Ablation of $\alpha_2\delta$-1 inhibits cell-surface trafficking of endogenous N-type calcium channels in the pain pathway in vivo

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The auxiliary $\alpha_2$ calcium channel subunits play key roles in voltage-gated calcium channel function. Independent of this, $\alpha_2\delta$-1 has also been suggested to be important for synaptogenesis. Using an epitope-tagged knockin mouse strategy, we examined the effect of $\alpha_2\delta$-1 on Ca$_{V}$2.2 localization in the pain pathway in vivo, where Ca$_{V}$2.2 is important for nociceptive transmission and $\alpha_2\delta$-1 plays a critical role in neuropathic pain. We find Ca$_{V}$2.2 is preferentially expressed on the plasma membrane of calcitonin gene-related peptide-positive small nociceptors. This is paralleled by strong presynaptic expression of Ca$_{V}$2.2 in the superficial spinal cord dorsal horn. EM-immunogold localization shows Ca$_{V}$2.2 predominately in active zones of glomerular primary afferent terminals. Genetic ablation of $\alpha_2\delta$-1 abolishes Ca$_{V}$2.2 cell-surface expression in dorsal root ganglion neurons and dramatically reduces dorsal horn expression. There was no effect of $\alpha_2\delta$-1 knockout on other dorsal horn pre- and postsynaptic markers, indicating the primary afferent pathways are not otherwise affected by $\alpha_2\delta$-1 ablation.

Calcium channel | primary afferent | auxiliary subunit | N-type | trafficking

The neuronal N-type voltage-gated calcium channel was first identified in primary afferent dorsal root ganglion (DRG) neurons (1, 2). Toxins from the Conus marine snails, ω-conotoxins GVIA and ω-conotoxin MVIIIC, are highly selective blockers of N-type channels (3, 4) and have been instrumental in dissecting their function (5, 6). A key role for N-type calcium channels was identified in primary afferent neurotransmission in the dorsal horn of the spinal cord, and these toxins were therefore pursued as a therapeutic target in the alleviation of chronic pain (7, 8). Indeed, the peptide ziconotide (synthetic ω-conotoxin MVIIIA) is licensed for intrathecal use in intractable pain conditions (9, 10).

Despite the functional importance of N-type channels in the pain pathway, a major hindrance to the study of their distribution and trafficking, in this system and elsewhere, has been the paucity of antibodies recognizing this channel. Although previous studies have used anti-peptide antibodies to intracellular Ca$_{V}$2.2 epitopes (for example, refs. 11 and 12), these have not shown plasma membrane localization of the endogenous channel in neurons and have not been rigorously examined against knockout tissue. For this reason, we developed a Ca$_{V}$2.2 construct with an exofacial epitope tag to detect its cell-surface expression and trafficking (13). This channel is observed on the plasma membrane, when expressed in DRGs and other neurons (13–15). We took advantage of our finding that the presence of the epitope tag did not affect function (13) to generate a knockin (KI) mouse line containing the hemagglutinin (HA) tag in the same position in the Cacna1b gene. This has allowed us to examine the distribution of native Ca$_{V}$2.2 protein in the intact nervous system.

N-type calcium channels are made up of the Ca$_{V}$2.2 pore-forming α1-subunit, which associates with auxiliary α2δ- and β-subunits (16). Many studies have indicated that α2δ-subunits are important for the correct trafficking and physiological function of the channels (for a review, see ref. 17). A significant role for $\alpha_2\delta$-1 in chronic neuropathic pain, which results from damage to peripheral sensory nerves, was identified as a result of two advances. First, it was shown that $\alpha_2\delta$-1 mRNA and protein are strongly up-regulated in somatosensory neurons following nerve damage (18–20). Second, $\alpha_2\delta$-1 was identified as the therapeutic target for the drugs gabapentin and pregabalin, which are used in neuropathic pain such as postherpetic neuralgia (21, 22).

