Cellular/Molecular

Constitutive KCC2 Cell- and Synapse-Specifically Regulates NMDA Receptor Activity in the Spinal Cord

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K^+-Cl^- cotransporter-2 (KCC2) critically controls neuronal chloride homeostasis and maintains normal synaptic inhibition by GABA and glycine. Nerve injury diminishes synaptic inhibition in the spinal cord via KCC2 impairment. However, how KCC2 regulates nociceptive input to spinal excitatory and inhibitory neurons remains elusive. Here, we show that basal GABA reversal potentials were significantly more depolarized in vesicular GABA transporter (VGAT)-expressing inhibitory neurons than those in vesicular glutamate transporter-2 (VGluT2)-expressing excitatory neurons in spinal cords of male and female mice. Strikingly, inhibiting KCC2 with VU0463271 increased currents elicited by puff NMDA and the NMDAR-mediated frequency of mEPSCs in VGluT2, but not in VGAT, dorsal horn neurons. Notably, VU0463271 had no effect on EPSCs monosynaptically evoked from the dorsal root in VGluT2 neurons. Furthermore, VU0463271 augmented α2δ-1–NMDAR interactions and their protein levels in spinal cord synaptosomes. In *Cacna2d1* KO mice, VU0463271 had no effect on puff NMDA currents or the mEPSC frequency in dorsal horn neurons. Disrupting α2δ-1–NMDAR interactions with α2δ-1 C-terminus mimicking peptide diminished VU0463271-induced potentiation in the mEPSC frequency and puff NMDA currents in VGluT2 neurons. Additionally, intrathecal injection of VU0463271 reduced mechanical and thermal thresholds in wild-type mice, but not in *Cacna2d1* KO mice. VU0463271-induced pain hypersensitivity in mice was abrogated by co-treatment with the NMDAR antagonist, pregabalin (an α2δ-1 inhibitory ligand), or α2δ-1 C-terminus mimicking peptide. Our findings suggest that KCC2 controls presynaptic and postsynaptic NMDAR activity specifically in excitatory dorsal horn neurons. KCC2 impairment preferentially potentiates nociceptive transmission between spinal excitatory interneurons via α2δ-1-bound NMDARs.

Key words: **dorsal root ganglion; gabapentinoid; neuropathic pain; spinal dorsal horn; synaptic plasticity; vesicular inhibitory amino acid transporter (VIAAT)**

Significance Statement

Impaired function of potassium-chloride cotransporter-2 (KCC2), a key regulator of neuronal inhibition, in the spinal cord plays a major role in neuropathic pain. This study unveils that KCC2 controls spinal nociceptive synaptic strength via NMDA receptors in a cell type- and synapse type-specific manner. KCC2 inhibition preferentially augments presynaptic and postsynaptic NMDA receptor activity in spinal excitatory interneurons via α2δ-1 (previously known as a calcium channel subunit). Importantly, spinal KCC2 impairment triggers pain hypersensitivity through α2δ-1-coupled NMDA receptors. These findings pinpoint the cell and molecular substrates for the reciprocal relationship between spinal synaptic inhibition and excitation in chronic neuropathic pain. Targeting both KCC2 and α2δ-1–NMDA receptor complexes could be an effective strategy in managing neuropathic pain conditions.

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Introduction

The spinal dorsal horn displays remarkable neuronal heterogeneity within lamina II, which predominantly consists of excitatory interneurons expressing vesicular glutamate transporter-2 (VGluT2) and inhibitory interneurons expressing vesicular GABA transporters (VGAT, also known as vesicular inhibitory amino acid transporter, VIAAT; Todd, 2010; Browne et al., 2020; Chen et al., 2022). VGluT2-expressing dorsal horn neurons

facilitate chronic pain caused by tissue inflammation and nerve injury (Wang et al., 2018), whereas VGAT-expressing neurons normally inhibit nociceptive transmission in the dorsal horn (Koga et al., 2017). Normal synaptic inhibition by GABA and glycine is essential for physiological processing of nociceptive transmission in the spinal dorsal horn. The synaptic inhibitory effect of GABA and glycine critically depends on intracellular Cl^- levels, controlled by two functionally distinct cation-chloride cotransporters: Na^+-K^+-$2Cl^-$ cotransporter-1 (NKCC1) and K^+-Cl^- cotransporter-2 (KCC2). KCC2 serves as the sole mechanism for Cl^- extrusion in mature CNS neurons, whereas NKCC1 is responsible for increasing intracellular Cl^- above equilibrium and opposes the action of KCC2 (Payne et al., 1996; Rivera et al., 1999). Therefore, changing the intracellular Cl^- concentration can profoundly alter the strength and polarity of GABA- and glycine-mediated synaptic inhibition. Peripheral nerve injury or spinal cord injury reduces KCC2 levels, where chemotherapy-induced painful neuropathy is associated with increased NKCC1 levels, in the spinal dorsal horn (Coull et al., 2003; Boulenguez et al., 2010; Zhou et al., 2012; Chen et al., 2014c). Inhibiting spinal KCC2 in uninjured animals causes pain hypersensitivity, whereas increasing KCC2 expression at the spinal cord level reverses pain hypersensitivity caused by nerve injury (Li et al., 2016; Mapplebeck et al., 2019). However, how the Cl^- homeostasis controls nociceptive input to spinal excitatory and inhibitory neurons remains elusive.

In addition to the loss of synaptic inhibition, another prominent feature of synaptic plasticity in neuropathic pain is augmented glutamatergic input to spinal dorsal horn neurons mediated by increased presynaptic and postsynaptic NMDAR activity (Zhou et al., 2011; Chen et al., 2014b, 2019; Zhang et al., 2021). Chemotherapy and traumatic nerve injury enhance synaptic NMDAR activity in VGluT2, but not in VGAT, neurons in the spinal cord (Huang et al., 2023b). There appears to be a reciprocal relationship between KCC2-mediated synaptic inhibition and aberrant synaptic NMDAR activity in the spinal cord. For example, pain hypersensitivity produced by intrathecal injection of glycine or $GABA_A$ receptor antagonists is alleviated by NMDAR antagonists (Yamamoto and Yaksh, 1993; Ishikawa et al., 2000). Also, disrupting KCC2 function leads to increased NMDAR responses in spinal dorsal horn neurons (Hildebrand et al., 2016; Dedek et al., 2019). Furthermore, NMDAR hyperactivity causes calpain-mediated KCC2 proteolysis and synaptic disinhibition in the spinal dorsal horn (Zhou et al., 2012), whereas increasing KCC2 expression at the spinal cord level reverses nerve injury-induced activation of presynaptic and postsynaptic NMDARs in the spinal cord (Li et al., 2016). Although an imbalance in excitatory and inhibitory inputs at the spinal cord level has been well recognized as a crucial mechanism underlying the worsening and maintenance of neuropathic pain, it is unclear exactly how synaptic disinhibition augments NMDAR-mediated excitatory glutamatergic input to the spinal dorsal horn.

α2δ-1 (encoded by the *Cacna2d1* gene), previously known as a voltage-activated calcium channel subunit, is a newly identified NMDAR regulatory protein and is essential for synaptic trafficking of NMDARs in the spinal cord in neuropathic pain conditions (Chen et al., 2018, 2019; Zhang et al., 2021). However, nothing is known about the role of α2δ-1 in regulating NMDAR activity caused by synaptic disinhibition in the spinal cord. To fill these gaps in knowledge, we determined whether intrinsic KCC2 activity regulates synaptic NMDARs in excitatory and inhibitory neurons in the spinal dorsal horn. Our study reveals that KCC2 impairment preferentially augments nociceptive transmission between VGluT2-expressing excitatory dorsal horn neurons via α2δ-1-bound NMDARs. This new knowledge advances our understanding of the cellular and molecular substrates that underlie the impact of synaptic disinhibition on spinal nociceptive circuitry.

Materials and Methods

Animals. All experimental procedures followed the Guide for the Care and Use of Laboratory Animals (National Institutes of Health) and were approved by the Institutional Animal Care and Use Committee at The University of Texas MD Anderson Cancer Center. Male and female mice (10–14 weeks old) were used in this study, and the genetic background of all mice was C57BL/6. The animals were housed in a controlled environment at 24°C under a 12 h light/dark cycle. Mice were kept at no more than 5 per cage, and they were provided *ad libitum* access to food and water. *Cacna2d1* knock-out (KO) mice (Stock #6900, Medical Research Council) were used in the experiments. The generation of conventional *Cacna2d1* KO mice was described previously (Fuller-Bicer et al., 2009). The $Cacna2d1^{+/-}$ heterozygous mice were bred to produce $Cacna2d1^{-/-}$ mice and $Cacna2d1^{+/+}$ wild-type (WT) littermates.

VGluT2-ires-Cre knock-in mice (#028863), *VGAT-ires-Cre* knock-in mice (#028862), and *tdTomato-floxed* mice (#007909) were obtained from The Jackson Laboratory. The $VGluT2^{Cre/+}$:$tdTomato^{flox/flox}$ and $VGAT^{Cre/+}$:$tdTomato^{flox/flox}$ mice were obtained by crossing female *tdTomato-floxed* mice with male *VGluT2-ires-Cre* and male *VGAT-ires-Cre* mice, respectively (Wang et al., 2018; Chen et al., 2022; Huang et al., 2022). Ear biopsies were used for mouse genotyping. The specificity of tdTomato-labeled VGluT2 and VGAT dorsal horn neurons has been validated previously (Koga et al., 2017; Wang et al., 2018; Browne et al., 2020).

Spinal cord slice preparation and electrophysiological recordings. Mice were anesthetized with inhalation of 3% isoflurane, and the lumbar spinal cords were quickly removed via laminectomy. Transverse slices (400 μm thick) of spinal cords were cut using a vibratome and immersed with sucrose-modified artificial cerebrospinal fluid containing (in mM) 234 sucrose, 3.6 KCl, 26 $NaHCO_3$, 1.2 NaH_2PO_4, 1.2 $MgCl_2$, 2.5 $CaCl_2$, and 25 glucose (oxygenated with 95% O_2 and 5% CO_2). The slices were immediately transferred into 95% O_2 and 5% CO_2 saturated Krebs' solution containing (in mM) 117 NaCl, 3.6 KCl, 25 $NaHCO_3$, 1.2 NaH_2PO_4, 1.2 $MgCl_2$, 2.5 $CaCl_2$, and 11 glucose for at least 1 h at 34°C before recording.

Whole-cell voltage-clamp recordings of spinal dorsal horn neurons were performed as we described previously (Chen et al., 2014a; Huang et al., 2019). Briefly, spinal cord slices were immobilized within a recording chamber with a glass bottom and subjected to a continuous perfusion of oxygenated Krebs' solution at 34°C, flowing at a rate of 3 ml/min. tdTomato-labeled neurons in spinal lamina II were identified using a combination of epifluorescence illumination and infrared and differential interference contrast optics on an upright microscope (Chen et al., 2022). Recordings were performed using 5–8 MΩ glass electrodes filled with the internal solution, which comprised the following (in mM): 135 potassium gluconate, 5 KCl, 2 $MgCl_2$, 0.5 $CaCl_2$, 5 HEPES, 5 EGTA, 5 ATP-Mg, 0.5 Na_2-GTP, and 10 lidocaine *N*-ethyl bromide (QX314), with an osmolality of 280–300 mOsm and a pH of 7.3. QX314 was used to block voltage-gated Na^+ channels to suppress action potentials. Recordings of excitatory synaptic currents (EPSCs) were obtained at a holding potential of −60 mV. Miniature EPSCs (mEPSCs) were recorded in the presence of 0.5 μM tetrodotoxin (#HB1035, Hello Bio). To elicit the release of glutamate from primary afferents, electrical stimulation (0.6 mA, 0.5 ms, and 0.1 Hz) of the ipsilateral dorsal root was carried out using a tungsten bipolar electrode. Monosynaptic EPSCs were discerned based on their consistent latency and the absence of conduction failure upon 20 Hz stimulation, in line with previous reports (Li et al., 2002; Chen et al., 2018). A pair of stimuli at 50 ms intervals was utilized to induce two EPSCs for calculating the

paired–pulse ratio (PPR), determined by the amplitude of the second synaptic response relative to the first one (Xie et al., 2016).

