VGLUT2 expression in primary afferent neurons is essential for normal acute pain and injury-induced heat hypersensitivity

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Edited* by Lily Yeh Jan, University of California, San Francisco, CA, and approved November 11, 2010 (received for review September 20, 2010)

Dorsal root ganglia (DRG) neurons, including the nociceptors that detect painful thermal, mechanical, and chemical stimuli, transmit information to spinal cord neurons via glutamatergic and peptidergic neurotransmitters. However, the specific contribution of glutamate to pain generated by distinct sensory modalities or injuries is not known. Here we generated mice in which the vesicular glutamate transporter 2 (VGLUT2) is ablated selectively from DRG neurons. We report that conditional knockout (cKO) of the *Slc17a6* gene encoding VGLUT2 from the great majority of nociceptors profoundly decreased VGLUT2 mRNA and protein in these neurons, and reduced firing of lamina I spinal cord neurons in response to noxious heat and mechanical stimulation. In behavioral assays, cKO mice showed decreased responsiveness to acute noxious heat, mechanical, and chemical (capsaicin) stimuli, but responded normally to cold stimulation and in the formalin test. Strikingly, although tissue injury-induced heat hyperalgesia was lost in the cKO mice, mechanical hypersensitivity developed normally. In a model of nerve injury-induced neuropathic pain, the magnitude of heat hypersensitivity was diminished in cKO mice, but both the mechanical allodynia and the microgliosis generated by nerve injury were intact. These findings suggest that VGLUT2 expression in nociceptors is essential for normal perception of acute pain and heat hyperalgesia, and that heat and mechanical hypersensitivity induced by peripheral injury rely on distinct (VGLUT2 dependent and VGLUT2 independent, respectively) primary afferent mechanisms and pathways.

nociceptor | inflammatory pain | electrophysiology | neuroanatomy

Primary afferent neurons of the dorsal root ganglia (DRG) detect a wide range of stimulus modalities and intensities (1). This is particularly true for nociceptors, which are the neurons specialized to detect noxious stimuli. Not only do subsets of nociceptors express different repertoires of neuropeptides, receptors, and ion channels, but they also project to different laminae in the spinal cord where they engage different CNS circuits (2–4). Importantly, studies in rodents in which different nociceptor populations have been deleted revealed remarkably selective behavioral deficits (e.g., heat, mechanical, or chemical pain), demonstrating the existence of behaviorally relevant peripheral-labeled lines for different modalities of pain (5–8).

Whether the modality-specific contribution of sensory neurons to acute pain and to injury-induced hypersensitivity states is also manifest at the level of the different neurotransmitters expressed by these neurons is still not known. Interestingly, previous pharmacological and genetic studies that disrupted neuropeptide function did not find a modality-specific loss of pain processing (9). Here we turned our attention to glutamate, which is presumed to be released by all DRG neurons to activate second-order spinal cord neurons.

Because pharmacological blockade of glutamate signaling would nonselectively inhibit the central effects arising from glutamate released by all primary afferent and CNS neurons, making it impossible to study the contribution of nociceptors specifically, we used a different approach, one that targeted the vesicular glutamate transporter (VGLUT). In glutamatergic neurons, glutamate is loaded into synaptic vesicles before its release into the synaptic cleft. This essential process is carried out by the VGLUTs, and inactivation of VGLUTs ablates glutamate release and neurotransmission (10–15). Three distinct VGLUTs have been described, and anatomical studies have established that VGLUT1, 2, and 3 are expressed by largely nonoverlapping and functionally distinct populations of glutamatergic neurons in the nervous system (16). In the DRG, however, the distribution of VGLUTs remains a matter of debate. Although some studies suggest that VGLUT2 is expressed by the majority of Aδ and C nociceptors, including peptidergic and IB4-binding unmyelinated C fibers (17–22), others found no expression of VGLUTs in these neurons (23), which questioned their glutamatergic identity (24).

To establish whether there is a functional association of glutamate release from nociceptors with particular pain modalities, we generated conditional knockout mice in which expression of VGLUT2 is disrupted specifically in nociceptors. We show here that VGLUT2-dependent glutamate signaling from nociceptors is, in fact, essential for normal acute pain and injury-induced heat hyperalgesia, but that mechanical hypersensitivity relies on distinct primary afferent mechanisms.

Results

VGLUT2 Expression Is Lost in Nociceptors of VGLUT2 cKO Mice. To ablate VGLUT2 specifically in DRG, we crossed mice bearing a conditional allele of the *Slc17a6* gene (VGLUT2 gene) that encodes VGLUT2 ($VGLUT2^{lox/lox}$ mice) (11) with transgenic mice that express Cre under the control of the peripherin gene promoter (Per-Cre mice) (25). In Per-Cre mice, Cre is expressed in most DRG nociceptors, including peptidergic (CGRP+) and TRPV1-expressing C and Aδ afferents and nonpeptidergic (IB4+) C fibers (Figs. S1 and S2). We generated conditional VGLUT2 knockout (cKO) mice and control littermates that lack the Per-Cre transgene (Fig. 1*A*). Excision of the second exon of the VGLUT2 gene in cells that express Cre generates a frameshift that disrupts VGLUT2 translation and function in cKO mice (11). cKO mice were born at the expected Mendelian ratios and showed no gross anatomical or behavioral defects, in distinct contrast to animals with a ubiquitous VGLUT2 deletion, which die neonatally from respiratory failure (26). To confirm the selective ablation of VGLUT2 in the DRG of cKO mice, we first analyzed the excision of exon 2 of the VGLUT2 gene. PCR using genomic DNA from DRG of cKO mice resulted

