2δ-1 mutant (R217A, also called R241A), or IRES-α2δ-1 (no tag) in HEK293 cells. Right: the GluN1/GluN2B heterodimer was cotransfected with YFP-α2δ-1, a YFP-α2δ-1 mutant (R217A, also called R241A), or IRES-α2δ-1 (no tag) in HEK293 cells. Data were from four independent experiments.
(D and E) Membrane surface protein analysis (D) and mean levels (E) show that gabapentin treatment reversed the α2δ-1 coexpression-induced increase in NMDAR surface expression. Immunoblotting was performed using antibodies against GluN1, GluN2A, GluN2B, and α2δ-1 for the membrane surface proteins isolated with biotinylation. HEK293 cells were cotransfected and treated with gabapentin or vehicle as indicated above the gel images. Na^+/K^+-ATPase, a known membrane protein marker, was used as an internal control. Data are expressed as means ± SEM (n = 5 independent experiments). *$p < 0.05$ (versus GluN1/GluN2A or GluN1/GluN2B alone), one-way ANOVA followed by Tukey's post hoc test; $^{\#}p < 0.05$ (versus GluN1/GluN2A/α2δ-1 or GluN1/GluN2B/α2δ-1 without gabapentin), two-tailed Student's t test. See also Table S1.

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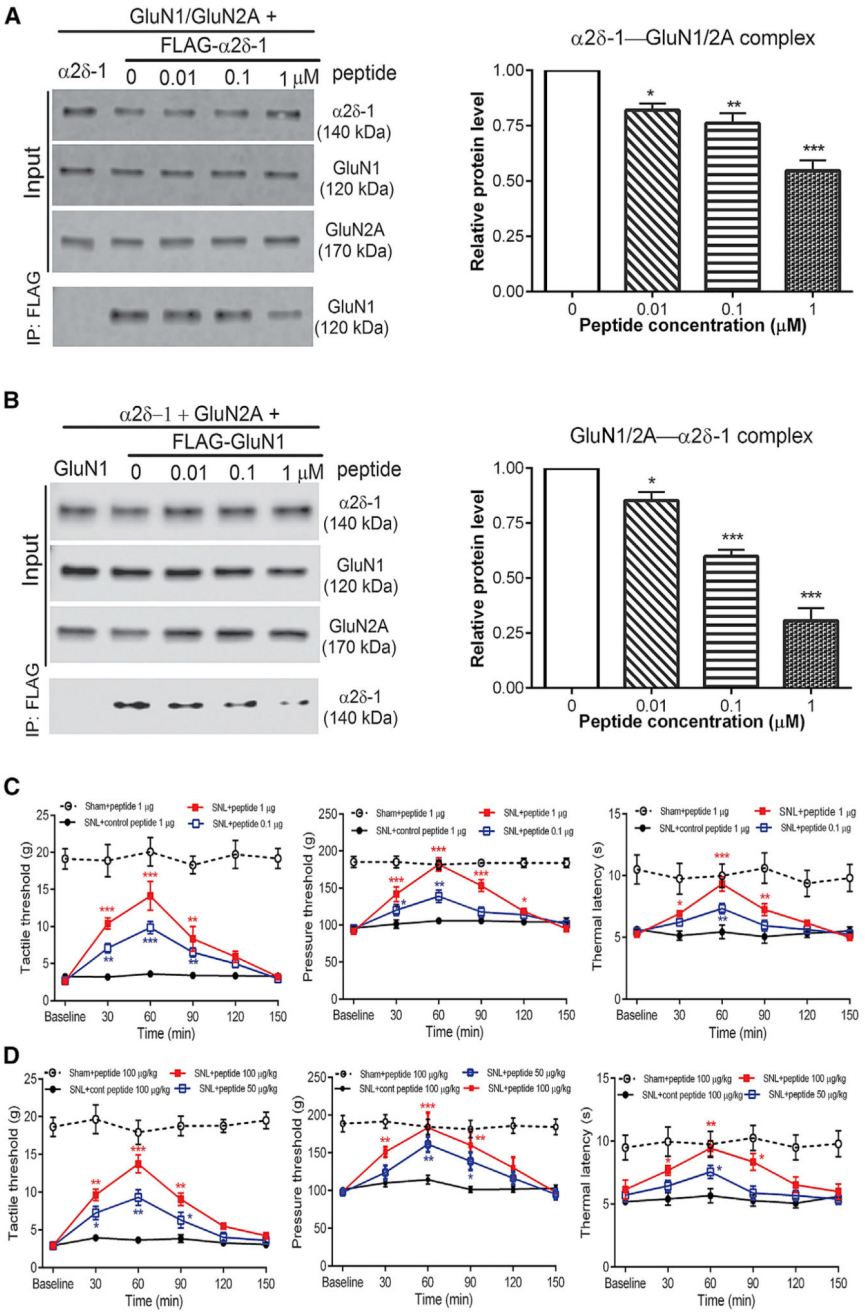