We recorded postsynaptic NMDAR currents elicited by puff application of 100 μM NMDA (#M3262, MilliporeSigma) onto recorded neurons at a distance of 150 μm through a positive pressure system (Toohey Company). The internal solution contained (in mM) 110 Cs_2SO_4, 2 $MgCl_2$, 0.5 $CaCl_2$, 10 HEPES, 5 EGTA, 2 Mg-ATP, 0.3 Na_2-GTP, and 10 QX314 with 280–300 mOsm and pH 7.3. Notably, $MgCl_2$ was substituted with $CaCl_2$ in the extracellular solution to remove the blocking effect of Mg^{2+} on NMDARs at negative holding potential (Chen et al., 2014a). VU0463271 (#4719) was purchased from Tocris Bioscience. The NMDAR antagonist 2-amino-5-phosphonopentanoate (AP5) was obtained from Hello Bio (#HB0252). All drugs were freshly prepared in Krebs' solution before recordings and delivered at the final concentrations via syringe pumps.

To record GABA reversal potential in tdTomato-labeled lamina II neurons, we employed the gramicidin-based perforated patch-clamp method, which prevents Cl^- permeation (Zhou et al., 2012; Chen et al., 2014c). The internal solution consisted of (in mM) 130 potassium acetate, 15 KCl, 5 NaCl, 1 $MgCl_2$, and 10 HEPES at 280–300 mOsm and a pH of 7.3. Gramicidin (#G5002, MilliporeSigma) was solubilized freshly in DMSO and then diluted to a final concentration of 50 μg/ml in the internal solution. Currents elicited by the puff application (15 ms) of 100 μM GABA (#A2129, MilliporeSigma) were recorded across a range of membrane potentials from −90 to −30 mV, incrementing in 10 mV intervals. We applied linear regression analysis to delineate the voltage dependence of chloride-mediated currents, deducing the GABA reversal potential (E_{GABA}) from the intercept with the abscissa (Ye et al., 2012; Zhou et al., 2012). All electrophysiological signals were filtered at 1–2 kHz and digitized at 10 kHz utilizing a DigiData 1550B and a MultiClamp 700A amplifier (Molecular Devices).

Nociceptive behavioral assessment. For the evaluation of tactile allodynia, individual mice were situated in separate chambers featuring a mesh floor and were allowed to acclimate for 30 min. A series of calibrated von Frey filaments (Stoelting) was applied vertically to the plantar surface of the hindpaw, exerting sufficient pressure to induce bending of the filaments. This pressure was maintained for 6 s, and a response was deemed positive in the case of a brisk paw withdrawal or flinching. Upon observation of a response, the subsequent filament with next smaller force was applied. If no response was elicited, the filament with next higher force was applied. Utilizing the "up-down" method as previously outlined (Chaplan et al., 1994; Chen et al., 2001), the tactile stimulus that resulted in a 50% probability of withdrawal was calculated.

For the quantification of mechanical nociception, the withdrawal threshold was evaluated in response to a noxious pressure stimulus using the Randall–Selitto test, following the method detailed in prior studies (Chen et al., 2009; Luo et al., 2020). Using the Analgesy-Meter device (Ugo Basile), a pressure stimulus was applied to the hindpaw through activation of a motor that steadily escalated the force. Once an observable withdrawal response from the animal was elicited, the device was promptly halted, and the nociceptive threshold was recorded.

Thermal sensitivity was assessed utilizing a radiant heat source (IITC Life Science), following established method described previously (Chen et al., 2022; Jin et al., 2022). Mice were placed in a plastic cylinder on the top of a glass plate that had been pre-warmed to 30°C, and an acclimatization of 30 min was allowed. A radiant light emitting noxious heat was directed toward the plantar surface of the hindpaw. The withdrawal latency was recorded on a timer when hindpaw was rapidly moved away from the heat source.

Pregabalin (#Y0001805) was purchased from MilliporeSigma. The α2δ-1 C-terminus mimicking peptide (VSGLNPSLWSIFGLQFILLWL VSGSRHYLW) fused with the cell-penetrating peptide Tat (YGRKKRRQRRR) and a scrambled control peptide (FGLGWQPWSL SFYLVWSGLILSVLHLIRSN) fused with Tat were synthesized by Synpeptide. Drugs or vehicle were intrathecally injected in mice via lumbar puncture as we described previously (Sun et al., 2019; Jin et al., 2022).

Co-immunoprecipitation and immunoblotting. Mice were anesthetized with 3% isoflurane before the lumbar spinal cords were removed via laminectomy. The dorsal spinal cord at lumbar L3–L5 levels were immediately collected for co-immunoprecipitation (co-IP) and immunoblotting as described previously (Chen et al., 2019; Zhang et al., 2021; Zhou et al., 2022). In brief, synaptosomes were extracted from dorsal spinal cord tissues using Syn-PER Synaptic Protein Extraction Reagent (#87793, Thermo Fisher Scientific) containing cocktails of protease and phosphatase inhibitors (#P8340, MilliporeSigma) following the instructions. The pellets were resuspended in ice-cold immunoprecipitation lysis buffer (#87788, Thermo Fisher Scientific) containing a cocktail of protease and phosphatase inhibitors, and the protein concentration of synaptosomes was determined by using a bicinchoninic acid protein assay kit. Equal amounts of protein samples were incubated with Protein G Agarose Beads (#16–266, MilliporeSigma) prebound to the rabbit anti-GluN1 antibody (#G8913, MilliporeSigma) or rabbit IgG beads (#A2909, MilliporeSigma) at 4°C overnight, then washed, and extracted using 4% sodium dodecyl sulfate solution.

For immunoblotting, the above rabbit anti-GluN1 antibody, mouse anti-α2δ-1 antibody (#sc-271697, Santa Cruz Biotechnology), and mouse anti-PSD-95 antibody (#MABN1190, MilliporeSigma) were used as primary antibodies. TrueBlot anti-rabbit IgG (#18-8816-31, Rockland) or horseradish peroxidase-conjugated anti-mouse IgG (#7076S, Cell Signaling Technology) was used as the secondary antibody. An ECL kit (#34580, Thermo Fisher Scientific) was used to visualize the protein bands. For quantification, input protein bands were normalized to the input PSD-95 protein band on the same blot. The IP protein bands were normalized to the GluN1 protein band on the same blot. The mean values of the band density in vehicle-treated samples were considered to be 1.

Study design and data analysis. Data were expressed as the means ± standard error of the mean (SEM). The investigators conducting the experiments were blinded to the mouse genotype and treatment. Data from male and female mice were pooled because no sex differences in the electrophysiological and behavioral data were observed in this study. The sample sizes of behavioral, electrophysiological, and biochemistry experiments were similar to those we published previously (Chen et al., 2014c; Sun et al., 2019; Huang et al., 2023a). For electrophysiological data, only one neuron was recorded from each spinal cord slice, and at least three animals were used for each recording protocol. The input resistance was monitored, and the recording was abandoned if it changed >15%. The amplitude of evoked EPSCs and puff NMDA currents was quantified by averaging six consecutive traces using Clampfit 11 software (Molecular Devices). The frequency and amplitude of mEPSCs were analyzed using the MiniAnalysis program (Synaptosoft). The Shapiro–Wilk test was used to assess the normality of data distribution. We used two-tailed Student's *t* tests to compare the differences between two groups. Two-way ANOVA, followed by Tukey's or Šidák's *post hoc* test, was used to determine the differences between more than two groups. All statistical analyses were performed using Prism software (version 9, GraphPad Software). A p value <0.05 was considered statistically significant.

Results

Functional KCC2 is present in both VGluT2 and VGAT neurons in the spinal dorsal horn

The spinal dorsal horn is a crucial site of sensory integration accomplished through a network of heterogeneous populations of neurons. VGluT2- and VGAT-expressing dorsal horn neurons have a distinct role in processing and integrating nociceptive input (Koga et al., 2017; Wang et al., 2018). The neuron-specific KCC2 is constitutively active under isotonic conditions (Mercado et al., 2006). VU0463271 is a potent and specific KCC2 inhibitor (Delpire et al., 2012), which diminishes GABAergic inhibition and causes neuronal hyperexcitability in the hippocampus (Sivakumaran et al., 2015) and neonatal spinal

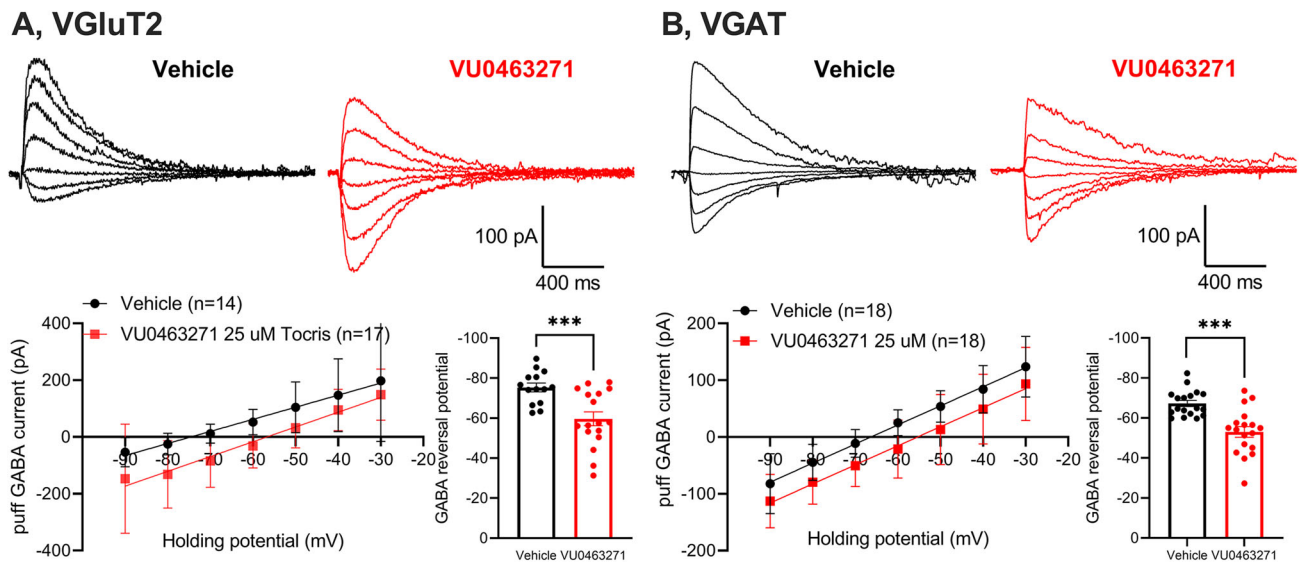

Figure 1. KCC2 inhibition induces a similar depolarizing shift in GABA reversal potentials in both VGluT2- and VGAT-expressing dorsal horn neurons. ***A***, Original traces, mean current–voltage plots, and mean data of GABA reversal potentials show puff application of GABA-elicited (100 μM, 15 ms) currents recorded at different holding potentials in lamina II VGluT2 neurons from vehicle-treated ($n = 14$ neurons from 4 mice) and VU0463271-treated ($n = 17$ neurons from 3 mice) slices. ***B***, Representative traces, mean current–voltage plots, and mean data of GABA reversal potentials show puff application of GABA-elicited (100 μM, 15 ms) currents recorded at different holding potentials in lamina II VGAT neurons from vehicle-treated ($n = 18$ neurons from 4 mice) and VU0463271-treated ($n = 18$ neurons from 6 mice) slices. Data are shown as means ± SEM. ***$p < 0.001$ (two-tailed Student's t test).