Author contributions: G.S., T.S.H., R.H.E., and A.I.B. designed research; G.S., S.A.L., X.W., J.Z., H.Y., R.U., C.S., B.H., and T.S.H. performed research; T.S.H. and R.H.E. contributed new reagents/analytic tools; G.S., S.A.L., X.W., J.Z., H.Y., R.U., C.S., B.H., T.S.H., R.H.E., and A.I.B. analyzed data; and G.S. and A.I.B. wrote the paper.

The authors declare no conflict of interest.

*This Direct Submission article had a prearranged editor.

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1013413108/-/DCSupplemental.

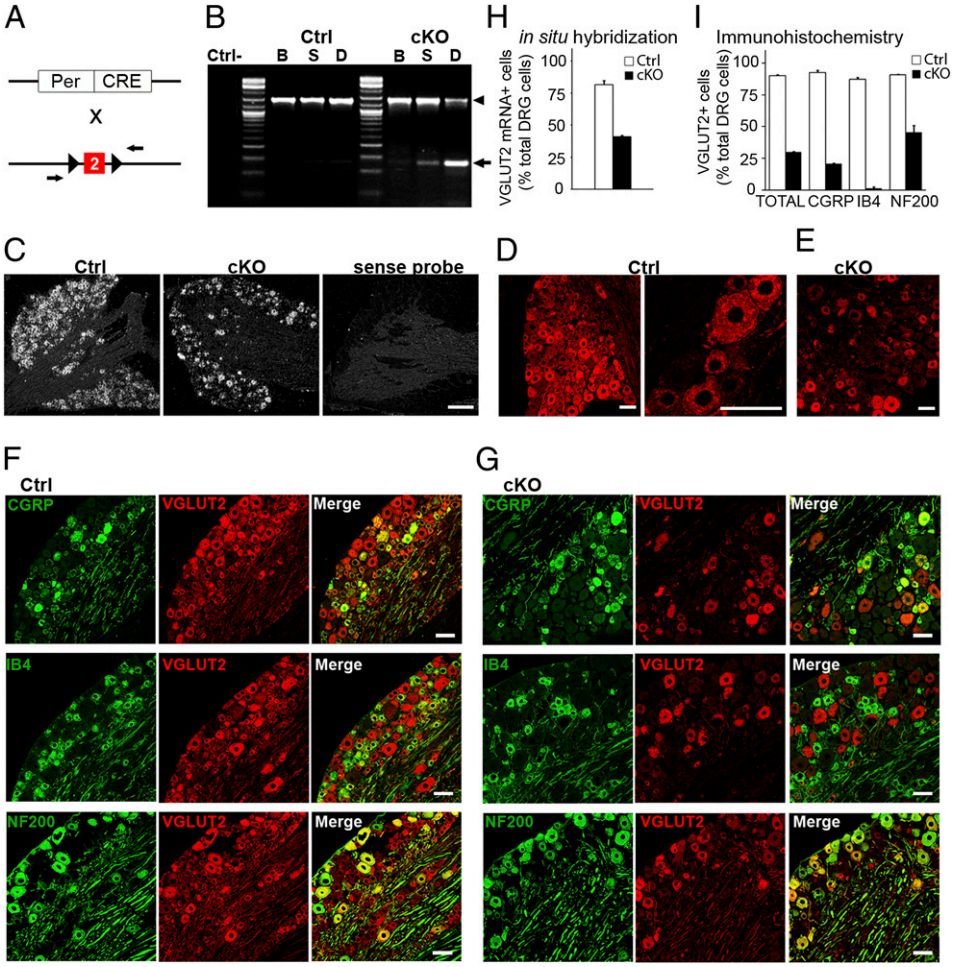

Fig. 1. Excision of the VGLUT2 second exon and depletion of VGLUT2 mRNA and protein in DRG from VGLUT2 cKO mice. (*A*) To ablate VGLUT2 selectively in DRG, we crossed Per-Cre mice with VGLUT2$^{lox/lox}$ mice (11) in which the second exon of VGLUT2 (red box) is flanked by loxP sites (black triangles) to obtain cKO mice. (*B*) PCR of genomic DNA using primers that hybridize to sequences flanking the second exon of VGLUT2 (black arrows in *A*) shows that Cre-mediated recombination of the VGLUT2 gene occurred in the DRG (lane D) from cKO mice, but not in the brain (lane B). Excision of the VGLUT2 second exon causes a shift in the size of the amplified DNA fragment [1,400 (arrowhead) and 220 (arrow) bp for control and recombined alleles, respectively]. The weak intensity 220-bp band obtained with spinal cord tissue (lane S) likely arises from Cre expression in spinal motoneurons during development (38) (Fig. S5). (*C*) In situ hybridization shows that VGLUT2 is expressed by 80.7 ± 1.0% and 40.8 ± 5.6% of DRG neurons in control and cKO mice, respectively. (Scale bar, 100 μm.) (*D*) 90.5 ± 0.5% of all DRG neurons show VGLUT2 immunoreactivity (VGLUT2-ir; n = 3). (*Right*) High-power image illustrating that the intensity of VGLUT2-ir varied greatly among positive cells. (Scale bars, 50 μm.) (*E*) In cKO mice, the percent of DRG neurons displaying VGLUT2-ir drops to 30.1 ± 0.3%. (*F* and *G*) Double-immunolabeling experiments show that VGLUT2 protein is present in 92.4 ± 1.6% of peptidergic (CGRP+) afferents in control mice, but only 21.1 ± 0.2% in cKO mice (n = 3). Expression of VGLUT2 in 87.4 ± 2.9% of nonpeptidergic C nociceptors (IB4+) in control mice is lost in cKO mice (1.0 ± 0.9%). VGLUT2-ir in cells with myelinated axons (NF200+) is reduced to a much smaller extent, from 90.9 ± 0.1% in control mice to 45.7 ± 5.2% in cKO mice. (Scale bars, 50 μm.) (*H*) Quantification of in situ hybridization experiments shown in *C* and Fig. S2. (*I*) Quantification of immunolabeling experiments shown in *D*–*G*.