Here we elucidate the cellular and subcellular localization of native Ca$_{V}$2.2 in neurons of the peripheral somatosensory nervous system. We reveal a dramatic effect of $\alpha_2\delta$-1 ablation on Ca$_{V}$2.2 distribution, particularly in a key subset of nociceptive sensory neurons. In contrast to an early study of the subunit composition of N-type channels (16), which showed an ~1:1 stoichiometry with $\alpha_2\delta$-1, a more recent study suggested that $\omega_\delta$-subunits were only associated with less than 10% of digitonin-solubilized Ca$_{V}$2.2 channels (25), although it cannot be ruled out that they became dissociated during solubilization. However, the present study reinforces the essential nature of the auxiliary $\alpha_2\delta$-1 subunit as a therapeutic target in the alleviation of chronic pain (7, 8). This open access article is distributed under Creative Commons Attribution License 4.0 (CC BY).
The αδ-1 protein for cell-surface expression of endogenous Ca\textsubscript{\textit{v}}v\textsubscript{2.2}, both in DRG neuronal cell bodies and in their presynaptic terminals. No effect of αδ-1 loss was observed on other pre- and postsynaptic markers in the dorsal horn, despite a previous study implicating postsynaptic αδ-1 in thrombospondin-mediated synaptogenesis. Our results therefore show that loss of synaptic Ca\textsubscript{\textit{v}}v\textsubscript{2.2} as a result of αδ-1 ablation is due to a reduction of Ca\textsubscript{\textit{v}}v\textsubscript{2.2} trafficking to synapses, rather than synapse loss.
Results

Generation of Ca\textsubscript{v}2.2_HA Knockin Mice. Mice containing a double-HA tag in constitutive exon 13 of the Cacna1b gene were generated in a C57BL/6 background, as described in Methods, such that every endogenous Ca\textsubscript{v}2.2 contained the double-HA tag in the position previously ascertained not to affect channel function (13) (Fig. 1A). The presence of the HA tag was confirmed by PCR (Fig. 1B). We confirmed that the HA-tagged Ca\textsubscript{v}2.2 protein is expressed in synaptosomes, since a 261-kDa band (the expected molecular mass of

