NEUROSCIENCE

A role for proprioceptors in sngception

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Proprioceptors are primary mechanosensory neurons to monitor the status of muscle contraction and/or body position (*1*). Although proprioceptors are known as non-nociceptive mechanoreceptors, they also express the pro-nociceptive acid-sensing ion channel 3 (ASIC3) (*2*–*5*). To probe the role for proprioceptors in sensing acidosis (or sngception) (*6*), we found that genetic deletion of *Asic3* in proprioceptors but not in nociceptors abolished acid-induced chronic hyperalgesia in mice. Chemo-optogenetically activating proprioceptors resulted in hyperalgesic priming that favored chronic pain induced by acidosis. In humans, intramuscular acidification induced acid perception but not pain. Conversely, in a spinal cord–injured patient who lost pain sensation in the right leg, proprioception and sngception were remaining somatosensory functions, associated with the spinal dorsal column. Together, evidence from both mouse and human studies suggests a role for proprioceptors in sngception.

INTRODUCTION

The perception of acid sensation can be regarded as one of the most mysterious somatosensory functions. Although soreness (e.g., muscle soreness) is commonly used to describe the perception of acid sensation, it may not precisely address the nature of acid sensation (*6*). The perception of acid sensation is most commonly felt after fatiguing exercise. This muscle soreness is defined as pain resulting from overuse in the English dictionary. However, although sometimes this soreness and pain are not defined by specific words in other languages, this soreness is a perception distinguishable from pain (e.g., "courbature" in French and "muskelkater" in German) (*7*).

Traditionally, tissue acidosis, which occurs in ischemia, inflammation, fatiguing exercise, etc., is a potent factor for activating proton-sensing ion channels/receptors to trigger pain, as has been demonstrated in humans and animal models (*5*, *8*–*13*). The location of the proton-sensing ion channels is, however, more paradoxical being found on a wide range of somatosensory neurons. These include not only nociceptors but also pruriceptors, and non-nociceptive mechanoreceptors (e.g., proprioceptors) (*4*, *14*, *15*). Thus, acidosis seems not only to be involved in nociception but also in pruriception, proprioception, and antinociceptive signaling (*6*). For instance, the acid-sensing ion channel 3 (ASIC3) is arguably the most acid sensitive of ion channels in somatosensory neurons and is involved in perception of acid-induced chronic pain in experimental animal models (*5*, *16*–*18*). Yet, intriguingly, ASIC3 is also expressed in proprioceptors where it functions as a mechanically sensitive ion channel involved in tether-mode (force-from-filament) mechanotransduction (*4*). In addition, a recent study showed another acid-sensitive ion channel, ASIC1a, can mediate antinociceptive effects in dextrose prolotherapy (*19*). Therefore, the role of acid signaling in non-nociceptive somatosensory neurons is of great interest for understanding the neurobiology of pain associated with tissue acidosis and ion channels involved in acid signaling are potential therapeutic targets.

To address the promiscuous nature of acid sensation, we have coined the term "sngception (sng - ception)" for this specific somatosensory function, to distinguish it from the nociceptor neuron-specific sensation of painful stimuli (nociception). "Sng" (pronounced as /səŋ/) is derived from a linguistic phenomenon where both "sour taste" and muscle soreness are encoded in the same word in the Taiwanese language (*7*). In Chinese, such acid-like discomfort is often described as sng or sng-pain, again using the sng Taiwanese word that represents the state of feeling sore. In the pain clinic, soreness (or sng) sensation is seen as a distinct and characteristic sensory phenotype of various acute and chronic pain syndromes (e.g., delayed-onset muscle soreness or DOMS, fibromyalgia, and radicular pain) (*6*, *20*–*24*). It is also a sign of successful analgesia for acupuncture and many physical therapies (*6*, *7*, *19*, *25*, *26*).

Thus, there is increasing evidence for a neurobiological basis for a separate sngception to be distinguished from nociception, which is detected and transmitted by specific somatosensory neurons. Here, we show the first evidence that sng and pain can be segregated and distinguished separately in humans and other animals. We also show in mouse models how sngception is transmitted and contributes to chronic hypersensitivity.