cord (Gackiere and Vinay, 2015). We first used VU0463271 to determine whether intrinsic KCC2 activity is differentially present in VGluT2 and VGAT neurons in the spinal dorsal horn. For this purpose, the spinal cord slices were incubated in vehicle or 25 μM VU0463271 for 30 min, and perforated whole-cell recordings were then conducted in tdTomato-tagged VGluT2 or VGAT neurons in lamina II. Interestingly, the basal E_{GABA} was significantly more depolarized in vehicle-treated VGAT neurons than that in vehicle-treated VGluT2 neurons (−67.28 ± 1.535 mV vs −75.32 ± 2.193 mV; $t_{(30)} = 3.095$; $p = 0.0042$; Fig. 1*A*,*B*). Treatment with VU0463271 caused a significant depolarizing shift in the E_{GABA} of VGluT2 neurons ($n = 14$ neurons in the vehicle group; $n = 17$ neurons in the VU0463271 group; $t_{(26.2)} = 3.821$; $p = 0.0007$; Fig. 1*A*). Similarly, in VGAT neurons, VU0463271 also caused a depolarizing shift in the E_{GABA} ($n = 18$ neurons per group; $t_{(26.93)} = 4.578$; $p < 0.0001$; Fig. 1*B*). These results suggest that basal KCC2 activity controls Cl^- homeostasis in both excitatory and inhibitory neurons in the spinal dorsal horn.

KCC2 inhibition potentiates postsynaptic NMDAR activity in VGluT2, but not VGAT, neurons in the spinal dorsal horn

To determine whether KCC2 constitutively regulates NMDAR activity in spinal VGluT2 and VGAT neurons in the spinal dorsal horn, we recorded NMDAR currents elicited by puff application of 100 μM NMDA to tdTomato-tagged VGluT2 or VGAT neurons in lamina II after incubation of spinal cord slices with vehicle or 25 μM VU0463271 for 30 min. The amplitude of currents elicited by puff application of NMDA to VGluT2 neurons was much greater in VU0463271-treated slices ($n = 14$ neurons) than that in vehicle-treated slices ($n = 13$ neurons) (275.3 ± 23.43 pA vs 125.2 ± 10.17 pA; $F_{(1, 57)} = 23.91$; $p < 0.0001$; Fig. 2*A*,*C*). In contrast, in VGAT neurons, the amplitude of puff NMDAR currents did not differ significantly between vehicle- and VU0463271-treated slices ($n = 15$ neurons per group; Fig. 2*B*, *C*). These findings suggest that KCC2 controls NMDAR activity in a cell type-specific manner and that normal KCC2 activity preferentially restrains postsynaptic NMDAR activity in VGluT2-expressing excitatory neurons in the spinal dorsal horn.

Inhibiting KCC2 potentiates presynaptic NMDAR activity in VGluT2, but not VGAT, neurons in the spinal dorsal horn

Both traumatic nerve injury and the chemotherapy drug paclitaxel potentiate presynaptic NMDAR activity in excitatory, but not inhibitory, spinal dorsal horn neurons (Huang et al., 2023b). We then determined whether KCC2 inhibition affects presynaptic NMDAR activity in VGluT2 and VGAT dorsal horn neurons. Whole-cell recordings showed that treatment with 25 μM VU0463271 for 30 min significantly increased the frequency, but not the amplitude, of mEPSCs in VGluT2 neurons (5.025 ± 0.742 Hz vs 2.436 ± 0.301 Hz; $F_{(1, 23)} = 7.578$; $p = 0.0157$; $n = 13$ neurons in VU0463271-treated slices; $n = 12$ neurons in vehicle-treated slices; Fig. 3*A*–*C*). Bath application of 50 μM AP5, a specific NMDAR antagonist, for 6 min had no effect on the frequency of mEPSCs in vehicle-treated VGluT2 neurons. In contrast, AP5 rapidly reduced the frequency of mEPSCs in VGluT2 neurons increased by VU0463271 treatment (5.025 ± 0.742 Hz vs 3.685 ± 0.641 Hz; $F_{(1.538, 35.37)} = 24.08$; $p < 0.0001$; Fig. 3*A*–*C*).

To determine whether KCC2 inhibition-induced presynaptic NMDAR activity in the spinal dorsal horn is cell-type specific, we also recorded mEPSCs in tdTomato-tagged VGAT neurons in lamina II after incubation with vehicle or 25 μM VU0463271 for 30 min. Remarkably, neither the frequency nor amplitude of mEPSCs in VGAT neurons differed significantly between vehicle-treated ($n = 15$ neurons) and VU0463271-treated ($n = 14$ neurons) slices (Fig. 4*A*–*C*). Furthermore, bath application of 50 μM AP5 had no effect on the frequency or amplitude of mEPSCs in VGAT neurons from vehicle-treated and VU0463271-treated slices (Fig. 4*A*–*C*). Together, these data suggest that normal KCC2 activity preferentially suppresses presynaptic NMDAR activity in VGluT2-expressing excitatory neurons in the spinal dorsal horn.

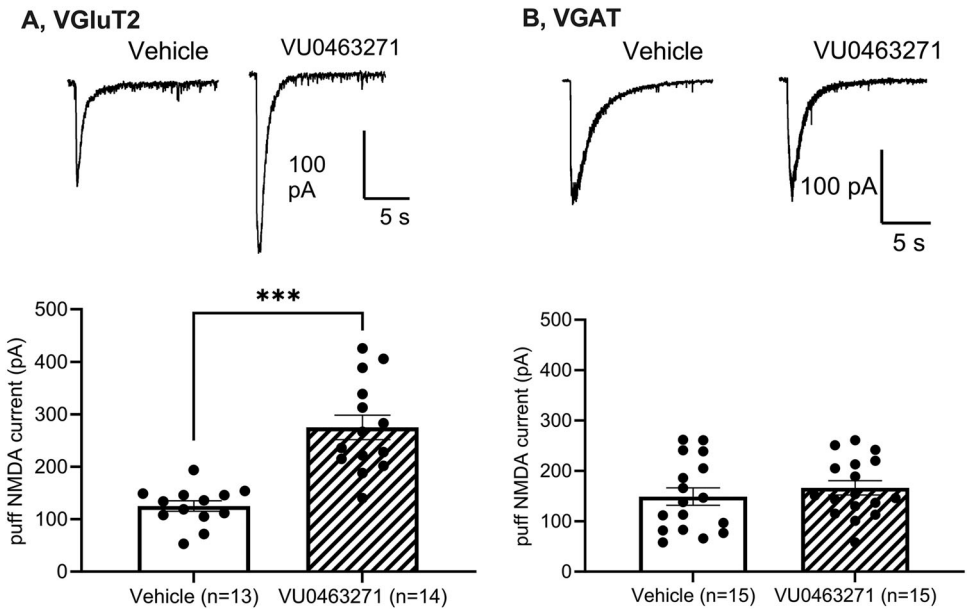

Figure 2. Inhibiting KCC2 increases postsynaptic NMDAR currents in VGluT2, but not in VGAT, spinal dorsal horn neurons. ***A***, ***B***, Representative recording traces show currents elicited by puff application of 100 μM NMDA to lamina II VGluT2 or VGAT neurons from vehicle-treated and VU0463271-treated slices. ***C***, Mean data show currents elicited by puff application of 100 μM NMDA in lamina II VGluT2 neurons from vehicle-treated ($n = 13$ neurons from 3 mice) and VU0463271-treated ($n = 14$ neurons from 3 mice) slices as well as VGAT neurons from vehicle-treated ($n = 15$ neurons from 4 mice) and VU0463271-treated ($n = 15$ neurons from 4 mice) slices. Two-way ANOVA showed a significant main effect for drug treatment ($p < 0.0001$; $F_{(1, 57)} = 23.91$) and a significant interaction between the drug treatment and cell types (($p = 0.0159$, $F(1,57) = 6.175$)). Data are presented as means ± SEM. ***$p < 0.001$ compared with vehicle group within the same cell type (two-way ANOVA followed by Šidák *post hoc* test).

Inhibiting KCC2 does not affect activity of presynaptic NMDARs at primary afferent terminals that form synapses with VGluT2 neurons

VGluT2 neurons in the spinal dorsal horn receive glutamatergic input from primary afferent terminals and interneurons (Santos et al., 2007; Wang et al., 2018; Chen et al., 2022; Huang et al., 2022). NMDARs are expressed at primary afferent terminals in the spinal superficial dorsal horn but are normally quiescent (Liu et al., 1994; Chen et al., 2018; Deng et al., 2019; Zhang et al., 2021; Huang et al., 2022, 2023b). In both nerve injury- and chemotherapy-induced neuropathic pain, presynaptic NMDARs become tonically activated at primary afferent central terminals that form synapses with VGluT2 spinal dorsal horn neurons (Huang et al., 2020). However, unlike NKCC1, KCC2 is not expressed in dorsal root ganglion (DRG) neurons (Sung et al., 2000; Li et al., 2016). We next determined specifically whether VU0463271-induced increases in presynaptic NMDAR activity occurs at primary afferent terminals that form synapses with excitatory neurons in the spinal dorsal horn. After treating spinal cord slices with vehicle or 25 μM VU0463271 for 30 min, we recorded EPSCs of tdTomato-tagged VGluT2 neurons in lamina II that were monosynaptically evoked from the dorsal root, which reflect glutamate release elicited from primary afferent terminals. The amplitude of evoked monosynaptic EPSCs in VGluT2 neurons did not differ significantly between the vehicle ($n = 13$ neurons) and VU0463271 ($n = 13$ neurons) groups (Fig. 5*A*,*B*). Also, bath application of 50 μM AP5 for 6 min did not affect significantly the amplitude of evoked EPSCs in VGluT2 neurons from vehicle-treated and VU0463271-treated slices (Fig. 5*A*,*B*).

In addition, we recorded the PPR of monosynaptically evoked EPSCs, a measure of probability of glutamate release from presynaptic terminals, in tdTomato-tagged VGluT2 neurons in lamina II. The baseline PPR of evoked EPSCs in VGluT2 neurons was similar between vehicle-treated ($n = 13$ neurons) and VU0463271-treated ($n = 14$ neurons) slices (Fig. 5*C*,*D*). In VGluT2 neurons from both vehicle-treated and VU0463271-treated slices, bath application of 50 μM AP5 for 6 min had no significant effect on the PPR of evoked EPSCs (Fig. 5*C*,*D*). Because KCC2 inhibition did not increase the activity of NMDARs at primary afferent terminal–excitatory neuron synapses, it suggests that KCC2 likely regulates NMDAR activity in a synapse type-specific manner. Thus, normal KCC2 activity predominantly restrains glutamatergic transmission between excitatory interneurons in the spinal dorsal horn.