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Fig. 2. Distribution of Ca\textsubscript{v}2.2_HA in intact dorsal root ganglia. (A) Immunostaining for HA (green) in Ca\textsubscript{v}2.2_HA\textsuperscript{KI/KI} (i–iv) and WT DRG neurons (v), costained with CGRP (i, ii, and v) or NF200 (iii and iv) (red). Nuclei are stained with DAPI (blue). Images ii and iv are enlargements of the ROIs shown in i and iii. (Scale bars: 20 μm (i, iii, and v) and 5 μm (ii and iv).) Lack of HA staining in Ca\textsubscript{v}2.2_HA\textsuperscript{WT/WT} sections (v) was observed in four additional independent experiments. (B) Surface Ca\textsubscript{v}2.2_HA intensity measured as a ratio of DRG neuronal perimeter/cytoplasmic staining for small (s; solid bars), medium (m; hatched bars), and large (l; shaded bars) DRG neurons that are either CGRP-positive (red) or CGRP-negative (black/dark gray). n = 313, 341, 122, 171, 169, and 164 DRG neurons, respectively, from sections from at least three mice. ****P < 0.0001, *P = 0.0135 (one-way ANOVA and Sidak’s post hoc test of all conditions with correction for multiple comparisons); ns, not significant. (C) Surface Ca\textsubscript{v}2.2_HA intensity measured as a ratio of DRG neuronal perimeter/cytoplasmic staining for small, medium, and large DRG neurons that are either NF200-negative (black) or NF200-positive (red). n = 213, 201, 3, 36, 97, and 192 DRG neurons, respectively, from sections from at least three mice. #: Note very few large DRG neurons are NF200-negative. ****P < 0.0001 (one-way ANOVA and Sidak’s post hoc test of all conditions with correction for multiple comparisons); ns, not significant. (D and E) Intracellular Ca\textsubscript{v}2.2_HA staining, quantified with respect to cell size, for CGRP-positive (red circles) and CGRP-negative (open black circles) DRG neurons (D), and for NF200-positive (red squares) and NF200-negative (open black squares) DRG neurons (E). Data are from a subset of experiments from B performed in parallel in which the absolute immunostaining levels are directly comparable. Linear regression analysis for data in D (red line, CGRP +ve: slope = 21.2, r\textsuperscript{2} = 0.03989, df = 168, P = 0.0012; black line, CGRP −ve: slope = 59.2, r\textsuperscript{2} = 0.362, df = 168, P < 0.0001).
**Fig. 3.** Effect of αδ-1 ablation on distribution of CaV.2.2_HA in intact dorsal root ganglia. (A) Immunostaining for αδ-1 (green) in CaV.2.2_HA^{WT/WT} (Top), CaV.2.2_HA^{KO/KO} (Middle), and CaV.2.2_HA^{WT/WT} (Bottom) DRG sections, costained with CGRP (red). Nuclei were stained with DAPI (blue). Scale bars: 20 μm. (B) Intracellular (ic) αδ-1 density with respect to cell size, for CGRP-positive (red circles) and CGRP-negative (black circles) DRG neurons from CaV.2.2_HA^{WT/WT} αδ-1^{WT/WT} (solid circles) and CaV.2.2_HA^{KO/KO} αδ-1^{KO/KO} (open circles) mice. Lines are linear fits for both CGRP-positive (red line; r^2 = -0.411; df 173, F = 120.9, P < 0.0001) and CGRP-negative (black line; r^2 = 0.380; df 177, F = 108.3, P < 0.0001) αδ-1^{WT/WT} DRG neurons. (C) Intracellular αδ-1 density for CGRP-positive (red bars) and CGRP-negative (black/gray bars) DRG neurons from CaV.2.2_HA^{WT/WT} αδ-1^{WT/WT} (solid bars) and CaV.2.2_HA^{KO/KO} αδ-1^{KO/KO} (open bars) mice. n = 175, 52, 179, and 72 DRG neurons, respectively, from three sections (one mouse per genotype). ****P < 0.0001 (one-way ANOVA and Bonferroni’s post hoc comparison of selected conditions). (D) Immunostaining for HA (green) in CaV.2.2_HA^{WT/WT} αδ-1^{WT/WT} DRG neurons, costained with CGRP (i) or NF200 (ii) (red). Nuclei were stained with DAPI (blue). (Scale bars: 20 μm.) (E) Surface CaV.2.2_HA intensity in αδ-1^{KO/KO} DRG neurons (ratio of perimeter/cytoplasmic staining) for small, medium, and large DRG neurons that are either CGRP-positive (red) or CGRP-negative (black/gray). n = 198, 197, 103, 134, 97, and 109 DRG neurons, respectively, from sections from at least three mice. ****P < 0.0001, ***P < 0.0022 (one-way ANOVA and Sidak’s post hoc test). (F) Surface CaV.2.2_HA intensity (ratio of perimeter/cytoplasmic staining) for small, medium, and large DRG neurons that are either NF200-negative (black/gray) or NF200-positive (red). n = 204, 136, 5, 37, 94, and 168 DRG neurons, respectively, from sections from at least three mice. *P = 0.0482 (one-way ANOVA and Sidak’s post hoc test). (G) Intracellular CaV.2.2_HA intensity for CGRP-positive (red) and CGRP-negative (black/gray) αδ-1^{WT/WT} (solid bars) and αδ-1^{KO/KO} (open bars) DRG neurons. n = 262, 232, 170, and 144 DRG neurons, respectively. Data are from a subset of experiments performed in parallel in which the absolute immunostaining levels are directly comparable. *P = 0.0308 (one-way ANOVA and Sidak’s post hoc test), ns, not significant. (H) Intracellular CaV.2.2_HA intensity for NF200-negative (black/gray) and NF200-positive (red) αδ-1^{WT/WT} (solid bars) and αδ-1^{KO/KO} (open bars) DRG neurons. n = 417, 345, 325, and 299 DRG neurons, respectively. ****P < 0.0001 (one-way ANOVA and Sidak’s post hoc test).

CaV.2.2_HA is recognized by anti-HA antibodies in Western blots of spinal cord tissue from CaV.2.2_HA^{WT/WT}, but not CaV.2.2_HA^{KO/KO}, mice (Fig. 1C).

CaV.2.2 mRNA Levels and Calcium Currents Are Unaltered in CaV.2.2_HA^{WT/WT} Compared with CaV.2.2_HA^{KO/KO} Mice. We next confirmed that the expression of CaV.2.2 did not differ between...
Cav2.2_HA<sup>KI/KI</sup> and Cav2.2<sup>WT/WT</sup> mice. The analyzed expression profiles at 2 and 10 wk postnatally showed that Cav2.2 mRNA levels were not altered in the Cav2.2_HA<sup>KI/KI</sup> compared with Cav2.2<sup>WT/WT</sup> mouse brains (Fig. 1D).