in the production of a 220-bp DNA fragment that was absent using tissues from control mice (Fig. 1*B*), demonstrating excision of exon 2 from the VGLUT2 gene. Consistent with this excision, in situ hybridization revealed a strong signal for VGLUT2 in 81.7% of all DRG neurons in control mice (Fig. 1 *C* and *H* and Fig. S3), and Cre expression dramatically decreased VGLUT2 mRNA in DRG from cKO mice. Only 40.8% of all DRG neurons were still labeled with the antisense probe. This profound, but incomplete, loss of VGLUT2 is consistent with the expression pattern of the Cre in Per-Cre mice. Importantly, although Cre-mediated recombination also occurs in motoneurons of Per-Cre mice, in situ hybridization for VGLUT2 in spinal cord did not differ between cKO and control littermates (Fig. S4).

We then assessed the expression of VGLUT2 protein in DRG from cKO and control mice by immunohistochemistry. In control tissues, we observed very low to very intense VGLUT2 immunoreactivity (VGLUT2-ir) in 90.6% of all DRG neurons (Fig. 1 *D* and *I*). Consistent with the in situ hybridization experiments, VGLUT2 deletion was readily apparent in DRG from cKO mice, in which only 30.3% of all DRG neurons were labeled for VGLUT2 (Fig. 1 *E* and *I*). Importantly, VGLUT2 expression was almost completely lost in peptidergc (CGRP+) and nonpeptidergic (IB4+) C nociceptors (Fig. 1 *G* and *I*). VGLUT2-ir was also significantly decreased in medium-diameter myelinated (NF200+) CGRP+ or TRPV1+ afferents, a pattern that illustrates the loss of expression in Aδ fibers (Fig. S5). We did not detect a significant change in distribution or intensity of VGLUT2-ir in the spinal cord of cKO mice (Fig. S6). This finding agrees with previous deafferentation studies (17, 18, 23) and suggests that the weaker VGLUT2 immunoreactivity at central terminals within the dorsal horn (22) reflects less-efficient labeling and/or the requirement for only modest VGLUT2 expression for synaptic glutamate release. Together, these results indicate that VGLUT2 is, in fact, expressed by the majority of nociceptors, and that the cKO mice lack VGLUT2 in these neurons.

Decreased Responses of Spinal Neurons to Noxious Stimuli After VGLUT2 Deletion. If release of glutamate from nociceptors is essential for the transmission of pain information, then ablation of VGLUT2 in the cKO mice should alter the responsiveness of spinal cord neurons to noxious stimulation. We therefore recorded the responses of lamina I cells to noxious stimuli in both control and cKO animals. Stimulus-evoked activity of these cells was strongly affected by the loss of VGLUT2 (Fig. 2 *A* and *D*). The total number of spikes produced by graded noxious mechanical stimulation of the receptive field was significantly lower in cKO mice than in controls (Fig. 2*B*). The respective peak firing rates to these mechanical stimuli were also greatly reduced in the cKO mice, relative to controls (Fig. 2*C*).

We also investigated the thermal responsiveness of these mechanosensitive lamina I neurons. We found that the total evoked activity and peak firing rates during a 10-s period of 40, 45, and 49 °C stimulation in cKO mice were significantly decreased compared with control animals (Fig. 2 *E* and *F*). A large fraction of the noxious mechanical and heat input to superficial dorsal horn nociresponsive lamina I neurons thus depends on glutamate release from VGLUT2-expressing nociceptors.