RESULTS

A previously unidentified role of proprioceptors in acid sensation

Although proprioceptors are non-nociceptive, low-threshold mechanoreceptors (*1*, *2*), the pro-nociceptive acid sensor ASIC3 is highly expressed in proprioceptors. While there is evidence that ASIC3 is involved in tether-mode mechanotransduction and associated proprioceptive behaviors in these neurons (*4*), it is not clear why proprioceptors need an acid sensor. Therefore, it is also possible that proprioceptors are involved in other somatosensory function. We hypothesize that this is sngception and specifically that they contribute to acid-induced pain chronicity. To test for such a role of

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proprioceptor ASIC3 in detection of acid, or in sngception, in mouse models we conditionally deleted the *Asic3* gene in either nociceptors or proprioceptors. We then tested its effect on the development of chronic mechanical hyperalgesia induced by repeated intramuscular acid injection, which is a widely used mouse model for chronic widespread pain and has been reported in the literature to mimic fibromyalgia (*5*). In this model, ASIC3 is the molecular determinant responding to intramuscular acid injections. This in turn leads to hyperalgesic priming and finally to chronic mechanical hyperalgesia (*17*, *18*).

As shown previously, in control *Asic3*$^{f/f}$ mice, the first acid injection (pH 4.0) induced a transient hyperalgesia that declined within 24 hours. A second acid injection 1 or 5 days later induced chronic hyperalgesia, lasting for more than 3 weeks (Fig. 1, A to D, and fig. S1). However, we found that conditional knockout of *Asic3* in nociceptors (*Nav1.8-Cre::Asic3*$^{f/f}$) either had no effect or it only partially attenuated the chronic hyperalgesia of the hind paws and muscle after the second acid injection. In contrast, conditional knockout of *Asic3* in proprioceptors (*Pv-Cre::Asic3*$^{f/f}$) substantially reduced the development of chronic hyperalgesia (Fig. 1, A to D, and figs. S1 and S2). Similar results were found in another mouse model, where fibromyalgia-like chronic widespread pain was induced by intermittent cold stress (ICS) (*27*). Again, conditional knockout of *Asic3* in proprioceptors, but not nociceptors, abolished the development of chronic muscle hyperalgesia (Fig. 1, E and F). These outcomes were not the effect of gene compensation by other ASIC subtypes, because individual gene knockouts of any of the other three functional ASIC channel subtypes in proprioceptors, ASIC1a, ASIC1b, and ASIC2a, did not affect the ICS-induced chronic widespread pain (fig. S3). Together, these data from two different mouse models of chronic widespread pain consistently indicate that ASIC3 expressed in proprioceptors contributes to the development of chronic widespread hyperalgesia. In contrast, the evidence for a role of nociceptors was much less consistent. Chronic hyperalgesia in mice lacking ASIC3 in nociceptors was only reduced when the second acid injection occurred 5 days after the first injection (fig. S1A), indicating that nociceptors may make a smaller contribution to hyperalgesia in this model. A caveat of using *Pv-Cre::Asic3*$^{f/f}$ mice is that ASIC3 is also expressed in *Pv*-positive mesencephalic trigeminal nucleus (Me5) neurons in the brain stem region, although these neurons are proprioceptors in the central nervous system (*28*, *29*). Still, it is unlikely that trigeminal neurons are involved in the acidosis-induced priming in the muscle afferents of gastrocnemius muscle in this study.