Figure 6. Uncoupling α2δ-1-NMDAR Interaction via the C Terminus of α2δ-1 Reduces Neuropathic Pain

(A and B) Original gel images and quantification data show the effect of α2δ-1Tat peptide on the α2δ-1-GluN1 interaction. (A) HEK293 cells were cotransfected with GluN1, GluN2A, and α2δ-1 or FLAG-α2δ-1. (B) HEK293 cells were cotransfected with α2δ-1, GluN2A, and GluN1 or FLAG-GluN1. Forty-eight hours after transfection, the transfected cells were incubated with 0.01, 0.1, or 1 μM α2δ-1Tat peptide for 30 min. The cell membranes were then isolated and used for coimmunoprecipitation using anti-FLAG antibody. Western blotting was conducted using (A) an anti-GluN1 antibody or (B) an anti-α2δ-1 antibody (n = 6 replicates). Data are expressed as means ± SEM. *p < 0.05; **p <

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0.01; ***p < 0.001 (versus control group), one-way ANOVA followed by Dunnett's post hoc test.

(C and D) Effects of a single intrathecal injection (C) or intraperitoneal injection (D) of α2δ-1Tat peptide on the tactile, pressure, and heat withdrawal thresholds in sham and SNL rats (n = 8 rats in each group). Data are expressed as means ± SEM. *p < 0.05; **p < 0.01; ***p < 0.001 (versus baseline before drug injection), one-way ANOVA followed by Dunnett's post hoc test.