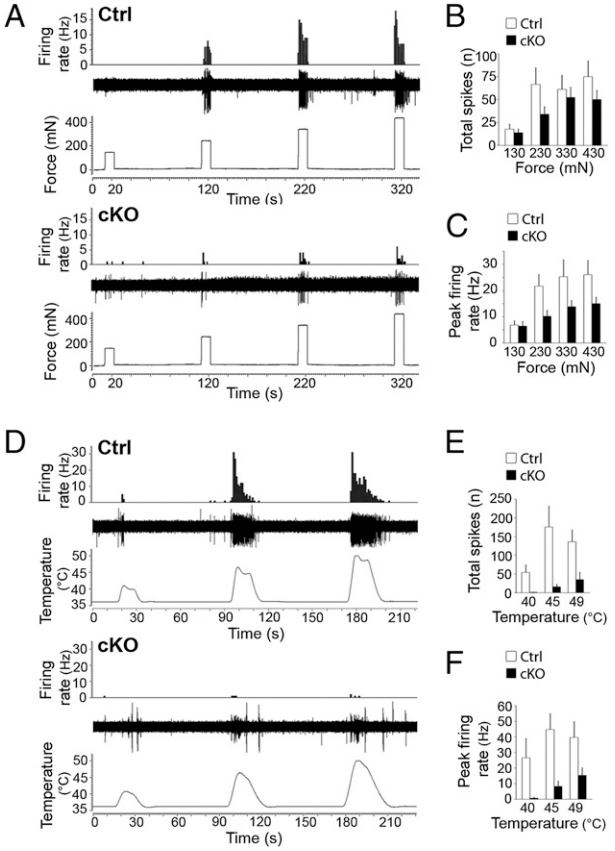

Fig. 2. Reduced firing of lamina I spinal cord neurons in response to noxious heat and mechanical stimulation in VGLUT2 cKO mice. (*A*) Representative mechanical responsiveness of lamina I neurons in control and cKO mice. Raw trace (*Middle*) and stimulus response histogram of an isolated unit (*Top*) obtained following a series of 10-s programmed forces of 130, 230, 330, and 430 mN (*Bottom*). (*B*) Total number of spikes of lamina I neurons induced by graded mechanical stimuli are significantly decreased in cKO mice compared with control mice ($P < 0.05$; $n = 9$ control and 20 cKO neurons). (*C*) Peak firing rate in response to mechanical stimulation was also significantly reduced in cKO mice ($P < 0.001$). (*D*) Representative heat responsiveness of lamina I neurons in control and cKO mice. Raw trace (*Middle*) and stimulus response histogram of an isolated unit (*Top*) in response to innocuous and noxious heat stimuli (40, 45, and 49 °C; *Bottom*). (*E*) As observed for mechanical stimulation, total number of spikes of lamina I neurons in response to graded heat stimuli were significantly reduced in cKO mice ($P < 0.001$; $n = 9$ control and 20 cKO neurons). (*F*) Peak firing rate was also significantly decreased in cKO animals ($P < 0.01$).

Altered Heat but Intact Cold Sensitivity in VGLUT2 cKO Mice. We next determined how the loss of VGLUT2 from afferents involved in nociception affects behavioral responses. We first evaluated motor function in cKO mice using the accelerating rotarod assay. cKO mice walked on the rotating rod with no obvious impairment and performed as well as control littermates (Fig. S7*A*), indicating normal motor function.

We tested sensitivity to noxious heat, using the hindpaw radiant heat (Hargreaves) hotplate and tail-immersion tests (Fig. 3*A*). When stimulated at temperatures ranging from 45 °C to 55 °C, cKO mice displayed nocifensive behaviors typical of these tests (withdrawal of the hindpaw, licking of the paws, withdrawal of the tail, respectively), but in all tests they did so at consistently longer latencies than control littermates. VGLUT2-dependent glutamate release from Cre-expressing nociceptors is thus required for normal sensitivity to noxious heat.

We also tested for cold sensitivity in the cKO animals using a two-platform preference assay. The cKO mice displayed a strong preference for a 30 °C platform over a second platform set at 20 °C, 10 °C, 5 °C, or 0 °C, i.e., at both cool (innocuous) and cold (noxious) temperatures (Fig. 3*B*). In each case, the percent of time spent by the cKO mice on the two platforms was