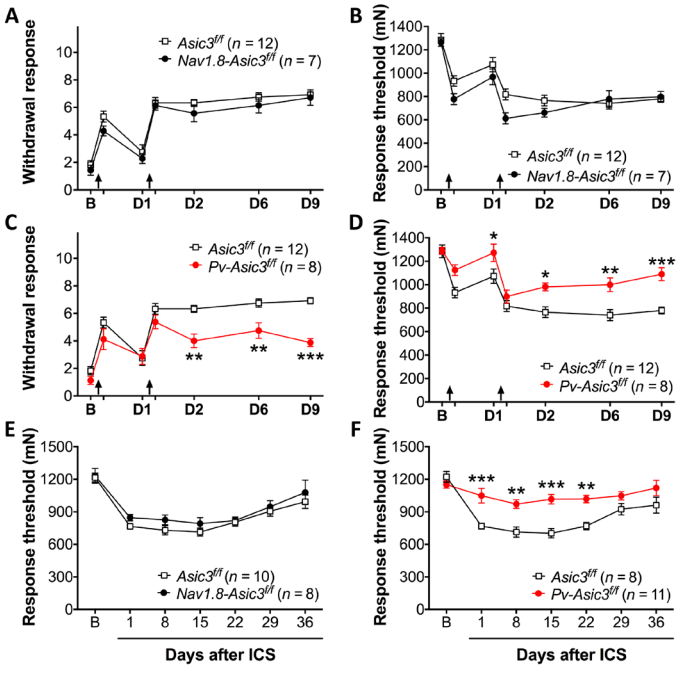

Fig. 1. Effect of conditional knockout of *Asic3* in proprioceptors and nociceptors on chronic sng pain. (**A** to **D**) In a mouse model of chronic widespread pain, wild-type (*Asic3*$^{f/f}$) mice developed chronic widespread mechanical hyperalgesia after two injections of acidic saline (pH 4.0) to unilateral gastrocnemius muscle. Conditional knockout of *Asic3* in nociceptors (*Nav1.8-Cre::Asic3*$^{f/f}$, as *Nav1.8-Asic3*$^{f/f}$) had no effect on the development of chronic hyperalgesia in hind paws and muscle [(A) and (B)], whereas conditional knockout of *Asic3* in proprioceptors (*Pv-Cre::Asic3*$^{f/f}$, as *Pv-Asic3*$^{f/f}$) significantly impaired the development of chronic hyperalgesia [(C) and (D)] (control, $n = 12$; *Nav1.8-Asic3*$^{f/f}$, $n = 7$; *Pv-Asic3*$^{f/f}$, $n = 8$). Black arrows indicate the time of acid intramuscular injection. (**E** and **F**) In a second mouse model of chronic widespread pain induced by intermittent cold stress, *Nav1.8-Cre::Asic3*$^{f/f}$ mice developed chronic widespread mechanical hyperalgesia in muscle comparable with wild-type controls (*Nav1.8-Asic3*$^{f/f}$, $n = 8$; *Asic3*$^{f/f}$, $n = 10$), whereas *Pv-Asic3*$^{f/f}$ mice did not develop muscle hyperalgesia as compared with wild-type control (*Pv-Cre::Asic3*$^{f/f}$, $n = 11$; control, $n = 8$). Data were analyzed by two-way ANOVA (*$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ versus *Asic3*$^{f/f}$).

To further differentiate between a causal role of proprioceptors and nociceptors in acid-induced pain chronicity, we adopted a chemo-optogenetics approach, using a chemical ligand, coelenterazine (CTZ), to activate luminopsin 3 (LMO3), a luciferase-channelrhodopsin fusion protein, via binding to its light-emitting luciferase moiety (Fig. 2A) (*30*). We used LMO3 due to its unique kinetics that allow induction of fast- and short-term chemogenetic effects. In this model, activation of extracellular signal–regulated kinase (ERK) by phosphorylation (pERK) in the soma of dorsal root ganglion (DRG) neurons is a surrogate marker to indicate CTZ-dependent neuron activation. In control experiments, we established that CTZ (800 ng) induced pERK expression at 1 and 5 min after injection in *Pv-Cre::LMO3* mice (fig. S4). In this chemo-optogenetic model, we were able to test the roles of the proprioceptors and nociceptors in the acid-induced pain chronicity. The dual intramuscular injection regimes used were either CTZ followed by acid (CTZ-acid), acid-CTZ, or CTZ-CTZ (Fig. 2B) for direct comparison with the standard acid-acid protocol (Fig. 2C). This approach meant that in *Pv-Cre::LMO3* and *Nav1.8-Cre::LMO3* mice, we could selectively activate proprioceptors or nociceptors, respectively, by an initial priming injection of CTZ into the muscle. We then tested the effects of a follow-up acid injection on the development of chronic hyperalgesia. In this CTZ-acid experimental paradigm, while the CTZ (800 ng) injection was not sufficient to induce the behavioral responses of transient hyperalgesia in either group of animals, it was sufficient for a hyperalgesic priming effect on the targeted sensory neurons. Notably, the intramuscular CTZ treatment only primed the induction of chronic hyperalgesia to a follow-up acid injection in *Pv-Cre::LMO3* mice (proprioceptor expression), but not in *Nav1.8-Cre::LMO3* mice (nociceptor expression; Fig. 2, D to G). Further, this CTZ-induced hyperalgesic priming effect was dose dependent, with a minimal effective dose of 400 ng (Fig. 2, H and I). In contrast, while the first acid injection induced transient hyperalgesia of hind paws and muscle, a follow-up CTZ injection on the next day failed to induce