The properties of calcium channel currents in cultured DRG neurons from 10- to 12-wk-old Cav2.2_HA<sup>KI/KI</sup> mice were not altered compared with those from Cav2.2<sup>WT/WT</sup> mice, both in terms of current density and voltage-dependent properties (Fig. 1E and F). We then examined whether Cav2.2_HA was detectable on the cell surface of cultured DRG neurons from Cav2.2_HA<sup>KI/KI</sup> mice (Fig. 1G). We found Cav2.2_HA to be present on the cell surface particularly of calcitonin gene-related peptide (CGRP)-positive peptidergic nociceptors, to a much greater extent than on isolectin-B4 (IB4)-positive non-peptidergic nociceptors (56.8%, compared with 11.3%; Fig. 1G and H). Furthermore, Cav2.2_HA was expressed on only a small...
proportion of neurofilament 200 (NF200)-positive DRG neurons (77.4% of HA-positive cells were NF200-negative; SI Appendix, Fig. S1 A and B). HA immunostaining was absent from DRG neurons cultured from wild-type mice (SI Appendix, Fig. S1C).

**Cell-Surface Expression of Ca$_{\alpha\delta}$2.2 HA in DRG Neurons in Vivo.** In agreement with the results from cultured DRG neurons, we found that Ca$_{\alpha\delta}$2.2 HA was clearly present on the cell surface of DRG neuronal somata in sections of ganglia from 10- to 12-wk-old Ca$_{\alpha\delta}$2.2 HA$^{K/K}$ mice (Fig. 2 A, i–iv), and absent from Ca$_{\alpha\delta}$2.2 WT/WT mice (Fig. 2 A, v). We stained with markers of DRG neuronal subtypes, including CGRP (Fig. 2 A, i, ii, and v) and NF200 (Fig. 2 A, iii and iv). Analysis of the ratio of Ca$_{\alpha\delta}$2.2 HA at the cell perimeter, relative to its cytoplasmic staining, shows that plasma membrane Ca$_{\alpha\delta}$2.2 HA is highest on the cell surface of small CGRP-positive DRG neurons (Fig. 2B). The small-cell surface Ca$_{\alpha\delta}$2.2 HA-positive DRG neurons were mainly NF200-negative (Fig. 2C). The absolute level of cytoplasmic staining of Ca$_{\alpha\delta}$2.2 HA was also negatively correlated with the size of DRG neurons (Fig. 2D), being higher in small-diameter neurons and in those which are CGRP-positive (Fig. 2D) and NF200-negative (Fig. 2E).

**Knockout of αδ-1 Abolishes Cell-Surface Expression of Ca$_{\alpha\delta}$2.2 HA on DRG Neurons in Vivo.** To determine the importance of αδ-1 in the cell-surface expression of Ca$_{\alpha\delta}$2.2 HA, we crossed Ca$_{\alpha\delta}$2.2 HA$^{K/K}$ mice with αδ-1$^{KO/WT}$ mice, and compared Ca$_{\alpha\delta}$2.2 HA$^{K/K}$ x αδ-1$^{KO/KO}$ with their Ca$_{\alpha\delta}$2.2 HA$^{K/K}$ x αδ-1$^{WT/WT}$ littermates. We first confirmed that DRG neurons from Ca$_{\alpha\delta}$2.2 HA$^{K/K}$ x αδ-1$^{WT/WT}$ mice have similar levels of αδ-1 to Ca$_{\alpha\delta}$2.2 HA$^{K/K}$ x αδ-1$^{WT/WT}$ (Fig. 3C, quantified in SI Appendix, Fig. S2A). We found the level of αδ-1 to be highest in CGRP-positive small DRG neurons (Fig. 3 A–C and SI Appendix, Fig. S2A). As expected, Ca$_{\alpha\delta}$2.2 HA$^{K/K}$ x αδ-1$^{KO/KO}$ DRG neurons show no staining for αδ-1 above background (Fig. 3 A–C).

The effect of genetic ablation of αδ-1 on Ca$_{\alpha\delta}$2.2 HA cell-surface expression was in general very marked (Fig. 3 D–F). We found that Ca$_{\alpha\delta}$2.2 HA was not concentrated on the cell surface in αδ-1$^{K/KO}$ DRG neurons (Fig. 3D), and this was true across all subtypes of DRG neuron examined (Fig. 3 E and F). Furthermore, there was an increase in mean intracellular Ca$_{\alpha\delta}$2.2 HA intensity in DRG neurons from αδ-1$^{K/KO}$ compared with αδ-1$^{WT/WT}$ mice, which was found in CGRP-positive DRG neurons (6.9% increase; Fig. 3G), and in both NF200-positive and NF200-negative DRG neurons (15.3 and 24.6% increase, respectively; Fig. 3H). The elevated intracellular Ca$_{\alpha\delta}$2.2 HA intensity in αδ-1$^{K/KO}$ DRG neurons was also inversely correlated with cell size (SI Appendix, Fig. S2B).