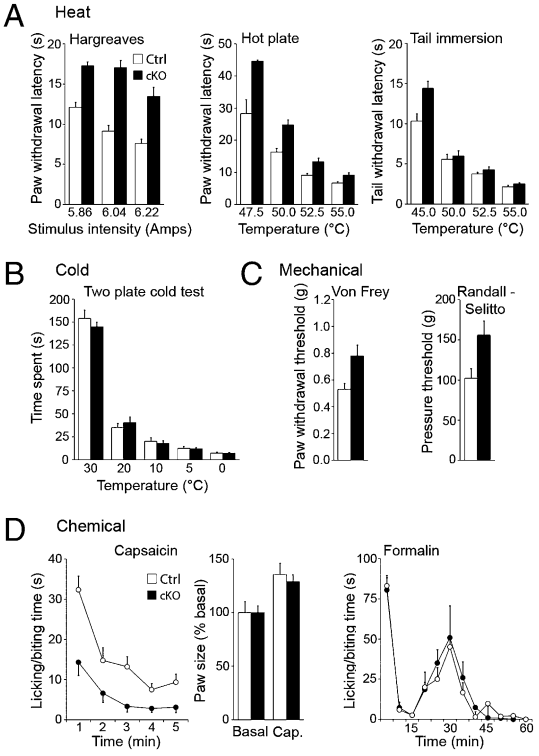

Fig. 3. Reduced heat, mechanical, and chemical acute pain in VGLUT2 cKO mice. (*A*) cKO mice responded with a significantly longer latency compared with controls in the Hargreaves ($P < 0.0001$, $n = 19$–27/group), hot plate ($P < 0.0001$, $n = 19$–27/group), and tail immersion ($P < 0.01$, $n = 19$–27/group) tests of heat pain. (*B*) Sensitivity to cool (innocuous) and cold (noxious) temperatures are unchanged in cKO mice ($P = 0.6765$, $n = 18$–19/group). (*C*) von Frey ($P < 0.05$, $n = 26$–30/group) and Randall–Selitto ($P < 0.05$, $n = 15$–16/group) tests of mechanical pain demonstrate that cKO mice are less sensitive to mechanical stimulation. (*D*) Nocifensive response to intraplantar capsaicin is reduced ($P < 0.01$) in cKO animals compared with controls. However, animals of both genotypes develop comparable edema ($P = 0.3353$; $n = 10$–15/group). cKO and control mice display similar nocifensive behaviors during phases I and II of the formalin test ($P = 0.7663$, $n = 7$–13/group).

similar to that recorded for littermate controls. Thus, sensitivity to noxious cold remains intact in cKO mice, providing evidence that acute heat and cold pain are generated through distinct primary afferent mechanisms.

Deficits in Acute Mechanical Pain and Capsaicin Response in VGLUT2 cKO Mice. We next tested whether VGLUT2-dependent glutamate release from Cre-expressing DRG neurons is also required for normal responsiveness to noxious mechanical and chemical stimuli (Fig. 3 *C* and *D*). Using von Frey filaments and the Randall–Selitto test, we found that the mechanical threshold in cKO mice was significantly higher relative to control littermates, indicating that the perception of both heat and mechanical noxious stimuli depends upon glutamate release by VGLUT2+ and Cre+ nociceptors.

We induced chemical pain in mice by intraplantar injection of capsaicin, a neurotoxin that acts on TRPV1-expressing peptidergic (i.e., substance P- and CGRP-containing) nociceptors. Following capsaicin injection into the hindpaw, control littermates immediately (0.4 s) started licking and biting the hindpaw vigorously, and continued throughout the 5-min test period (Fig. 3*D* and Fig. S7*B*). Strikingly, the response to capsaicin was markedly reduced in cKO mice. Initiation of the nocifensive behaviors was also significantly delayed in the cKO mice, up to 14.2 s on average (Fig. S7*B*). Glutamate released from TRPV1-expressing nociceptors thus contributes to capsaicin-evoked acute chemical pain, and the release of neuropeptides is not sufficient to generate a normal behavioral response. However, there was no difference between cKO mice and littermate controls in the edema produced by capsaicin injection (Fig. 3*D*), indicating that VGLUT2 is not required to trigger a neurogenic inflammatory response, and that release of vasoactive neuropeptides from the peripheral terminals of the TRPV1-expressing afferents occurs normally in mice lacking glutamate release from the majority of nociceptors. In contrast to the reduced capsaicin response, however, we found that neither phase I nor phase II of the formalin test, another model of chemical pain, was affected by the loss of VGLUT2 (Fig. 3*D*). This finding is consistent with the engagement of all primary afferents by formalin (27), making the VGLUT2+ cell population dispensable. In summary, cKO mice showed reduced responsiveness to acute stimulation with noxious heat, mechanical, and chemical stimuli. We conclude that glutamate signaling by VGLUT2+ nociceptors is essential for normal, physiological pain.

Essential Contribution of VGLUT2+ Afferents to Injury-Induced Heat Hypersensitivity. We next assessed whether the persistent, pathological pain that results from tissue or nerve injury (inflammatory and neuropathic pain, respectively) also depends on glutamate release from VGLUT2+ and Cre+ afferents. We tested the cKO mice in a model of inflammatory pain in which injury to peripheral tissue is triggered by intraplantar injection of carrageenan. In control littermates, carrageenan produced a dramatic increase in sensitivity to heat and mechanical stimuli, as measured with the hindpaw radiant heat and von Frey tests, respectively (Fig. 4*A* and Fig. S8*A*). In cKO mice, however, heat hypersensitivity was completely lost, whereas carrageenan-induced mechanical hypersensitivity developed normally. Similar results were obtained using the complete Freund's adjuvant (CFA) model of inflammatory pain, in which hypersensitivity persists for several days (Fig. 4*B* and Fig. S8*B*). These results indicate clear differences in the mediators of hypersensitivity to heat and mechanical stimuli after peripheral tissue injury. Glutamate signaling by VGLUT2+ afferents is essential for tissue injury-induced heat hyperalgesia, but not for mechanical hypersensitivity.

Nerve Injury-Induced Microglial Activation and Mechanical Hypersensitivity Develop Normally in VGLUT2 cKO Mice. Finally, we addressed the contribution of glutamate release from VGLUT2+ and Cre+ afferents to hypersensitivity produced by nerve injury. Fig. 4*C* and Fig. S8*C* shows that control mice displayed prolonged hypersensitivity to heat and mechanical stimuli in the chronic constriction model of nerve injury ($P < 0.0001$). In contrast to the results obtained using models of tissue injury, we found that heat hyperalgesia does develop in cKO mice ($P = 0.0331$), albeit to a lesser extent than in controls. However, as for tissue injury, VGLUT2 deletion changed neither the magnitude nor the time course of development of the mechanical hypersensitivity that follows nerve injury.