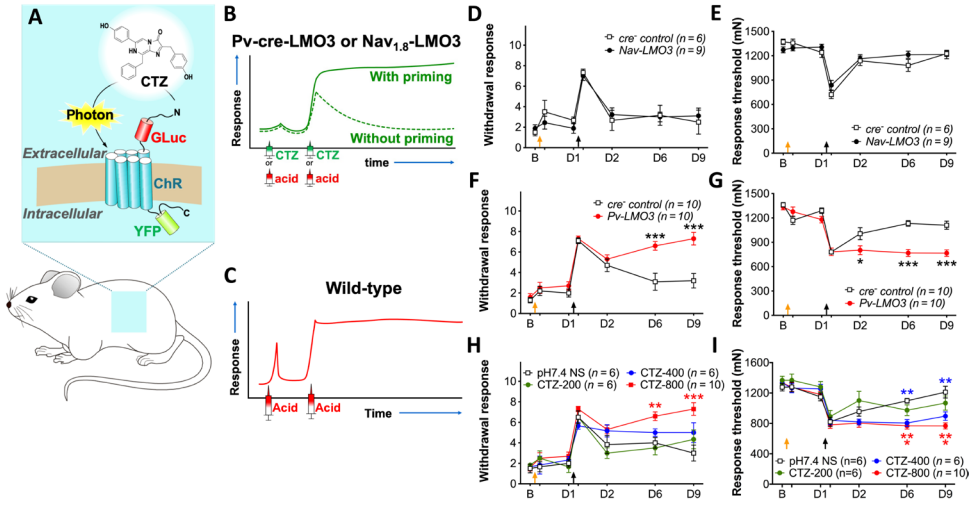

Fig. 2. Effect of chemo-optogenetics activation of proprioceptors and nociceptors on the hyperalgesic priming to trigger acid-induced chronic hyperalgesia. (**A**) Scheme illustrating the chemo-optogenetic design. (**B** and **C**) Experimental design of the dual intramuscular injections of CTZ-acid, CTZ-CTZ, acid-CTZ, and acid-acid. (**D** to **G**) The mechanical sensitivity of *Nav1.8-Cre::LMO3* (*Nav1.8-LMO3*) and *Pv-Cre::LMO3* (*Pv-LMO3*) mice was monitored before and after CTZ-acid injection to gastrocnemius muscle. One day after CTZ injection, an acid injection induced only transient hyperalgesia of hind paws and muscle in *Nav1.8-LMO3* mice [(D) and (E), *Nav1.8-LMO3*, $n = 9$; control $n = 6$], but induced a long-lasting hyperalgesia in *Pv-LMO3* mice [(F) and (G), *Pv-LMO3*, $n = 10$; control $n = 10$]. (**H** and **I**) CTZ had a dose-dependent effect on the hyperalgesic priming that allowed a follow-up acid injection to induce chronic hyperalgesia of hind paws and muscle in *Pv-LMO3* mice ($n = 6$ to 10). Black arrows indicate intramuscular acid injection (pH 4.0). Orange arrows indicate intramuscular CTZ injection. All data presented as mean ± SEM. Data were analyzed by two-way ANOVA (*$P < 0.05$ and ***$P < 0.001$ versus control).

chronic hyperalgesia in either *Pv-Cre::LMO3* (fig. S5, A and B) or *Nav1.8-Cre::LMO3* mice (fig. S5, C and D), suggesting that both proprioceptors and nociceptors are engaged in the development of chronic hyperalgesia. In agreement, dual injections of CTZ in *Pv-Cre::LMO3* mice failed to induce chronic hyperalgesia (fig. S5, E and F). Last, it does not seem that group II Aβ muscle afferents were involved in this proprioceptor-mediated hyperalgesic priming, because flagellin-QX314–mediated Aβ blockade did not affect the development of chronic hyperalgesia induced by the CTZ-acid injection regime in the *Pv-Cre::LMO3* mice (fig. S6).

Collectively, these results indicate that only activated *Pv*-positive proprioceptors, but not Nav1.8-positive nociceptors, contribute to the hyperalgesic priming. It is possible that the second CTZ injection was insufficient to activate proprioceptors to induce chronic hyperalgesia. However, since the primary injection always induced priming, this seems unlikely. Thus, our results are most easily explained by a model in which both proprioceptors and nociceptors are engaged in the subsequent development of chronic hyperalgesia induced by a follow-up acid challenge.