**Ca$_{\alpha\delta}$2.2 HA Is Localized in the Dorsal Horn of the Spinal Cord.** Next, we examined the distribution of Ca$_{\alpha\delta}$2.2 HA in the spinal cord, and found strong immunoreactivity for the channel subunit in the dorsal horn (Fig. 4A). There was very little Ca$_{\alpha\delta}$2.2 HA in the ventral horn (Fig. 4A), and no specific staining in Ca$_{\alpha\delta}$2.2 WT/WT spinal cord (Fig. 4 A, i). Taking regions of interest (ROIs) perpendicular to the pial layer (Fig. 4 A, ii), we found that within the dorsal horn, Ca$_{\alpha\delta}$2.2 HA was most abundant in superficial laminae I and II (Fig. 4B). Here Ca$_{\alpha\delta}$2.2 HA shares topographic distribution with both the presynaptic markers CGRP, which is present in peptidergic primary afferent terminals in laminae I and II-outer (Fig. 4C and SI Appendix, Fig. S3A), and with IB4, which is present in nonpeptidergic terminals, mainly in lamina II-inner (Fig. 4D and SI Appendix, Fig. S3B). Ca$_{\alpha\delta}$2.2 HA was also associated with a postsynaptic marker of excitatory synapses, Homer (Fig. 4E).

**Ablation of αδ-1 Reduces Ca$_{\alpha\delta}$2.2 HA in the Dorsal Horn Without Effect on Other Synaptic Markers.** The distribution of Ca$_{\alpha\delta}$2.2 HA in the dorsal horn was markedly reduced in αδ-1$^{K/KO}$ mice (Fig. 4 F–H), particularly in the superficial layers (Fig. 4I). Following subtraction of nonspecific signal found in wild-type Ca$_{\alpha\delta}$2.2 sections (Fig. 4B), the reduction in Ca$_{\alpha\delta}$2.2 HA was 72.7, 65.9, 64.6, and 44.7% in layers I, II-outer, II-inner, and III, respectively (Fig. 4I). This decrease provides clear evidence for the essential role of αδ-1 for Ca$_{\alpha\delta}$2.2 trafficking to the primary afferent presynaptic terminals. In contrast, in the deeper layers of the dorsal horn (laminae IV and V), there was no effect of the ablation of αδ-1 on the low level of Ca$_{\alpha\delta}$2.2 HA present (Fig. 4I).

Next, we investigated whether the αδ-1-mediated loss of Ca$_{\alpha\delta}$2.2 HA in the dorsal horn was concomitant with a reduction in density or distribution of synaptic markers, since αδ-1 has also been implicated in synaptogenesis (26). In contrast to the marked reduction in Ca$_{\alpha\delta}$2.2 HA in the absence of αδ-1 (Fig. 4I), there was no effect of αδ-1 ablation on the overall immunostaining intensity or distribution in the dorsal horn of three primary afferent presynaptic markers, CGRP (Fig. 4J), IB4 (Fig. 4K), and vesicular glutamate transporter-2 (vGlut2) (Fig. 4L), and no effect on postsynaptic Homer immunostaining (Fig. 4M).

**Dorsal Rhizotomy Reduces Ca$_{\alpha\delta}$2.2 HA in the Dorsal Horn of the Spinal Cord.** In light of the marked reduction in Ca$_{\alpha\delta}$2.2 HA, without loss of synaptic markers, in the dorsal horn of αδ-1$^{K/KO}$ mice (Fig. 4I), we wished to examine further the extent of its origin in presynaptic primary afferent terminals. To investigate this, we performed unilateral dorsal rhizotomy (Fig. 5A). This resulted in a significant reduction of Ca$_{\alpha\delta}$2.2 HA in the ipsilateral dorsal horn (Fig. 5 B–D). In the central ROI, the reduction was 52.7% in the superficial layers I and II, and there was also a substantial depletion (by 44.7%) in layers III to V (Fig. 5D). Rhizotomy is generally found to be incomplete, as longitudinal fibers remain intact (27). To determine the extent of the rhizotomy, we also examined the level of CGRP, as a marker of loss of presynaptic peptidergic afferents (27). A very similar extensive reduction of CGRP was observed, by 53.1% in layers I and II and 58.6% in layers III to V (Fig. 5 E and F). The correspondence between the reduction of Ca$_{\alpha\delta}$2.2 HA and that of CGRP, whose origin is entirely presynaptic in the dorsal horn, confirms the main presynaptic localization of the Ca$_{\alpha\delta}$2.2 HA signal in this region. Following dorsal rhizotomy, there was also a 20.7% decrease of αδ-1 in central laminae I and II (SI Appendix, Fig. S4), which is expressed both in primary afferents and in intrinsic neurons (20). In contrast, there is no reduction in the NPY signal in the same region (SI Appendix, Fig. S4), this peptide being expressed mainly by dorsal horn interneurons (for a review, see ref. 28).