After nerve injury, activated microglia accumulate in the vicinity of the dorsal horn terminals of damaged sensory neurons and contribute to pathological hypersensitivity (28–31). To assess the requirement of VGLUT2 for the mobilization of microglia, we made a complete transection of the sciatic nerve in cKO and control mice. In control mice, as previously described (29, 30), microglial cells were activated in the topographically appropriate region of the superficial dorsal horn as well as around motoneuron cell bodies, the axons of which were concurrently transected (Fig. 4*D*). Consistent with the preservation of nerve injury-induced mechanical hypersensitivity, we found that microglia also accumulate in the spinal cord of cKO mice with sciatic transection, indicating that glutamate release from VGLUT2+ and Cre+ afferents is not essential for activation of spinal microglia after nerve injury.

Discussion

In the present study, we found that VGLUT2 is expressed by the great majority of nociceptors and that deletion of VGLUT2 from these neurons reduced acute heat, mechanical, and chemical pain

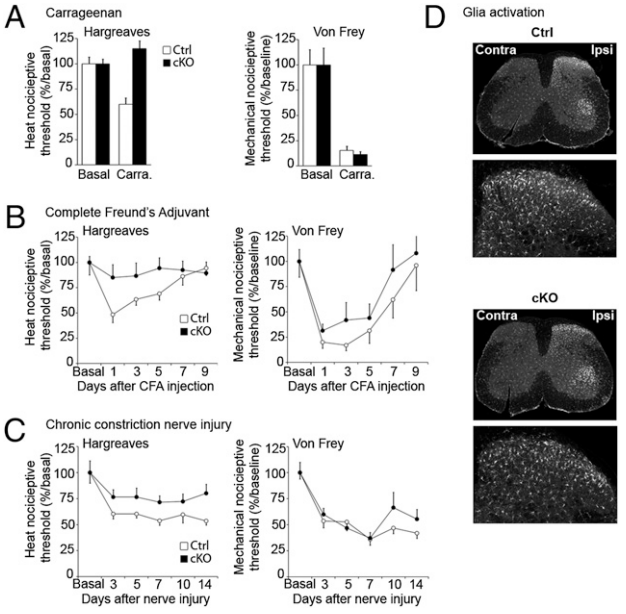

Fig. 4. Decreased injury-induced heat but not mechanical hypersensitivity in VGLUT2 cKO mice. (*A*) Intraplantar injection of carrageenan generates heat hyperalgesia in control mice ($P = 0.0005$) but not in cKO mice ($P = 0.3635$). In distinct contrast, mechanical allodynia develops in both control and cKO animals ($n = 11$–16/group). (*B*) Results obtained using a different model of tissue injury, the intraplantar injection of CFA, confirms those obtained with the carrageenan model. Although both control and cKO mice show mechanical hypersensitivity after CFA injection ($P < 0.0001$ and $P < 0.002$, respectively), only control ($P < 0.0001$), but not cKO animals ($P = 0.8419$), display sensitization (decreased latency to withdraw the hindpaw) to heat stimuli. (*C*) In the chronic constriction model of nerve injury, control mice show robust and prolonged heat and mechanical hypersensitivity ($P < 0.0001$ for both stimuli). cKO mice developed comparable mechanical allodynia ($P < 0.0001$); however, the magnitude of heat hyperalgesia was reduced ($P = 0.0331$). (*D*) Complete transection of the sciatic nerve induces comparable activation of microglia in the spinal cords of cKO and control mice, as visualized using an antibody against Iba-1. Microglia are activated in the dorsal and ventral horns ipsilateral to the nerve injury, i.e., in regions where primary afferents terminate and motoneurons are present, respectively. (*Lower*) High-power images.

responsiveness, and largely eliminated the heat hypersensitivity associated with tissue or nerve injury (inflammatory and neuropathic pain, respectively), but did not alter injury-induced mechanical hypersensitivity. Several important conclusions follow from these observations. First, glutamate release from nociceptors is unquestionably required for full sensitivity to acute noxious stimuli, and VGLUT2 could be targeted to alleviate injury-induced heat hypersensitivity. Our findings suggest that haplosufficiency must have masked the function of VGLUT2 in nociceptors in a previous study that used VGLUT2 heterozygous global KO mice (26). Second, the peptide cotransmitters, substance P and CGRP, are not sufficient to sustain full responsiveness to noxious stimuli, which is consistent with our previous studies showing no change in heat or mechanical pain thresholds in mice lacking substance P (9). Third, neither the mechanical hypersensitivity nor the microgliosis that occur following peripheral injury require glutamatergic neurotransmission from the two major subsets of unmyelinated nociceptors. An important corollary of the latter conclusion is that injury-induced heat and mechanical hypersensitivity rely on distinct (VGLUT2-dependent and -independent, respectively) primary afferent mechanisms and pathways.