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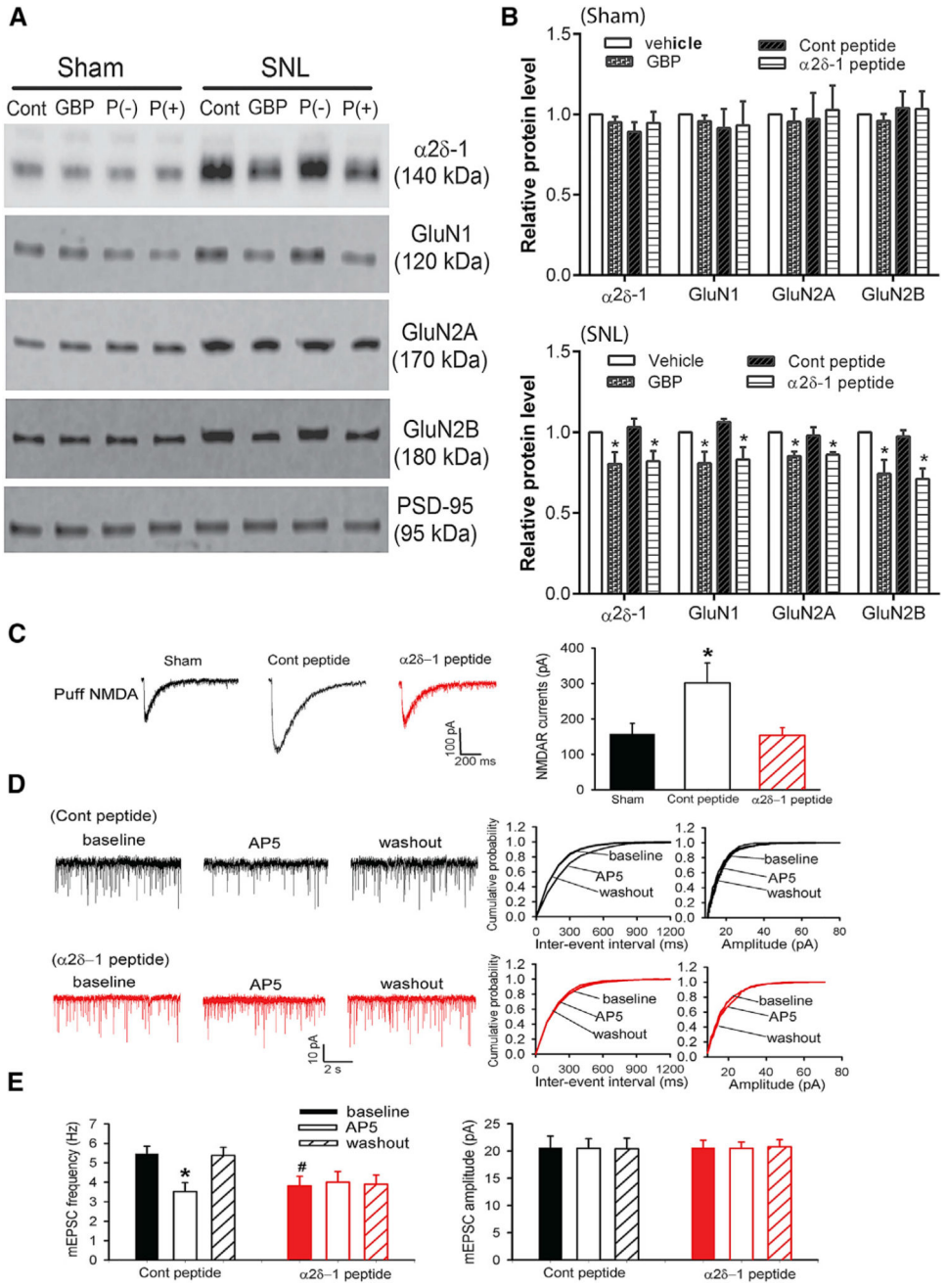

Figure 7. α2δ-1-Bound NMDARs Are Critically Involved In Nerve-Injury-Induced Synaptic Targeting and Activity of NMDARs

(A and B) Representative gel images (A) and quantification data (B) show the protein levels of α2δ-1 and NMDAR subunits in spinal cord synaptosomes of sham and SNL rats (n = 6 samples from 6 rats in each group). The spinal cord slices were incubated with control vehicle (Cont), 100 μM gabapentin (GBP), 1 μM α2δ-1Tat peptide (P+), or 1 μM scrambled control peptide (P−) for 30 min. PSD-95, a known postsynaptic protein, was used as an internal control. Data are expressed as means ± SEM. *$p < 0.05$ (versus vehicle control), one-way ANOVA followed by Dunnett's post hoc test.

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(C) Original traces and mean effects of the α2δ-1Tat peptide or scrambled control peptide (1 μM for 30 min) on currents elicited by puff application of 100 μM NMDA to spinal dorsal horn neurons in SNL (n = 12 neurons in each group) rats 3 weeks after surgery. Data are expressed as means ± SEM. *$p < 0.05$ (versus sham rats), one-way ANOVA followed by Tukey's post hoc test.

(D and E) Representative traces and cumulative probabilities (D), and mean changes (E) of baseline values and the AP5 effect on the frequency and amplitude of mEPSCs of spinal dorsal neurons recorded from SNL rats (n = 12 neurons in the control peptide group; n = 11 neurons in the α2δ-1Tat peptide group). Data are expressed as means ± SEM. *$p < 0.05$ (versus respective baseline value); #$p < 0.05$ (versus baseline in the control peptide group), one-way ANOVA followed by Tukey's post hoc test.