KCC2 inhibition potentiates presynaptic and postsynaptic NMDAR activity in spinal VGluT2 dorsal horn neurons via α2δ-1

α2δ-1 is a newly identified regulatory protein of NMDARs and is essential for augmented NMDAR activity in the spinal dorsal horn in neuropathic pain (Chen et al., 2018; Huang et al., 2022). We thus examined whether α2δ-1 is required for elevated presynaptic NMDAR activity in lamina II neurons induced by VU0463271 treatment. Spinal lamina II predominantly consists of VGluT2 neurons, with a small population of VGAT neurons (Chen et al., 2022). Spinal cord slices from mice lacking α2δ-1 (*Cacna2d1* KO) were incubated with vehicle or 25 μM VU0463271 for 30 min, and whole-cell patch-clamp recordings were then performed to record mEPSCs in lamina II neurons. Both the baseline frequency and amplitude of mEPSCs did not differ in vehicle-treated ($n = 14$ neurons) and VU0463271-treated ($n = 13$ neurons) lamina II neurons from mice (Fig. 6*A*–*C*). Bath application of 50 μM AP5 for 6 min had no effect on either the frequency or amplitude of mEPSCs in vehicle-treated and VU0463271-treated spinal lamina II neurons (Fig. 6*A*–*C*).

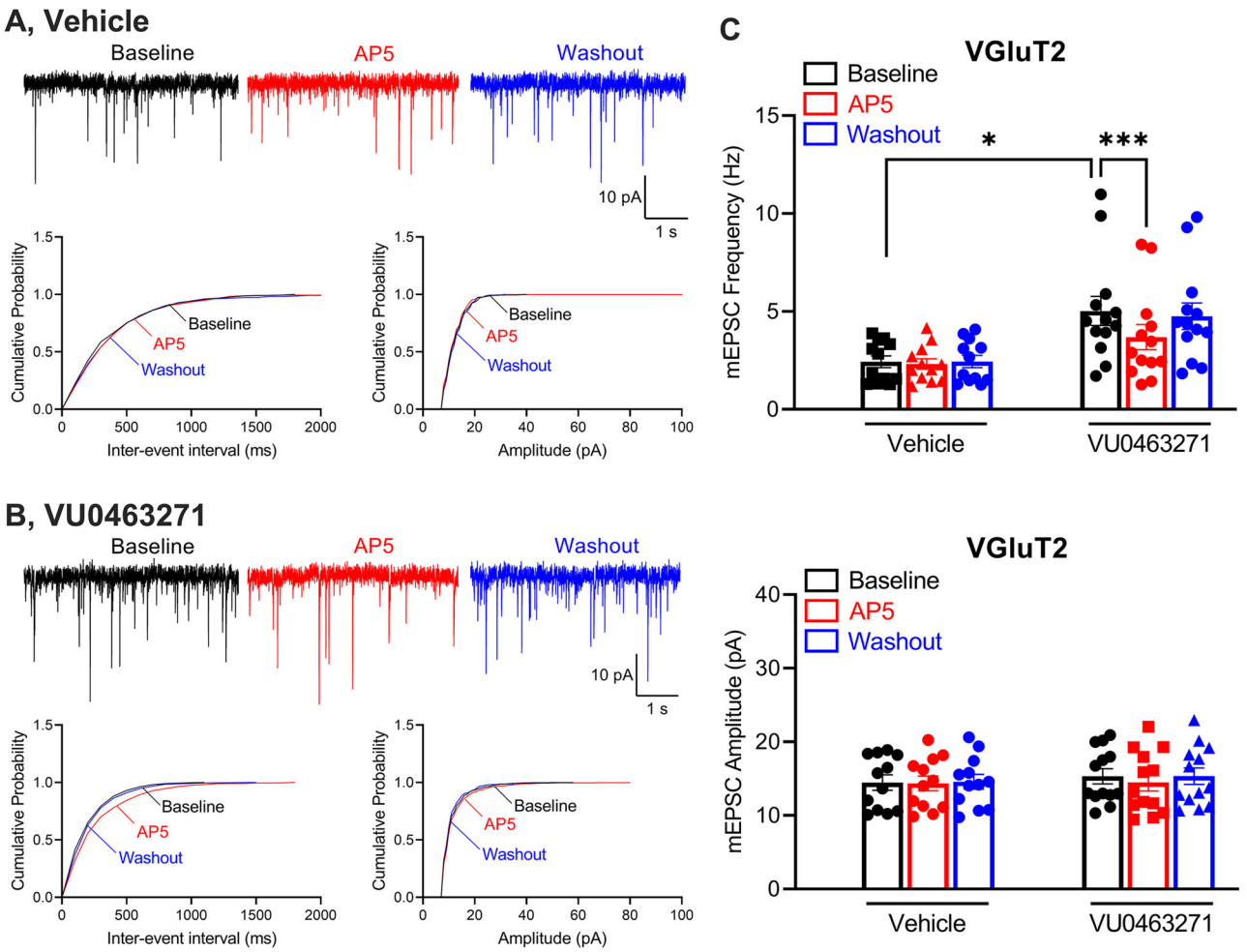

Figure 3. KCC2 inhibition increases presynaptic NMDAR activity in VGluT2 spinal dorsal horn neurons. ***A***, ***B***, Representative recording traces and cumulative probability plots show the effect of bath application of 50 μM AP5 on mEPSCs of lamina II VGluT2 neurons from vehicle-treated and VU0463271-treated slices. ***C***, Mean data show the effect of AP5 on the frequency and amplitude of mEPSCs of lamina II VGluT2 neurons from vehicle-treated ($n = 12$ neurons from 3 mice) and VU0463271-treated ($n = 13$ neurons from 3 mice) slices. Two-way ANOVA showed that there were a significant main effect for VU0463271 treatment ($p = 0.0113$; $F_{(1, 23)} = 7.578$) and AP5 treatment ($p < 0.0001$; $F_{(1.538, 35.37)} = 24.08$) and a significant interaction between the VU0463271 and AP5 treatment ($p < 0.0001$; $F_{(2, 46)} = 16.11$). Data are shown as means ± SEM. $*p < 0.05$, $***p < 0.001$ (two-way ANOVA followed by Tukey's or Šidák *post hoc* test).

To further determine whether α2δ-1 is essential for postsynaptic NMDAR activity in spinal dorsal horn neurons potentiated by KCC2 inhibition, we measured currents elicited by puff application of NMDA to lamina II neurons in *Cacna2d1* KO mice after treatment with vehicle or 25 μM VU0463271 for 30 min. The amplitude of puff NMDAR currents in lamina II neurons did not differ significantly between vehicle- and VU0463271-treated slices from *Cacna2d1* KO mice ($n = 14$ neurons per group; Fig. 6*D*). Together, these findings indicate that α2δ-1 is requisite for the KCC2 inhibition-induced tonic activity of presynaptic and postsynaptic NMDARs of spinal dorsal horn neurons.

KCC2 inhibition increases α2δ-1–NMDAR interactions and their synaptic expression in the spinal cord

Because α2δ-1 is integral to increased synaptic NMDAR activity caused by KCC2 inhibition, we next determined whether KCC2 controls the interaction of α2δ-1 with NMDARs in the spinal cord. We obtained synaptosomes from dorsal spinal cord tissues in mice 30 min after intrathecal injection of vehicle or 5 μg VU0463271. We used co-IP to determine the interaction between α2δ-1 and GluN1, the obligatory subunit of NMDARs. Immunoblotting analysis of spinal synaptosomes showed that both the GluN1 ($t_{(12)} = 5.253$; $p = 0.0002$) and α2δ-1 ($t_{(12)} = 5.317$; $p = 0.0002$) protein levels were significantly higher in mice treated with VU0463271 than those in mice treated with vehicle ($n = 7$ mice per group; Fig. 7*A*,*B*). In addition, co-IP assays showed that the amount of α2δ-1 proteins pulled down by the GluN1 antibody in spinal synaptosomes was much greater in mice treated with VU0463271 than that in mice treated with vehicle ($n = 7$ mice per group; $t_{(12)} = 4.714$; $p = 0.0005$; Fig. 7*A*,*C*). These results suggest that KCC2 inhibition potentiates the physical interaction between α2δ-1 and NMDARs and synaptic trafficking of α2δ-1-bound NMDARs in the spinal dorsal horn.

Inhibiting KCC2 augments glutamatergic synaptic transmission in spinal VGluT2 neurons via α2δ-1-bound NMDARs

α2δ-1 interacts with NMDARs predominantly via its C terminus, and the α2δ-1 C-terminus mimicking peptide effectively disrupts the α2δ-1–NMDAR interaction in vitro and in vivo (Chen et al., 2018; Zhou et al., 2018). Therefore, we utilized the α2δ-1 C-terminus mimicking peptide to determine the role of α2δ-1-bound NMDARs in KCC2 inhibition-induced

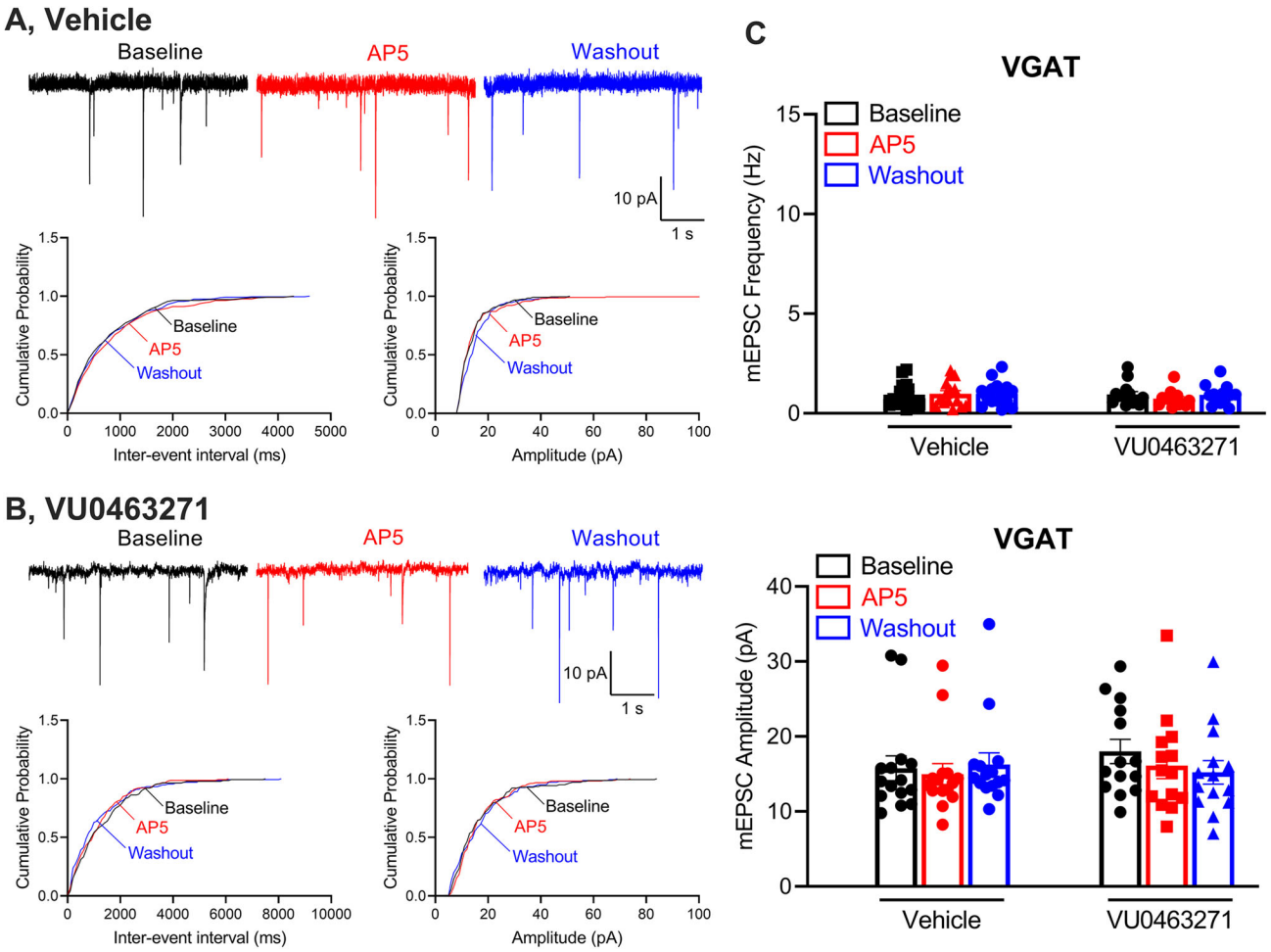

Figure 4. Inhibiting KCC2 has no effect on presynaptic NMDAR activity in VGAT dorsal horn neurons. ***A***, ***B***, Original recording traces and cumulative probability plots show the effect of bath application of 50 μM AP5 on mEPSCs of lamina II VGAT neurons from vehicle-treated and VU0463271-treated slices. ***C***, Mean data show the effect of AP5 on the frequency and amplitude of mEPSCs of lamina II VGluT2 neurons from vehicle-treated ($n = 15$ neurons from 4 mice) and VU0463271-treated ($n = 14$ neurons from 4 mice) slices. Data are shown as means ± SEM.