Mechanism underlying the proprioceptor-mediated chronic hyperalgesia induced by intramuscular acidosis

We next assessed how proprioceptors could participate in the hyperalgesic priming, since repeated activation of proprioceptors by CTZ was not sufficient for the development of chronic hyperalgesia in the dual-injection model. Previous studies have shown that peripheral terminals of proprioceptors can release glutamate that acts as an autocrine mediator to activate the phospholipase D (PLD)–coupled mGluR, a metabotropic GluK2 receptor, to enable and potentiate proprioceptor firing (*31*, *32*). We tested whether this GluK2-mediated signaling is involved in the proprioceptor-mediated hyperalgesic priming. When PCCG-13, a selective antagonist of the GluK2 receptor (*33*), was coinjected with CTZ, it dose-dependently inhibited the acid injection-induced chronic hyperalgesia in the *Pv-Cre::LMO3* mice (Fig. 3, A and B). Consistent with this finding, PCCG-13 dose-dependently inhibited CTZ-induced pERK expression in DRG neurons of *Pv-Cre::LMO3* mice (Fig. 3, C and D). However, PCCG-13 (2 nmol, intramuscular) did not inhibit the mechanical hyperalgesia in *Pv-Cre::LMO3* mice after the establishment of CTZ/acid-induced hyperalgesia (fig. S7).

We have previously shown that substance P signaling is antinociceptive in muscle afferents, and that it can counterbalance the acid-induced hyperalgesia and chronic pain (*19*, *34*–*37*). Thus, here, we probed whether the CTZ/acid regime-induced chronic hyperalgesia could be disrupted by activating intramuscular substance P signaling. Coinjection of CTZ and [Sar^9, $Met(O_2)^{11}$]–substance P (SM-SP), a substance P analog specific for the NK1 receptor, inhibited the hyperalgesic priming and development of chronic hyperalgesia by a follow-up acid challenge in *Pv-Cre::LMO3* mice (Fig. 4, A and B). This indicates that substance P signaling does disrupt proprioceptor-mediated hyperalgesic priming. We hypothesized this might be through hyperpolarization of the target neuron. We thus examined whether SM-SP induces an outward current to hyperpolarize *Pv*-positive proprioceptors. Whole-cell patch-clamp recordings revealed SM-SP induced an outward current in 82.6% ($n = 23$) of *Pv*-positive DRG neurons, but only in 7.8% ($n = 51$) of *Nav1.8*-positive DRG neurons (Fig. 4C), suggesting that proprioceptor hyperpolarization is the mechanism by which SM-SP blocked the hyperalgesic priming. These results corroborate the crucial role of proprioceptors for hyperalgesic priming.

Since PCCG-13 injection blocked the hyperalgesic priming, we next examined whether activation of proprioceptor ASIC3 is what triggered the priming effect. We therefore tested the effects of ASIC3-specific activation on the acid-induced chronic pain. We injected an