**Ca$_{\alpha\delta}$2.2 HA Subcellular Localization in the Spinal Cord: Effect of αδ-1 Ablation.** At higher resolution, we observed that Ca$_{\alpha\delta}$2.2 HA, present in the superficial dorsal horn laminae, was distributed in rosette structures consisting of Ca$_{\alpha\delta}$2.2 HA puncta surrounding a central core containing vGlut2 and often (but not always) associated with either CGRP (SI Appendix, Fig. S5 A and B) or IB4 (SI Appendix, Fig. S5 C and D), resembling glomerular synapses (29).

To improve resolution of these structures, we then obtained superresolution Airyscan images of Ca$_{\alpha\delta}$2.2 HA together with vGlut2 and Homer in regions of the dorsal horn in both αδ-1$^{WT/WT}$ (Fig. 6A) and αδ-1$^{K/KO}$ mice (Fig. 6B). The rosette-shaped clusters of Ca$_{\alpha\delta}$2.2 HA consisted of groups of four or five puncta (Fig. 6C). These puncta may each correspond to individual active zones of primary afferent terminal glomerular synapses, because they are usually organized around a central core containing vGlut2, and also frequently apposed to the postsynaptic marker Homer (Fig. 6C).

We found the density of Ca$_{\alpha\delta}$2.2 HA was markedly reduced in αδ-1$^{K/KO}$ dorsal horn (Fig. 6 B and C), and we quantified the effect on several parameters associated with Ca$_{\alpha\delta}$2.2 HA puncta (for a method, see SI Appendix, Fig. S6). The density of Ca$_{\alpha\delta}$2.2 HA was reduced in individual clusters of puncta in αδ-1$^{K/KO}$ dorsal horn (Fig. 6C).
dorsal horn, by 47.7% (Fig. 6D), but the cluster areas were not significantly affected (Fig. 6E). In contrast, neither the area nor the intensity of vGlut2 or Homer clusters was affected by loss of α2δ-1 (Fig. 6D and E). In estimating the pairwise association between CaV2.2_HA and Homer (Fig. 6F), or CaV2.2_HA and vGlut2 (Fig. 6G), we found that the intensity of vGlut2 and Homer in these associated clusters was not affected in α2δ-1KO KO dorsal horn (Fig. 6F and G). However, as expected, the intensity of CaV2.2_HA in the associated clusters was reduced by 50.0% for CaV2.2_HA puncta overlapping with Homer (Fig. 6F), and by 50.7% for those overlapping with vGlut2 (Fig. 6G).

Subcellular Localization of CaV2.2_HA. To determine the subcellular localization of the CaV2.2_HA channels, we used preembedding immunogold labeling. For electron microscopic investigation, tissue blocks were taken from the dorsal horn of the spinal cord. Immunoreactivity for CaV2.2_HA was predominantly found in presynaptic elements, namely on axon terminals of presumed primary afferents (Fig. 7A–C). Single or small clusters of immunogold particles were mainly localized to the active zone of boutons on individual glomeruli (Fig. 7B and C), and also appeared at the edge of presynaptic membrane specializations (Fig. 7A–C) and along the extrasynaptic plasma membrane (Fig. 7A–C) of axon terminals making asymmetrical putative glutamatergic synapses with dendritic shafts and spines of postsynaptic neurons. The specificity of the immunolabeling was confirmed by the absence of immunoreactivity for CaV2.2_HA in tissues obtained from control animals (Fig. 7D).
**Discussion**