presynaptic NMDAR activity in spinal excitatory neurons. We recorded mEPSCs of tdTomato-tagged VGluT2 neurons in lamina II from spinal cord slices incubated with 25 μM VU0463271 in the presence of 1 μM control peptide or 1 μM α2δ-1 peptide for 30 min. The frequency of mEPSCs in VGluT2 neurons was much lower in slices treated with α2δ-1 peptide plus VU0463271 than that in slices treated with control peptide plus VU0463271 ($n = 12$ neurons per group; 2.403 ± 0.220 Hz vs 5.970 ± 0.342 Hz; $F_{(1, 22)} = 59.46$; $p < 0.0001$; Fig. 8*A–C*), whereas the mEPSC amplitude in VGluT2 neurons was similar between the two groups. In addition, bath application of AP5 for 6 min largely reduced the frequency of mEPSCs of VGluT2 neurons in slices treated with control peptide plus VU0463271 (5.970 ± 0.342 Hz vs 3.929 ± 0.328 Hz; $F_{(1.613, 35.48)} = 20.37$; $p = 0.0005$; Fig. 8*A–C*). In contrast, AP5 had no significant effect on the frequency of mEPSCs in VGluT2 neurons in slices treated with α2δ-1 peptide plus VU0463271.

To determine whether α2δ-1-bound NMDARs are involved in postsynaptic NMDAR activity of VGluT2 neurons potentiated by KCC2 inhibition, we recorded currents elicited by puff NMDA onto tdTomato-tagged VGluT2 neurons in lamina II from spinal cord slices incubated with 25 μM VU0463271 in the presence of 1 μM control peptide or 1 μM α2δ-1 peptide for 30 min. The amplitude of puff NMDAR currents in VGluT2 neurons was much smaller in slices treated with α2δ-1 peptide plus VU0463271 than that in slices treated with control peptide plus VU0463271 (112.3 ± 9.70 pA vs 276.9 ± 21.76 pA; $t_{(19.23)} = 6.909$; $p < 0.0001$; Fig. 8*D*). Collectively, these findings suggest that KCC2 constitutively restrains glutamatergic transmission in excitatory dorsal horn neurons via α2δ-1-bound NMDARs at both presynaptic and postsynaptic sites.

Spinal cord α2δ-1 and NMDARs are critical for KCC2 inhibition-induced pain hypersensitivity

Because KCC2 inhibition augments presynaptic and postsynaptic NMDAR activity in spinal dorsal horn excitatory neurons, we subsequently determined whether KCC2 inhibition at the spinal cord level induces pain hypersensitivity via NMDARs. To this end, we measured nociceptive thresholds in mice after intrathecal administration of vehicle or 5 μg VU0463271 in WT mice. Intrathecal injection of VU0463271, but not vehicle, resulted in a large reduction in tactile, pressure, and heat withdrawal thresholds within 30 min ($n = 10$ mice per group; Fig. 9*A*). Remarkably, intrathecal injection of 5 μg AP5 15 min prior to VU0463271 injection diminished the reduction in tactile, pressure, and heat withdrawal thresholds caused by VU0463271 injection in WT mice ($n = 10$ mice; Fig. 9*B*).

Furthermore, intrathecal injection of 5 μg pregabalin, a clinical used α2δ-1 inhibitory ligand, 15 min before intrathecal treatment with 5 μg VU0463271 similarly abolished VU0463271-

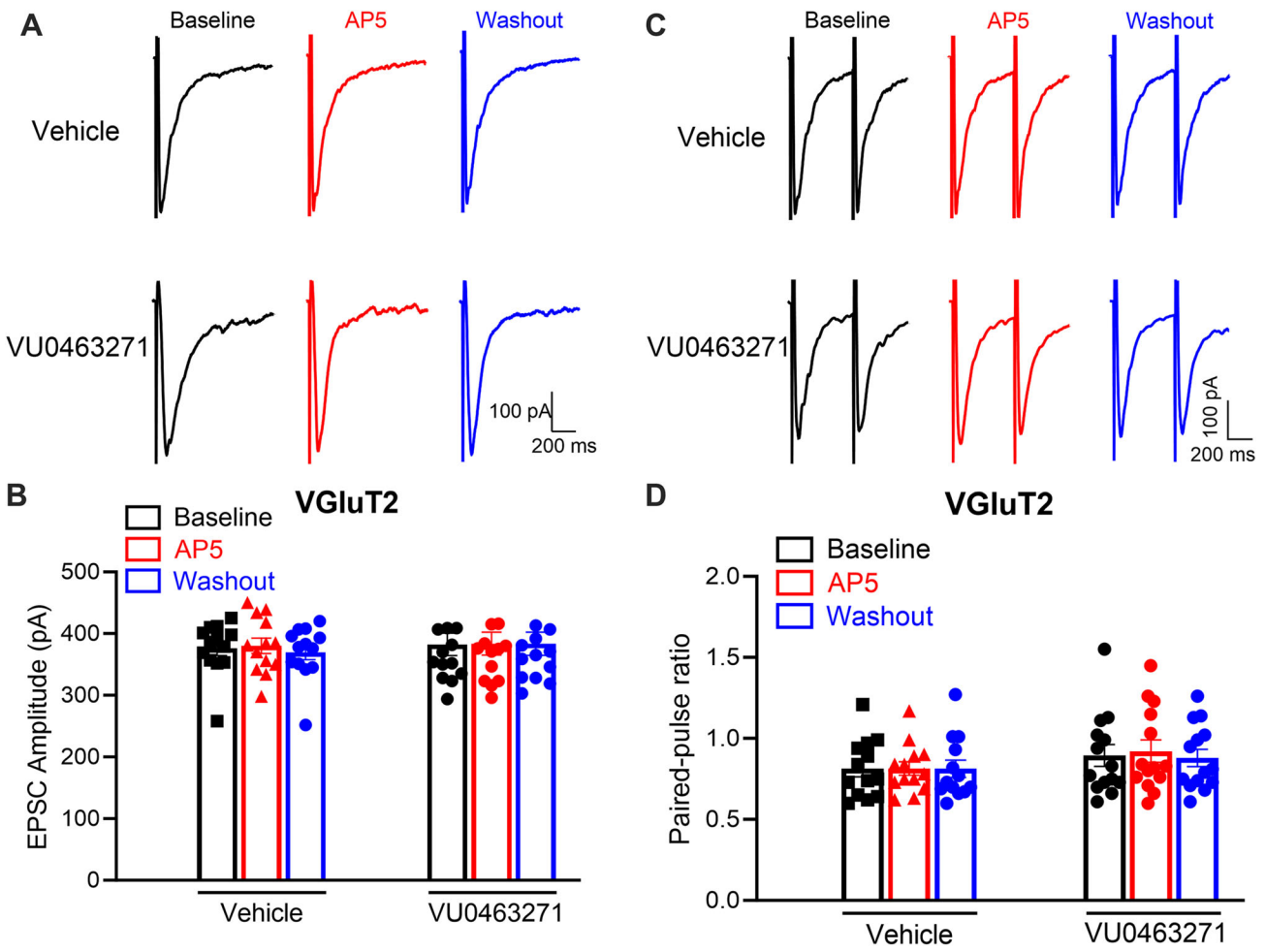

Figure 5. KCC2 inhibition has no effect on presynaptic NMDARs at primary afferent terminals that synapse VGluT2 dorsal horn neurons. ***A***, ***B***, Original recording traces (***A***) and summary data (***B***) show the effect of bath application of 50 μM AP5 on the amplitude of EPSCs in lamina II VGluT2 neurons evoked monosynaptically from the dorsal root in vehicle-treated ($n = 13$ neurons from 6 mice) and VU0463271-treated ($n = 14$ neurons from 6 mice) slices. ***C***, ***D***, Representative recording traces (***C***) and mean data (***D***) show the effect of bath application 50 μM AP5 on the paired–pulse ratio (PPR) of EPSCs in lamina II VGluT2 neurons evoked monosynaptically from the dorsal root in vehicle-treated ($n = 13$ neurons from 6 mice) and VU0463271-treated ($n = 14$ neurons from 6 mice) slices. Data are means ± SEM.

induced reduction in tactile, pressure, and heat withdrawal thresholds in WT mice ($n = 10$ mice; Fig. 9*B*). In addition, intrathecal injection of 5 μg VU0463271 failed to significantly alter the tactile, pressure, or heat withdrawal thresholds in *Cacna2d1* KO mice ($n = 10$ mice; Fig. 9*B*). These in vivo results strongly suggest that KCC2 regulates spinal nociceptive transmission via both NMDARs and α2δ-1.

α2δ-1-bound NMDARs at the spinal cord level mediate pain hypersensitivity caused by KCC2 inhibition

Lastly, we used the α2δ-1 C-terminus peptide to determine whether KCC2 inhibition at the spinal cord level induces pain hypersensitivity through α2δ-1-bound NMDARs. WT mice received intrathecal injection of 5 μg control peptide 15 min prior to injection of 5 μg VU0463271 exhibited a large reduction in tactile, pressure, and heat withdrawal thresholds. In contrast, intrathecal injection of 5 μg α2δ-1 C-terminus mimicking peptide fully blocked the reduction in tactile, pressure, and heat withdrawal thresholds induced by VU0463271 in WT mice ($n = 10$ mice per group; Fig. 9*C*). These data suggest that pain hypersensitivity induced by KCC2 inhibition at the spinal cord level depends on α2δ-1-bound NMDARs.