In VGLUT2 cKO mice we found that VGLUT2 was ablated in the vast majority of C nociceptors (peptidergic and IB4 binding). Cre-mediated recombination also occurred in a population of myelinated afferents, as well as in motoneurons, consistent with expression of peripherin in these neurons during development (32). Analysis of expression of CGRP, TRPV1, and VGLUT2 in medium-diameter myelinated neurons in cKO mice indicates that VGLUT2 is also lost in the majority of peptidergic and capsaicin-sensitive Aδ afferents. VGLUT2 might also be lost in nonpeptidergic Aδ fibers or in a subset of Aα/β afferents, but the lack of specific markers for these neuronal subpopulations made it difficult to test this possibility. However, because cKO mice showed normal motor coordination, it appears that VGLUT2 is either not expressed in or not essential for the function of proprioceptors or motoneurons, consistent with VGLUT1-dependent glutamatergic and cholinergic neurotransmission, respectively. Together, these immunohistochemical and electrophysiological experiments provide strong support for the conclusion that the pain deficits in cKO mice result from loss of VGLUT2-dependent glutamate release from C and Aδ nociceptors. We cannot rule out the possibility that the deficits reflect a contribution of VGLUT2 protein to the development of nociceptive circuits. However, as the anatomy of the peptidergic and nonpeptidergic nociceptors appears normal in cKO mice, this seems unlikely. As to the residual pain responsiveness, several systems could contribute, including VGLUT2+ and Cre-negative afferents, VGLUT2-negative afferents implicated in nociceptive processing (notably the NF200+ VGLUT3-expressing afferents) (33), and perhaps peptidergic function in nociceptors from which VGLUT2 was deleted.

Defining the Pain Modalities Mediated by Subsets of DRG Neurons. We show here that VGLUT2 is expressed in most peptidergic and nonpeptidergic (IB4 binding) C nociceptors, confirming previous studies (17–22). With the exception of a very small number of IB4+ neurons that express VGLUT3 (Fig. S9) (33), it appears that VGLUT2 is the only VGLUT present in these two major nociceptor populations. As a consequence, the present findings provide important information about the contribution of VGLUT2-expressing nociceptors to the perception of specific pain modalities (Fig. S10). Our finding that the cKO mice are less sensitive to acute heat stimuli and intraplantar capsaicin, and do not develop heat hyperalgesia after tissue injury, is consistent with VGLUT2 expression in the peptidergic C fibers, which in the mouse also express the heat- and capsaicin-sensitive TRPV1 channel. In fact, destruction of TRPV1-expressing DRG neurons or their central terminals in the mouse produces a remarkably similar behavioral phenotype—namely, loss of both acute heat pain responsiveness and the sensitization to heat stimuli that occurs with tissue injury (6–8). We propose, therefore, that heat deficits observed in the cKO mice arise from loss of VGLUT2-dependent glutamate signaling from TRPV1-expressing peptidergic C or Aδ nociceptors.

The mechanical deficits produced by interfering with primary afferent neurons have a more complicated basis, as both VGLUT2+ and VGLUT3+ nociceptors appear to contribute. Thus, we recently reported that a subset of VGLUT3+ DRG neurons is critical to normal perception of acute mechanical pain (32). We now show that VGLUT2 deletion also reduces sensitivity to noxious mechanical stimuli. Furthermore, specific deletion of a large fraction of IB4+ C nociceptors (6), or intrathecal administration of an agonist of the delta opioid receptor, which is expressed by IB4+ nociceptors (8), also reduces acute mechanical pain responsiveness. Taken together, we suggest that glutamate release from these VGLUT2-expressing IB4+ afferents is critical for—but not the only contributor to—normal responsiveness to noxious mechanical stimuli.

Our findings partly agree with a study showing that deletion of DRG neurons that express the Nav1.8 subtype of sodium channel, which includes most C nociceptors, resulted in loss of mechanical pain and heat hyperalgesia after tissue injury (5). However, these authors also reported no significant change in sensitivity to acute noxious heat, a surprising result given the strong association of peptidergic C fibers and TRPV1 in the mouse, and given the authors' own finding that deletion of the Nav1.8 channel in the mouse (rather than the Nav1.8-expressing population of nociceptors) does reduce heat pain (34). Conceivably there exists a population of VGLUT2- and TRPV1-expressing nociceptors that lacks Nav1.8 but is nevertheless critical to heat pain processing.

Of particular interest is our finding that despite showing reduced mechanical pain sensation, cKO mice develop normal mechanical hypersensitivity following either tissue or nerve injury. It follows that VGLUT2-dependent glutamate signaling by nociceptors is not required for establishment of the central sensitization and touch-evoked pain induced after peripheral injury. Consistent with that conclusion, we found that nerve injury-induced microgliosis in the spinal cord, a major correlate of injury-induced central sensitization (28–31), remains intact in cKO mice. It thus appears that primary afferents that depend on VGLUT2 to replenish glutamate in their axon terminals do not engage spinal circuits that contribute to touch-evoked pain (allodynia) after injury. In this regard, large-diameter myelinated Aβ fibers, or low-threshold C fibers, which preferentially express VGLUT1 and VGLUT3, respectively, appear better positioned to contribute. Both of these afferent populations target the inner part of lamina II and the dorsal part of lamina III, a region critical for the development of mechanical allodynia/hypersensitivity after injury (33, 35–37). In fact, mice with a deletion of VGLUT3 show reduced mechanical allodynia (33), with no effect on heat hyperalgesia, suggesting complementary roles of the VGLUT2- and VGLUT3-expressing afferent populations following injury. Our findings clearly emphasize the functional diversity of primary afferent neurons and provide further support for the existence of behaviorally relevant peripheral labeled lines for the perception of distinct pain modalities. As a corollary, the segregation of vesicular glutamate transporters into these distinct afferent populations enables the selective regulation of glutamatergic signaling from subsets of nociceptors, and the potential for specific pharmacological intervention that could not be achieved by targeting the common pre- and postsynaptic receptors with which glutamate interacts.