In this study, we have been able to visualize native N-type Ca$_{2.2}$ channels on the cell surface of neurons in vivo. We have concentrated here on the primary afferent neuronal pathway, because of the importance of Ca$_{2.2}$ in synaptic transmission in this system and its therapeutic importance as a drug target (7, 30). We show that Ca$_{2.2}$ is very strongly expressed on the cell surface, particularly of CGRP-positive small DRG neurons, because of the importance of Ca$_{2.2}$ in synaptic transmission in the presynaptic Ca$^{2+}$ channel (29). The Ca$_{2.2}$ puncta are also adjacent to puncta containing the postsynaptic density protein Homer. The localization of Ca$_{2.2}$ in DRG neurons is paralleled by striking expression of Ca$_{2.2}$ in the dorsal horn of the spinal cord, predominantly in laminae I and II. Here the presynaptic Ca$_{2.2}$ puncta are associated with the primary afferent markers CGRP, vGlut2, and IB4, present in glomerular primary afferent presynaptic terminals as described previously (29). The Ca$_{2.2}$ puncta are also adjacent to puncta containing the postsynaptic density protein Homer. The presynaptic localization of Ca$_{2.2}$ in primary afferents is confirmed through their ablation by dorsal rhizotomy. Furthermore, from the high-resolution immunoelectron-microscopic localization of Ca$_{2.2}$, we confirm that these rosette structures formed by the Ca$_{2.2}$ puncta are likely to represent Ca$_{2.2}$ in active zones of individual glomerular terminals.
The αδ-1 auxiliary subunit has been shown to be important for calcium channel trafficking in expression systems (13). It plays a major role in pain pathways and is up-regulated following neuropathic injury (17–20, 23). Furthermore, knockout of αδ-1 caused a marked delay in the development of neuropathic mechanical hypersensitivity (24), and overexpression of αδ-1 mimics features of neuropathic injury (24). In rats, αδ-1 is expressed in all DRG neurons with highest expression in small neurons (20), and this distribution is confirmed here, in mice. However, until now it has not been possible to examine the effect of αδ-1 on the trafficking of the relevant endogenous N-type channels in vivo.

Our results using Cav2.2_HA^Kuki mice crossed with αδ-1^KokO mice, in which αδ-1 is globally ablated, highlight the essential role of αδ-1 in directing Cav2.2_HA to the cell surface in DRG neurons and in targeting Cav2.2_HA to presynaptic terminals in the dorsal horn. Accompanying the complete loss of DRG neuronal cell-surface Cav2.2_HA, there was also a significant increase in cytoplasmic Cav2.2_HA in CGRP-positive αδ-1^KokO DRG neurons, indicating a defect in cell-surface trafficking.

The calcium currents in DRG neuronal somata in culture are found to be composed of between 20 and 50% N-type current, depending on the species, developmental stage, culture conditions, and subtype of DRG neuron examined (24, 32–35). One comprehensive study showed the proportion of N-type current was about 40% in cultured mouse DRG neurons with a diameter of less than 30 μm, and 20% in those larger than 30 μm (35), which is in agreement with the differential distribution of Cav2.2_HA found here in small DRG neurons. We found previously that in cultured DRG neurons from αδ-1 knockout mice the calcium channel current was only reduced by about 30% compared with wild-type DRG neurons, and the N-type current was reduced proportionately (24), which is in contrast to the marked effects of αδ-1 knockout on Cav2.2_HA localization described here. It is highly likely that even short-term cultured DRG neurons do not fully represent the in vivo situation, and that rapid changes occur in cell-surface expression of receptors and channels when cells are enzymatically dissociated and maintained in culture, allowing neurite outgrowth (36). Since evoked synaptic currents in laminae I and II are 74% N-type (37), there is likely to be a differential synaptic localization of these channels in vivo.

It has been found that there are other synaptic roles for αδ-subunits unrelated to calcium channel function; for example, an association of the extreme C terminus of αδ-1 with NMDA receptors has been identified (38). Furthermore, postsynaptic αδ-1 has been implicated in central neurons as the binding partner of thrombospondins to promote synaptogenesis induced by this secreted protein family, independent of its role as a calcium channel subunit (26, 39). Thrombospondins alone promote the formation of silent synapses, lacking postsynaptic elements (40). However, we did not detect robust binding of thrombospondin-4 to αδ-1 (41). By contrast, in cultured hippocampal neurons, neurogin was also identified as a binding partner of thrombospondins mediating an increase in the rate of synaptogenesis (42).