Discussion

We have demonstrated that intrinsic KCC2 activity is present in both VGluT2-expressing excitatory and VGAT-expressing inhibitory neurons in the spinal dorsal horn. In this study, we found that inhibiting KCC2 with VU0463271 caused a similar depolarizing shift (~15 mV) of E_{GABA} in both VGluT2 and VGAT neurons, which closely resembles the level observed in dorsal horn neurons of mice subjected to nerve injury (Huang et al., 2023b). Computational analysis suggests that even a ~10 mV shift can have a substantial effect on the strength of postsynaptic inhibition and neuronal excitability (Doyon et al., 2011). The subsequent depolarizing shift in the GABA/glycine reversal potential weakens synaptic inhibition and promotes neuronal excitability (Sivakumaran et al., 2015; Lee et al., 2019). Normal KCC2 activity is crucial for the maintenance of intracellular Cl^- homeostasis and the Cl^- gradient across the plasma membrane in neurons (Payne et al., 1996; Rivera et al., 1999; Mahadevan and Woodin, 2016). KCC2 functional impairment through genetic knockdown, pharmacological inhibition, or nerve injury-induced downregulation consistently leads to weakened inhibitory strength (Coull et al., 2003; Zhou et al., 2012; Gackiere and Vinay, 2015).

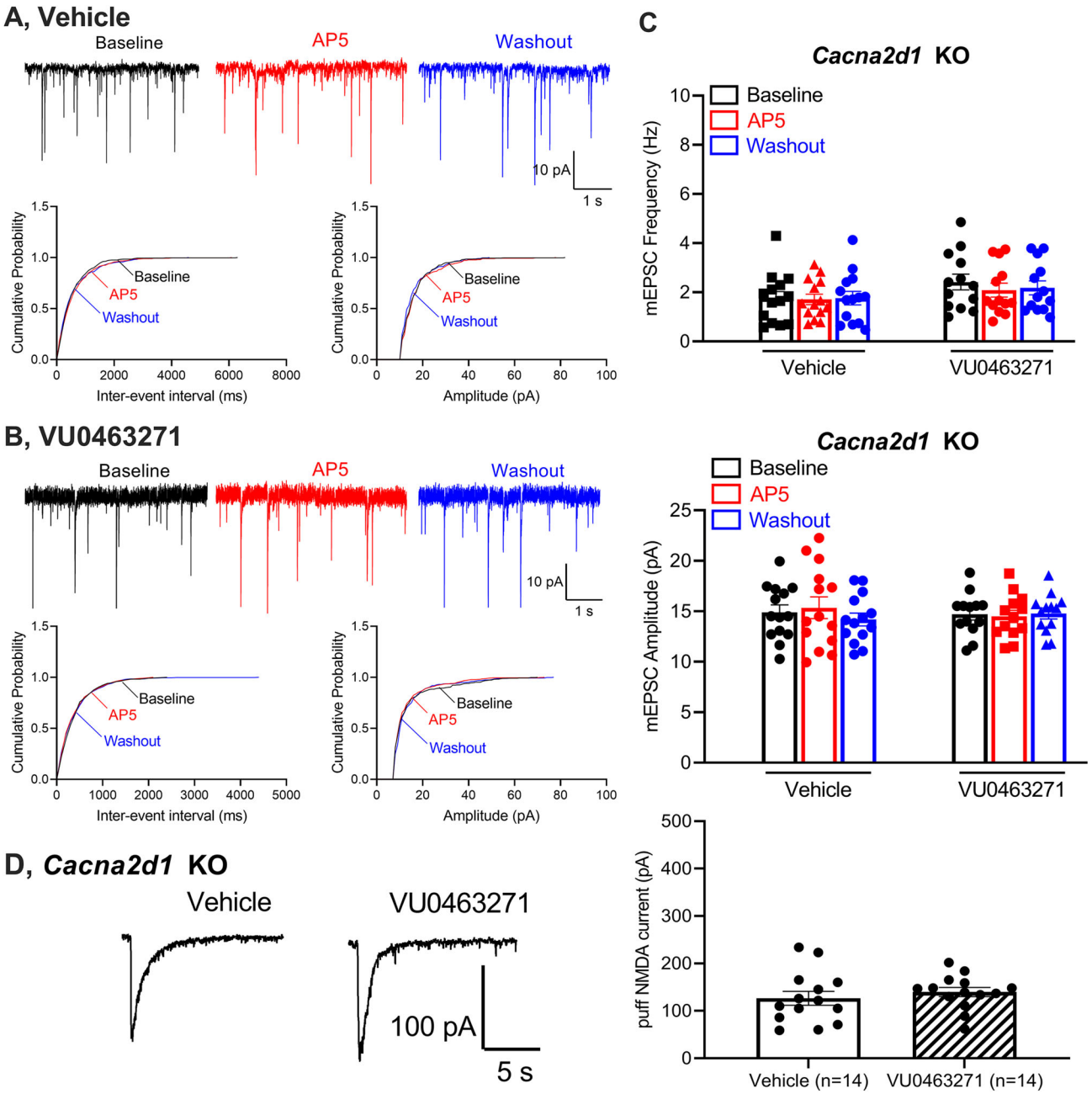

Figure 6. Inhibiting KCC2 augments presynaptic and postsynaptic NMDAR activity in dorsal horn neurons via α2δ-1. ***A***, ***B***, Representative recording traces and cumulative probability plots show the effect of bath application of 50 μM AP5 on mEPSCs of lamina II neurons from vehicle-treated (***A***) or VU0463271-treated (***B***) slices of *Cacna2d1* KO mice. ***C***, Mean data show the effect of AP5 on the frequency and amplitude of mEPSCs of lamina II neurons from vehicle-treated ($n = 14$ neurons from 3 mice) and VU0463271-treated ($n = 13$ neurons from 3 mice) slices of *Cacna2d1* KO mice. ***D***, Original recording traces and mean data show currents elicited by puff application of 100 μM NMDA in lamina II neurons from vehicle-treated ($n = 14$ neurons from 3 mice) and VU0463271-treated ($n = 14$ neurons from 3 mice) slices of *Cacna2d1* KO mice. Data are presented as means ± SEM.

Remarkably, we found that the basal E_{GABA} was significantly more depolarized in VGAT neurons compared with VGluT2 neurons, suggesting that the intracellular Cl^- level, co-regulated by NKCC1 and KCC2, is higher in VGAT neurons than in VGluT2 neurons. Consequently, compared with VGAT neurons, VGluT2 neurons may receive greater synaptic inhibition by GABA and glycine, relying more heavily on inhibition to counterbalance strong excitation in the normal spinal cord (Lee et al., 2019; Spoljaric et al., 2019).

A major finding of our study is that KCC2 inhibition with VU0463271 potentiates presynaptic and postsynaptic NMDAR activity in VGluT2, but not VGAT, neurons in the spinal lamina II, suggesting a critical role of KCC2 in selectively restraining synaptic NMDAR activity in excitatory interneurons. In a basal state, the participation of NMDARs in synaptic transmission is likely suppressed by synaptic inhibition, which maintains a negative resting membrane potential and the Mg^{2+} block of NMDARs. However, loss of synaptic inhibition by KCC2 impairment allows even low-intensity stimulation to induce neuronal firing and central sensitization (Coull et al., 2003; Zhou et al., 2012). The interplay between spinal KCC2 and NMDARs has been implicated in the context of neuropathic pain conditions.

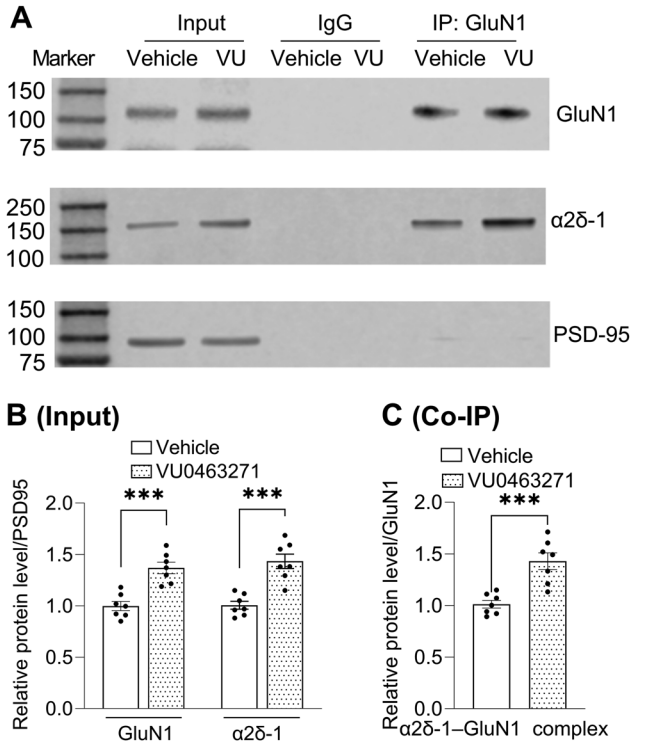

Figure 7. KCC2 inhibition increases physical interactions between α2δ-1 and NMDARs and their synaptic trafficking in the dorsal spinal cord. ***A–C***, Representative blotting images (***A***) and quantification (***B***, ***C***) show the effects of VU0463271 on the protein levels of GluN1, α2δ-1, and α2δ-1–GluN1 complexes in mouse spinal cord synaptosomes. Mice were intrathecally injected with vehicle or VU0463271 (VU), and the spinal cords were obtained 30 min later ($n = 7$ mice per group). Synaptosomal proteins from the dorsal spinal cord were extracted for immunoprecipitation with an anti-GluN1 antibody. Immunoblotting was then performed using primary antibodies against GluN1, α2δ-1, and PSD-95 (a synaptic protein marker). Input protein levels were normalized to PSD-95, and α2δ-1–GluN1 protein complexes were normalized to GluN1 on the same blot. Data are mean ± SEM. $***p < 0.001$ (two-tailed Student's *t* test).

For instance, KCC2 overexpression restores synaptic inhibition and reinstates normal synaptic NMDAR activity in the spinal dorsal horn caused by nerve injury (Li et al., 2016). On the other hand, nerve injury-induced excessive NMDAR activation causes Ca^{2+} influx and induces calpain-mediated KCC2 proteolytic cleavage, impairing Cl^- homeostasis and synaptic inhibition in the spinal dorsal horn (Zhou et al., 2012). It is possible that reduced KCC2 activity, in turn, amplifies NMDAR activity in spinal excitatory neurons. This mechanism could explain how peripheral nerve injury leads to persistent NMDAR hyperactivity in spinal dorsal horn neurons in neuropathic pain.

Our findings unveil a pronounced asymmetry in the influence of KCC2 impairment-induced synaptic disinhibition on synaptic NMDAR activity in spinal excitatory and inhibitory neurons. $GABA_A$ or glycine receptor-mediated synaptic inhibition of spinal dorsal horn neurons typically curtails nociceptive transmission by dampening signals from primary afferents (Grudt and Henderson, 1998; Zhang et al., 2005; Zhou et al., 2012; Chen et al., 2014c). Although lamina II excitatory neurons receive synaptic input directly from DRG neurons, inhibitory and excitatory interneurons as well as supraspinal descending fibers, lamina II inhibitory neurons are largely innervated by presynaptic terminals from DRG neurons and supraspinal descending fibers (Pan and Pan, 2004; Zhou et al., 2008; Foster et al., 2015). In this regard, spinal inhibitory neurons expressing the glycine transporter GlyT2 mainly receive monosynaptic input from large DRG neurons expressing NF200 (Foster et al., 2015). The presynaptic terminals engaging VGAT neurons in our recordings likely originate from primary afferent central terminals devoid of KCC2 expression, thus explaining the lack of effect of KCC2 inhibition on mEPSCs of VGAT neurons.