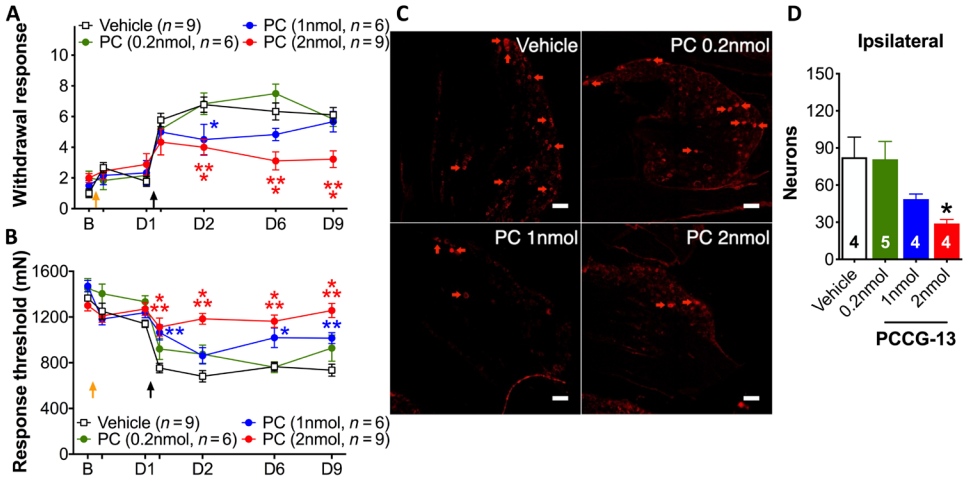

Fig. 3. Involvement of PLD-coupled mGluR in the proprioceptor-mediated pain chronicity. (**A** and **B**) The effect of PLD-coupled mGluR antagonism on CTZ-pH 4.0–induced chronic mechanical hyperalgesia was evaluated in *Pv-LMO3* mice. In the CTZ-acid regime, mice were intramuscularly injected with CTZ with or without PCCG-13 (PC, 0.2, 1, or 2 nmol) followed by an acid (pH 4.0) injection, 1 day later. Orange arrows indicate the time point of CTZ injection, and black arrows indicate the time point of pH 4.0 injection. Mechanical responses were monitored via paw withdrawal responses (A) and muscle withdrawal threshold (B) at different time points (vehicle, $n = 9$; PCCG-13, 0.2 nmol, $n = 6$; 1 nmol, $n = 6$; and 2 nmol, $n = 9$). (**C**) PCCG-13 dose-dependently reduced CTZ-induced pERK expression in sensory neurons of *Pv-LMO3* mice. Immunofluorescence images of pERK expression in L4 sensory neurons were analyzed 5 min after coinjection of CTZ (800 ng) with vehicle ($n = 4$) or different doses of PCCG-13 (0.2 nmol, $n = 5$; 1 nmol, $n = 4$; and 2 nmol, $n = 4$). Red arrows indicate pERK-positive neurons. Scale bars, 100 μm. (**D**) Summary data of pERK-positive neurons. Data are expressed as mean ± SEM and were analyzed by one-way ANOVA (*$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ versus vesicle).

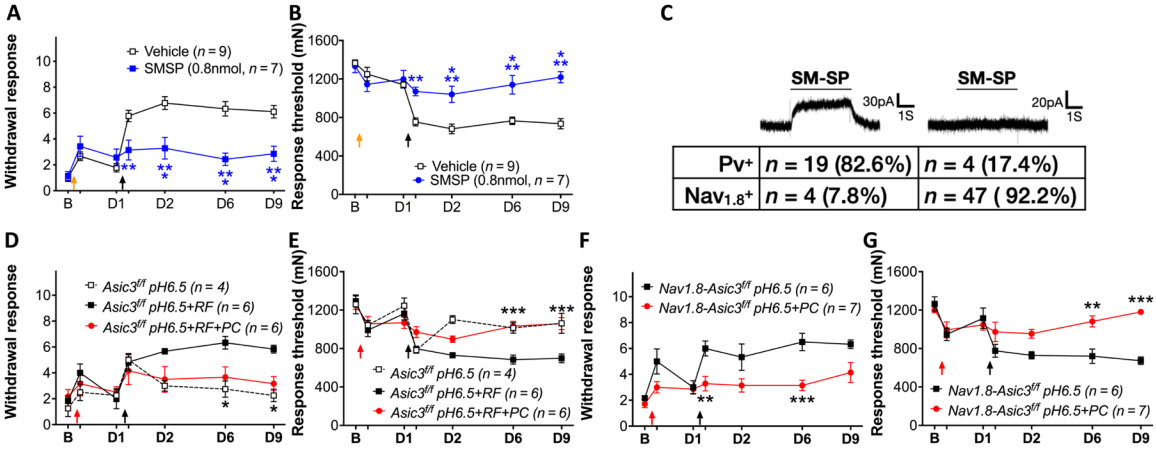

Fig. 4. Effect of *Pv-Asic3* activation in acid-induced sng-pain chronicity. (**A** and **B**) Coinjection of CTZ and [Sar^9, Met(O$_2$)^{11}]-substance P (SM-SP; 0.8 nmol) diminished the CTZ-pH 4.0–induced chronic mechanical hyperalgesia of hind paws (A) and muscle (B) in *Pv-Cre::LMO3* mice. Control group, $n = 9$; and SM-SP group, $n = 7$. (**C**) SM-SP–induced electrophysiological responses resulting from superfusion of 3 μM SM-SP for 4 s in *Pv*-positive and *Nav1.8*-positive DRG neurons. The number of neurons and percentage for each type of responses are shown. (**D** and **E**) In wild-type (*Asic3$^{f/f}$*) mice, dual acid injections of pH 6.5 and pH 4.0 1 day apart did not induce chronic hyperalgesia, whereas injection of pH 6.5 + RPRFamide (0.5 nmol) and pH 4.0 1 day later induced chronic hyperalgesia in hind paws (D) and muscle (E). The effect of pH 6.5 + RPRFamide was inhibited by coinjection of 2 nmol of PCCG-13. *Asic3$^{f/f}$* pH 6.5 group, $n = 3$; *Asic3$^{f/f}$* pH 6.5 + RF group, $n = 6$; *Asic3$^{f/f}$* pH 6.5 + RF + PC group, $n = 6$. (**F** and **G**) In *Nav1.8-Cre::Asic3$^{f/f}$* (as *Nav1.8-Asic3$^{f/f}$*) mice, the dual injections of pH 6.5 and pH 4.0 acidic saline 1 day apart induced chronic hyperalgesia in hind paws (F) and muscle (G), whereas coinjection of PCCG-13 (2 nmol) with the first acid injection significantly inhibited the development of chronic hyperalgesia. Orange arrows indicate the time point of CTZ + SM-SP injection. Red arrows indicate the time point of injection of pH 6.5 + RF (RPRFamide, 500 pmol) in wild-type or pH 6.5 in *Nav1.8-Asic3$^{f/f}$* mice. Black arrows indicate the time point of pH 4.0 intramuscular injection. *Nav1.8-Asic3$^{f/f}$* pH 6.5 + PC group, $n = 7$; all other groups $n = 6$. Data are expressed as mean ± SEM and were analyzed by two-way ANOVA (*$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ versus vehicle control). RF, RPRFamide; PC, PCCG-13.