Both presynaptic αδ-3 (43) and αδ-4 (44) have also been implicated in determining synaptic morphology in the auditory system and retina, respectively, although in these cases the synaptic abnormalities resulting from knockout of the respective αδ-subunits are likely related to calcium channel dysfunction. In the present study, despite the effect of global ablation of αδ-1, which strongly disrupted Cav2.2_HA cell-surface localization, particularly of CGRP-positive small DRG neurons, and markedly reduced presynaptic terminal localization of Cav2.2_HA in the dorsal horn of the spinal cord, we did not observe any reduction in other presynaptic markers of these primary afferents, CGRP, vGlut2, and IB4, or the postsynaptic marker, Homer. At the level of individual synapses, we did not find a reduction in area of Cav2.2_HA–positive puncta clusters, but there was a very clear reduction in intensity of Cav2.2 in each cluster, in the absence of αδ-1. This result suggests that, if these puncta represent presynaptic active zones in primary afferent glomerular synapses, αδ-1 has not affected the density of synapses in the dorsal horn, despite a large reduction in presynaptic Cav2.2_HA intensity. However, whether there are changes in synaptic morphology will require more detailed examination at the EM level in the future.

Methods

Generation of Cav2.2_HA Epitope-Tagged Knockin Mice. The CaV2.2_HA mouse line was generated by Taconic Artemis in the C57BL/6 background by homologous recombination with the targeting vector, which included the genomic region around exon 13 of the Cav2.2_HA gene from clones of a C57BL/6J RPCIB-731 BAC library into which the sequence coding for the 2.2_HA tag was cloned. The targeting vector also carried the puromycin resistance gene (PuRor) as a positive-selection marker in intron 13 between two Flipper recombination sites and the negative-selection marker thymidine kinase outside the homologous regions. The targeting vector was linearized and transfected into embryonic stem cells. The homologous recombinant clones were isolated by positive and negative selection and injected into blastocysts from BALB/c. Highly chimeric mice were crossed with C57BL/6, and transmission to the germ line was confirmed by black offspring. The positive selective marker was removed by Flipper recombinase after crossing the first generation of knockin mice with Flip deleter transgenic mice. Subsequent backcrossing with wild-type C57BL/6 mice allowed us to select mice without the 2.2_HA tag in intron 13. Genotyping PCR was performed with the primers forward, 5'-CACACACAGTACATACGCTCG-3' and reverse, 5'-TCCAGGCTCACTACGTCG-3', that bind to the intronic sequences just before and after exon 13 to generate...
ampliﬁcions of 279 and 345 for the wild-type and knockin allele, respectively. The Ca_{2.2,2} HA\textsuperscript{WT} mice showed no difference compared with Ca_{2.2,2} \textsuperscript{kI/kI} mice in weight to body weights (SI Appendix, Table S1).

The α2-δ1 knockout C57BL/6 mouse line described previously (24, 25) was crossed with the Ca_{2.2,2} HA knockin mice to generate double-transgenic Ca_{2.2,2,2} HA\textsuperscript{KIKO} α2-δ1\textsuperscript{KIKO} mice. It should be noted that male α2-δ1 knockout mice on a different genetic background showed a susceptibility to diabetes (46), although we had not noted excessive urination up to 11 wk of age in male double-transgenic mice. Both male and female mice were used in the present study. There was a small reduction of body weight in Ca_{2.2,2,2} HA\textsuperscript{KIKO} α2-δ1\textsuperscript{KIKO} compared with Ca_{2.2,2,2} HA\textsuperscript{KIKO} α2-δ1\textsuperscript{WT/WT} mice for both sexes (SI Appendix, Table S1).

Mice were housed in groups of no more than five on a 12-h:12-h light-dark cycle; food and water were available ad libitum. All experimental procedures were covered by UK Home Office licenses, had local ethical approval by University College London (UCL) Bloomsbury Animal Welfare and Ethical Review Body, and followed the guidelines of the International Association for the Study of Pain (47).

Additional Methods. Methods for quantitative synapsosome preparation, immunoblotting, DRG neuronal cultures, electrophysiology, immunocytochemistry, and cultured DRG neurons, dorsal rhizotomy, immunohistochemistry, confocal image acquisition and analysis, and preembedding immunoelectron microscopy are included in SI Appendix.

Statistical Analysis. Data were analyzed with Prism 5.0 or 7.0 (GraphPad Software) or OriginPro 2015 (OriginLab). Where error bars are shown, they are SEM; “n” refers to the number of cells or sections, unless indicated otherwise. Statistical signiﬁcance between two groups was assessed by Student’s t test or paired t test, as stated. One-way ANOVA and stated post hoc analysis were used for comparison of means between two or more groups. All box and whisker plots show box (25 to 75%) and whisker (10 to 90%) plots with median (line) and mean (1).

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