Our findings indicate that increased postsynaptic NMDAR activity in spinal excitatory neurons by KCC2 inhibition requires α2δ-1. Recent studies indicate that α2δ-1 is essential for NMDAR synaptic trafficking and activity in the brain and spinal cord irrespective of its conventional role as a calcium channel subunit (Chen et al., 2018; Luo et al., 2018; Zhou et al., 2018). In this study, we showed that treatment with VU0463271 augmented α2δ-1–NMDAR interactions and their synaptic trafficking in the spinal cord. We also demonstrated that in *Cacna2d1* KO mice, treatment with VU0463271 failed to induce activation of postsynaptic NMDARs in spinal dorsal horn neurons. Furthermore, disrupting α2δ-1–NMDAR interactions with the α2δ-1 C-terminus peptide abolished the effect of VU0463271 on postsynaptic NMDAR activity in VGluT2 neurons. Because α2δ-1 predominantly resides in VGluT2-expressing neurons in the spinal dorsal horn (Koga et al., 2023), this may explain why KCC2 inhibition augmented postsynaptic NMDAR activity in VGluT2, but not in VGAT, neurons in the spinal dorsal horn. The KCC2 inhibition-induced potentiation in α2δ-1–NMDAR interactions likely results from the increase in neuronal activity. In this regard, inhibition of calcineurin, a Ca^{2+}/calmodulin-activated protein phosphatase, enhances α2δ-1–NMDAR interactions and augments synaptic NMDAR activity in the spinal cord (Chen et al., 2014a; Huang et al., 2020), suggesting an important role of protein phosphorylation in the interaction between α2δ-1 and NMDARs. Also, α2δ-1 is indispensable in protein kinase C (PKC)-induced NMDAR synaptic trafficking (Zhou et al., 2021). Accordingly, α2δ-1 functions as a phosphobinding protein and mostly interacts with phosphorylated NMDARs. Because NMDAR phosphorylation and activity are regulated by numerous protein kinases (e.g., PKC, CK2, and Fyn; Chen et al., 2014b; Hildebrand et al., 2016; Zhou et al., 2021) and phosphatases (e.g., calcineurin and STEP61; Dedek et al., 2019; Huang et al., 2020, 2022), neuronal/synaptic hyperactivity can augment kinase activity and/or attenuate phosphatase activity, thereby potentiating NMDAR phosphorylation and promoting the α2δ-1–NMDAR interaction. This event eventually enhances the synaptic trafficking and activity of α2δ-1-bound NMDARs in spinal excitatory neurons.

Another salient finding of our study is that α2δ-1 is essential for the KCC2 control of presynaptic NMDAR activity in spinal excitatory neurons. We found that inhibition of KCC2 increased the AP5-sensitive mEPSC frequency only in VGluT2 neurons, which was blocked by *Cacna2d1* KO or α2δ-1 C terminus-interfering peptide. Hence, it is plausible that KCC2 inhibition primarily affects VGluT2 dorsal horn neurons that form synapses with α2δ-1-expressing presynaptic terminals from other excitatory neurons. Notably, loss of synaptic inhibition and potentiated NMDAR activity at the spinal level has largely been regarded as separate, yet dependent, events in neuropathic pain. Our study reinforces the crucial role of KCC2-mediated Cl^- homeostasis in the reciprocal relationship between synaptic inhibition and NMDAR activity. An imbalance in this interplay likely initiates a vicious cycle, sustaining central sensitization and perpetuating chronic neuropathic pain. Our findings also indicate that spinal synaptic disinhibition directly augments glutamatergic input among excitatory interneurons

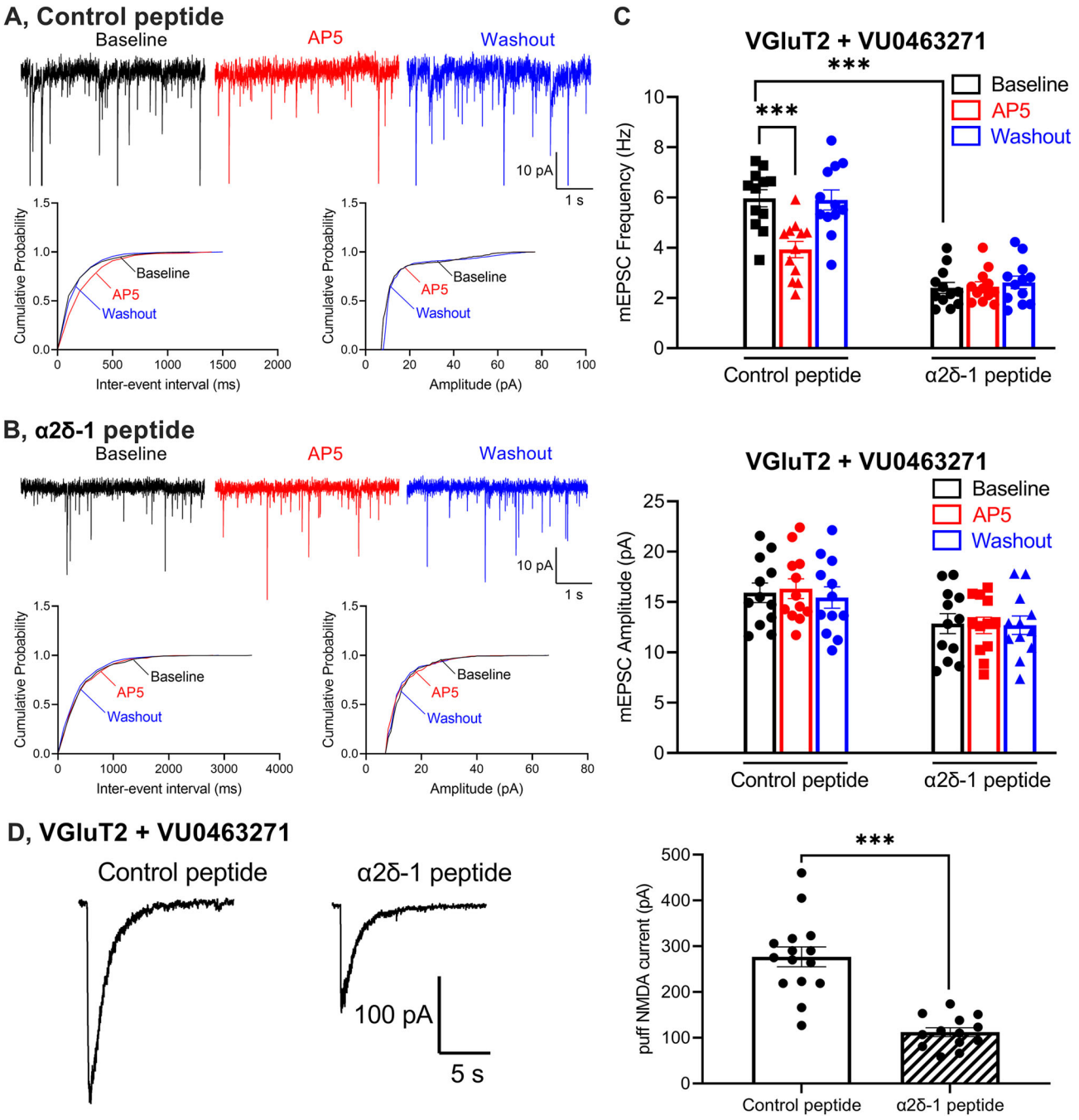

Figure 8. Inhibiting KCC2 increases presynaptic and postsynaptic NMDAR activity in VGluT2 dorsal horn neurons via α2δ-1-bound NMDARs. ***A***, ***B***, Original recording traces and cumulative probability plots show the effect of bath application of 50 μM AP5 on mEPSCs of lamina II VGluT2 neurons from slices co-treated with VU0463271 plus control peptide (***A***) or VU0463271 plus α2δ-1 peptide (***B***). ***C***, Mean data show the effect of AP5 on the frequency and amplitude of mEPSCs of lamina II VGluT2 neurons from slices co-treated with VU0463271 plus control peptide ($n = 12$ neurons from 3 mice) or VU0463271 plus α2δ-1 peptide ($n = 12$ neurons from 3 mice). Two-way ANOVA showed that there were a significant main effect for α2δ-1 peptide treatment ($p < 0.0001$; $F_{(1, 22)} = 59.46$) and AP5 treatment ($p < 0.0001$; $F_{(1.613, 35.48)} = 20.37$) and a significant interaction between the α2δ-1 peptide and AP5 treatment ($p < 0.0001$; $F_{(2, 44)} = 18.46$). ***$p < 0.001$ (two-way ANOVA followed by Tukey's or Šidák *post hoc* test). ***D***, Representative recording traces and mean data show currents elicited by puff application of 100 μM NMDA in lamina II VGluT2 neurons from slices co-treated with VU0463271 plus control peptide ($n = 15$ neurons from 3 mice) or VU0463271 plus α2δ-1 peptide ($n = 13$ neurons from 3 mice). Data are mean ± SEM. ***$p < 0.001$ (two-tailed Student's *t* test).

via α2δ-1-bound NMDARs at both presynaptic and postsynaptic sites. This provides novel insights into the mechanisms of synaptic plasticity in the nociceptive circuitry.

Our study reveals that KCC2 impairment selectively potentiates NMDAR-mediated glutamatergic transmission between excitatory interneurons, but not NMDARs at primary afferent-excitatory neuron synapses, in the spinal dorsal horn. This is supported by our finding that inhibiting KCC2 had no effect on EPSCs monosynaptically evoked from the dorsal root, indicating nonprimary afferent terminals as the origin of presynaptic NMDAR activity increased by KCC2 inhibition. While various molecular mechanisms are implicated in evoked and spontaneous/quantal glutamate release, our study specifically determined the KCC2 inhibition-induced increase in synaptic