ASIC3 potentiator, RPRFamide, in solution of a normally nonactivating pH condition, pH 6.5 (*38*). This would selectively activate ASIC3 in different DRG neuron subpopulations. We then tested the effect of a subsequent pH 4.0 acid injection on the development of chronic hyperalgesia. In wild-type (*Asic3$^{f/f}$*) mice, coinjection of RPRFamide in pH 6.5 saline, but not pH 6.5 saline alone, was sufficient for the following pH 4.0 acid injection to induce chronic hyperalgesia. Conversely, including PCCG-13 with the first injection inhibited the ASIC3-specific effect (Fig. 4, D and E). Unexpectedly, in *Nav1.8-Cre::Asic3$^{f/f}$* conditional knockout mice, where ASIC3 is still expressed in proprioceptors but not nociceptors, the first injection of pH 6.5 acid saline was sufficient for the hyperalgesic priming

and the second injection of pH 4.0 acid induced chronic hyperalgesia (Fig. 4, F and G). Again, PCCG-13 inhibited the pH 6.5–induced priming in the *Nav1.8-Cre::Asic3*$^{f/f}$ mice. Also, coinjection of pH 6.5 with either ASIC3 blocker, APETx2, or SM-SP inhibited the priming effect in *Nav1.8-Cre::Asic3*$^{f/f}$ mice (fig. S8, A to D). This suggests that proprioceptor ASIC3 is hypersensitive to acidosis in the context of the absence of nociceptor ASIC3, in which the inhibitory signal of substance P is likely missing. In contrast, in *Pv-Cre::Asic3*$^{f/f}$ mice, neither an acid injection of pH 6.5 saline nor coinjection of pH 6.5 saline containing RPRFamide induced chronic hyperalgesia with a pH 4.0 acid injection the next day (fig. S9, A to D). Moreover, we also tested whether nociceptor ASIC3 activation by the second injection is sufficient for inducing chronic hyperalgesia after proprioceptor-mediated priming. In *Pv-Cre::LMO3::Asic3*$^{f/f}$ mice, after CTZ-induced activation of proprioceptors (priming), coinjection of pH 6.5 saline with RPRFamide at the second injection failed to induce chronic hyperalgesia, whereas combined application of CTZ with RPRF-containing pH 6.5 acidic saline induced chronic hyperalgesia (fig. S10). Therefore, these data are most consistent with a model in which both proprioceptors and nociceptors are engaged in the subsequent development of chronic hyperalgesia, but only after proprioceptor-mediated priming.

Together, these data suggest that activation of proprioceptor ASIC3 mediates acid-induced local glutamate release. The local glutamate will act as an autocrine mediator to activate GluK2 to promote further glutamate release (*31*). This in turn could act as a paracrine mediator to prime nearby nociceptors, probably by activation of a glutamate receptor, such as mGluR5, on the nociceptor terminal (*32*, *39*), promoting the induction of chronic hyperalgesia by a following acid insult (*39*, *40*). Inhibition of the GluK2 receptor on the sensory terminal would inhibit the ASIC3-mediated glutamate release, preventing the paracrine activation of glutamate receptors on the sensory terminal. This would, therefore, block the normal induction of chronic hyperalgesia in the nociceptor (fig. S11).