Materials and Methods

Genomic DNA Analysis. We prepared genomic DNA (gDNA) from DRG, spinal cord, and brain from control and cKO mice. Deletion of exon 2 of the VGLUT2 gene was confirmed by PCR using 100 ng of gDNA with forward (CAGTGTGCTGTAACTGAGATAGT) and reverse (AAAGGTCCTGGATCAGAGCAGG) primers (Fig. 1 *A* and *B*).

Histology. Mice were deeply anesthetized with 100 mg/kg sodium pentobarbital and perfused transcardially with 0.1 M PBS followed by 10% formalin in PB. Tissues were postfixed for 4 h, cryoprotected, and sectioned on a cryostat. Standard procedures were used for immunohistochemistry and in situ hybridization (*SI Experimental Procedures*). For VGLUT2 immunostaining, tissues were incubated with a guinea pig anti-VGLUT2 (1:20,000) antibody (kindly provided by Masahiko Watanabe, Hokkaido University School of Medicine, Sapporo, Japan) for 3 d at 4 °C in the absence of detergent. Analysis of ex-

pression and size distribution of neurochemical markers in DRG was performed by an investigator blind to genotypes using Zeiss LSM 510 Meta and ImageJ softwares (stereology was not used).

Spinal Cord Lamina I Extracellular Recording. We performed extracellular single unit recording from neurons of the superficial dorsal horn of the spinal cord (38) blind to the genotypes. Briefly, mice were anesthetized by i.p. injection of 1.5 g/kg urethane. A laminectomy was performed at vertebral levels T13 to L1, corresponding to spinal segments L4–L5. A pool was formed with 5% agar and then filled with 37 °C mineral oil. A fine-tipped tungsten microelectrode (impedance of 6–8 MΩ at 1 kHz; FHC) was used to record unit activity. To search for units, we applied brief, moderate pressure with a blunt glass probe to different regions of the glabrous skin of the ipsilateral hindpaw. Once a mechanical receptive field was identified, we tested the unit for its responsiveness to brush, pressure, pinch, and 50 °C water. Next, we applied graded mechanical and heat stimuli using a custom-built mechanical stimulator (ESTIMEC; Cibertec) or a contact Peltier device (kindly provided by Merck, Sharpe, and Dohme), respectively. Unit activity was amplified (CyberAmp380; Axon Instruments), digitized (Micro1401; CED), and discriminated (Spike2; CED).

Behavior. All animal experiments were approved by the Institutional Animal Care and Use Committee at University of California at San Francisco and were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. For all experiments, the investigator performing the behavioral tests was blind to genotype. To assay for heat pain with the Hargreaves test, a radiant heat source was focused on the hindpaw, and latency to withdraw the paw was measured. For the hotplate test, latency to lick/flinch the hindpaws or to jump was recorded. For the tail immersion test, mice were gently restrained, the tip of the tail immersed in hot water, and then monitored the latency for tail withdrawal. To test for cold sensitivity, we used the two-plate choice assay. Mice were placed in a chamber containing identical adjacent floor plates, with one set to 30 °C and the other adjusted to various colder temperatures. Mice were free to explore, and the total time spent on each surface was recorded over a 5-min period. For the von Frey assay, mice were stimulated with von Frey filaments, and thresholds were measured using the up-and-down method. For the Randall–Selitto test (Ugo Basile), mice were gently restrained and increasing pressure was applied to the tail until the animal displayed significant struggling, which we interpreted as discomfort and considered to be the noxious mechanical threshold. For the capsaicin and formalin tests, we made intraplantar injections of 3 μg capsaicin or 5% formalin and recorded the time spent licking/biting the injected hindpaw. For tissue injury-induced hypersensitivity, we made intraplantar injections of 20 μL of a 3% carrageenan solution or 10 μL of a saline/CFA emulsion. For nerve injury-induced hypersensitivity, we used the chronic constriction injury model of neuropathic pain.

Note Added in Proof. Very recently, two papers reported that conditional deletion of VGLUT2 from primary afferent neurons produces spontaneous scratching, leading to severe skin lesions (39, 40). We did not observe this spontaneous scratching phenotype or skin lesions despite deleting VGLUT2 from a comparable, although not identical, population of primary afferent neurons.

ACKNOWLEDGMENTS. This work was supported by National Institutes of Health Grants NS14627 and NS48499 (to A.I.B.) and MH50712 and DA10154 (to R.H.E.), a Fondation pour la Recherche Médicale postdoctoral grant (to G.S.), and the Pell Family Foundation. We thank Dr. Masahiko Watanabe (Hokkaido University School of Medicine, Sapporo, Japan) for providing the anti-VGLUT2 antibody, Dr. Pablo Brumovsky for advice about VGLUT2 immunohistochemistry, Drs. Clement Cheung and Holly Ingraham (University of California, San Francisco) for plasmids and help with VGLUT2 in situ hybridization, and Ian Oldenburg for his contribution to this project as an Amgen Scholar at University of California, San Francisco.

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