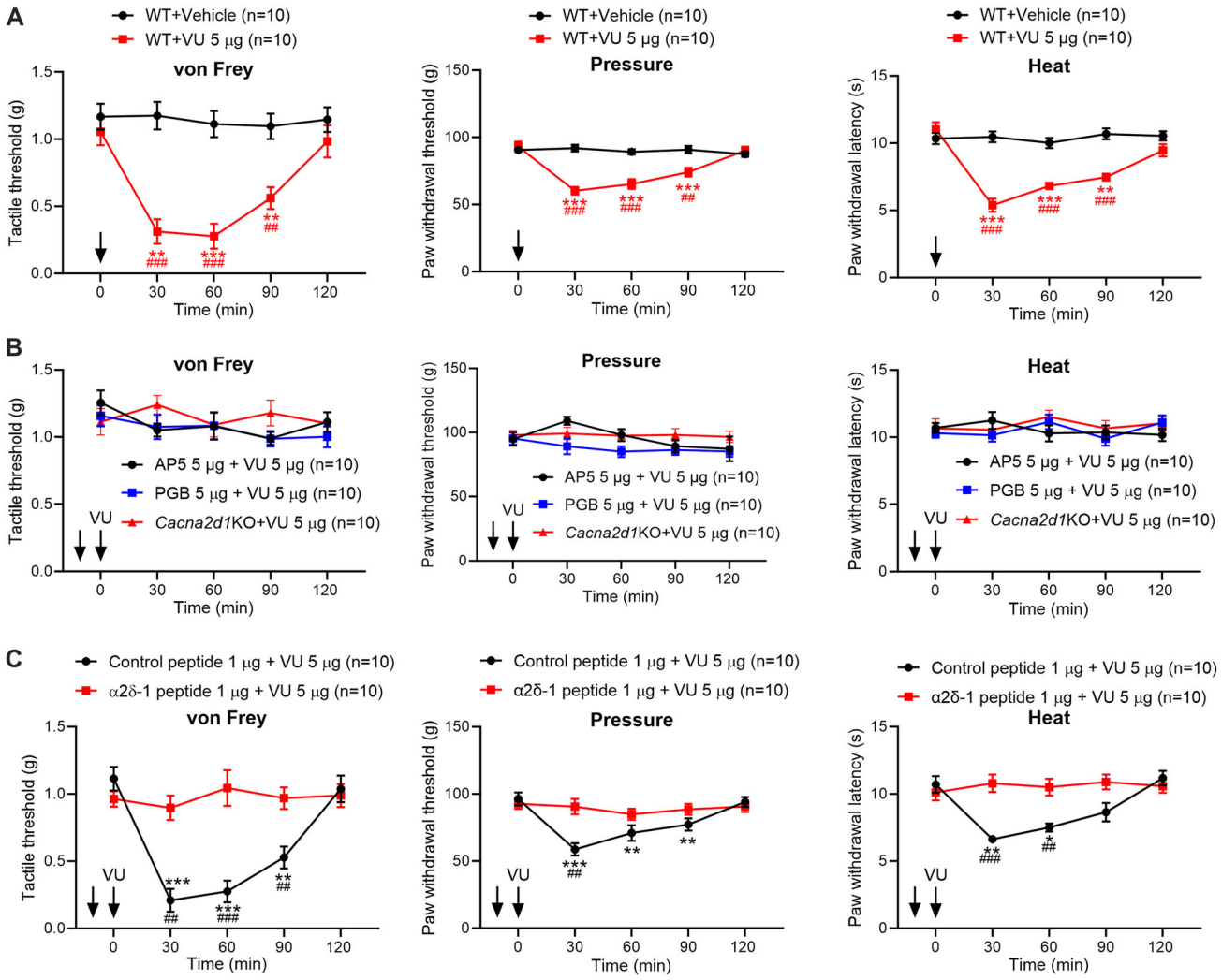

Figure 9. Spinal KCC2 inhibition induces pain hypersensitivity in mice via NMDARs and α2δ-1. ***A***, Time course of changes in withdrawal thresholds in response to von Frey filaments, pressure, and noxious heat stimuli in WT mice after intrathecal injection with vehicle or 5 μg VU0463271 (VU; $n = 10$ mice per group). ***B***, Time course of changes in withdrawal thresholds in response to von Frey filaments, pressure, and noxious heat stimuli in WT mice after intrathecal injection of 5 μg AP5 or 5 μg pregabalin (PGB) prior to intrathecal injection of 5 μg VU and in *Cacna2d1* KO after intrathecal injection of 5 μg VU ($n = 10$ mice per group). ***C***, Time course of changes in withdrawal thresholds in response to von Frey filaments, pressure, and noxious heat stimuli in WT mice after intrathecal administration with 1 μg control peptide or 1 μg α2δ-1 peptide prior to intrathecal treatment with 5 μg VU ($n = 10$ mice per group). Down arrows indicate the timing of drug treatments. Data are shown as means ± SEM. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ compared with the baseline (time 0) within the same group; $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ compared with control peptide group at the same time point (two-way ANOVA followed by Tukey's or Šidák *post hoc* test).

NMDAR activity, which augments both the amplitude of evoked EPSCs and the frequency of mEPSCs in spinal dorsal horn neurons (Xie et al., 2016; Chen et al., 2018; Zhang et al., 2021; Huang et al., 2023b). Traumatic nerve injury potentiates the activity of presynaptic and postsynaptic NMDARs in the spinal dorsal horn, including those at primary afferent central terminals (Chen et al., 2014b, 2018; Huang et al., 2023b). Intriguingly, removing presynaptic NMDARs in primary sensory neurons has a minor effect on chronic pain caused by traumatic nerve injury or small-fiber neuropathy (Zhang et al., 2021; Huang et al., 2023b). α2δ-1 is expressed in primary sensory neurons and their central terminals, but the KCC2 transcripts and proteins are not present in primary sensory neurons (Sung et al., 2000; Li et al., 2016). DRG neurons and their central terminals generally maintain high Cl^- levels through NKCC1 (Sung et al., 2000), which is critical for presynaptic inhibition of input from primary afferent nerves (Rudomin and Schmidt, 1999; Yuan et al., 2009). In comparison, paclitaxel treatment upregulates NKCC1 and augments the activity of presynaptic NMDARs at primary central terminals, without affecting postsynaptic NMDAR activity and KCC2 protein levels in the spinal cord (Chen et al., 2014c). Remarkably, KCC2 gene transfer via intrathecal injection reinstates Cl^- homeostasis by increasing KCC2 expression in both DRG and dorsal horn neurons, thereby normalizing presynaptic and postsynaptic NMDAR activity in the spinal cord potentiated by nerve injury (Li et al., 2016). This synapse type-specific effect of KCC2 inhibition suggests that KCC2 preferentially regulates glutamatergic synaptic transmission between excitatory interneurons in the spinal dorsal horn.

Additionally, in this study, we have demonstrated that inhibiting KCC2 at the spinal cord level readily provokes pain hypersensitivity via α2δ-1-bound NMDARs. We found that intrathecal injection of VU0463271 rapidly induced pain hypersensitivity, which was blunted by the NMDAR antagonist, pregabalin, *Cacna2d1* KO, or α2δ-1 C terminus-interfering peptide. KCC2 inhibition likely diminishes synaptic inhibition and increases

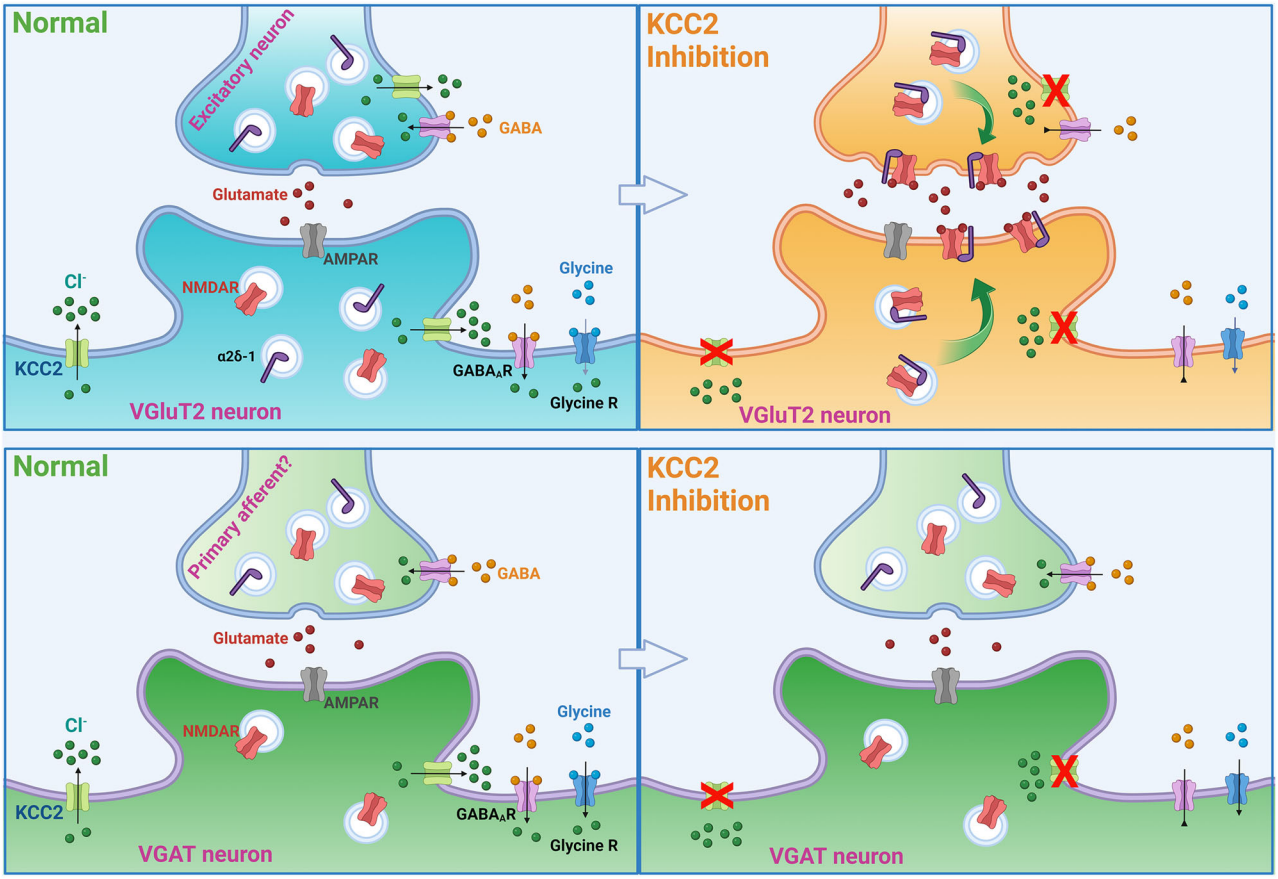

Figure 10. Schematic illustrates that KCC2 preferentially controls spinal nociceptive transmission between excitatory interneurons through α2δ-1-bound NMDARs. In the spinal dorsal horn, NMDARs are minimally active at presynaptic and postsynaptic sites under normal conditions due to tonic synaptic inhibition, which is sustained by $GABA_A$ and glycine receptors and by KCC2-mediated chloride homeostasis. Although functionally active KCC2 is present in both VGluT2 (upper panels) and VGAT (lower panels) neurons only VGluT2 neurons and their terminals possess both KCC2 and α2δ-1. KCC2 inhibition enhances α2δ-1–NMDAR physical interactions and their synaptic expression in VGluT2 neurons, leading to increased presynaptic and postsynaptic NMDAR activity and augmented glutamatergic transmission between excitatory neurons. In contrast, VGAT neurons lack α2δ-1, and primary afferent terminals are devoid of KCC2. Consequently, KCC2 inhibition does not impact synaptic NMDARs in VGAT neurons or NMDARs at primary afferent terminals.

neuronal excitability and NMDAR phosphorylation, leading to increased α2δ-1–NMDAR interactions and synaptic trafficking. As a result, α2δ-1-bound NMDARs play a pivotal role in KCC2 inhibition-induced enhancement in spinal nociceptive transmission. These signaling events likely explain how diminished synaptic inhibition could promote nociceptive neuronal network excitation within the dorsal horn in neuropathic pain conditions. By strengthening nociceptive transmission between excitatory interneurons within the spinal dorsal horn through α2δ-1-bound NMDARs, KCC2 impairment probably serves as a key signaling mechanism for maintaining central sensitization in neuropathic pain conditions. This new insight shows a novel dimension of the interplay between synaptic inhibition and excitation in the nociceptive spinal circuitry.

In summary, our study provides substantial new information that KCC2 controls spinal nociceptive transmission via NMDARs in a cell type- and synapse type-specific manner. This differential regulation is attributed to the distinct co-expression of KCC2 and α2δ-1 in spinal excitatory interneurons. KCC2 inhibition leads to hyperactivity of both presynaptic and postsynaptic NMDARs via α2δ-1, thereby potentiating glutamatergic transmission among excitatory interneurons in the dorsal horn (Fig. 10). These findings advance our mechanistic understanding of how the mutual interaction between KCC2 and α2δ-1-bound NMDARs promotes central sensitization and contributes to the development of chronic pain. Given the unique role of KCC2 in regulating the strength of synaptic inhibition and NMDAR activity-mediated glutamatergic input in the spinal cord, restoring KCC2 activity holds the therapeutic potential for neuropathic pain. Enhancing KCC2 activity and inhibiting α2δ-1-bound NMDARs with gabapentinoids or α2δ-1 C-terminus peptides could rebalance excitatory and inhibitory tone, offering an effective strategy for treating neuropathic pain.

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