Revisiting the human studies of "acid-induced pain"

Previous studies have shown acid stimuli induce "pain" in humans in a dose- and flow-dependent manner (*8*–*13*). However, since our studies now show that intramuscular acidosis can activate proprioceptors, we hypothesized that sensing acidosis is also a separate somatosensory function from nociceptive pain in humans. Therefore, we revisited the human studies of "acid-induced pain" to determine whether perception of intramuscular acidosis could be discerned independently (Fig. 5A).

In a randomized, controlled, double-blinded study, we recruited 14 healthy volunteers (nine male and five female) who reported no observable acute or chronic pain before the trial. All volunteers understood that the acid-like or soreness perception (referred as sng in Taiwanese) is distinguishable from pain. The age, gender, physical component summary, mental component summary, and scores on questionnaires for disabilities [Oswestry disability index (ODI)], and anxiety and depression [hospital anxiety and depression scale (HADS)] were not significantly different between the two groups (table S1). Three males and four females received normal pH phosphate-buffered saline (PBS; pH 7.4) injection and six males and one female received acidic PBS (pH 5.2).

The participants indicated that intramuscular injection of pH 5.2 saline to the left tibialis anterior (TA) muscle induced sng but not pain, whereas injection of pH 7.4 saline did not evoke significant sng or pain (Fig. 5B). As the flow rate increased, the pH 5.2 acid injection induced an increase of sng score on the visual analog scale (VAS) (Fig. 5B). We used a general estimate equation (GEE) to find the factor affecting the VAS of sng and pain. This analysis revealed that pH (Wald's $\chi^2 = 8.310$, degrees of freedom = 1, and $P = 0.004$), flow rate (Wald's $\chi^2 = 79.332$, degrees of freedom = 7, and $P < 0.001$),

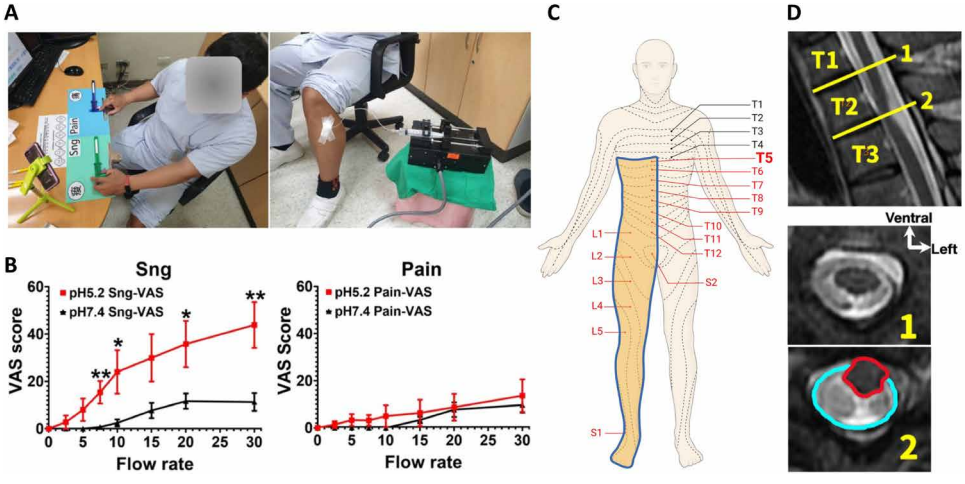

Fig. 5. Segregation of sng from pain in humans. (**A**) The setup of intramuscular acid injection and sng-pain assessment. (**B**) Intramuscular acid injection induces sng but not pain in humans ($n = 7$ in each group). Data presented as mean ± SEM; $^*P < 0.05$ and $^{**}P < 0.01$ versus control. (**C**) A patient with ventral thoracic spinal cord herniation senses nothing but sng and proprioception in his right leg. The sensory examination revealed total sensory loss to light touch, pinprick, and temperature, as well as decreased sensation to vibration below the right T5 dermatome (orange area). In contrast, the motor function and proprioception were well maintained. Of note, the patient was not able to feel deep pain when his right first distal phalanx fractured, but he could feel sng in his right leg. (**D**) The T2-weighted MRI in sagittal view showed the ventral herniation of the spinal cord at T2–3 levels. The axial view at the level of the line marked with a "1" showed the spinal cord at T1–2 level was normally located within the dura matter. The axial view at the level of the line marked with a "2" showed that the ventral part of the spinal cord (red line) was out of the confinement of the dura matter (blue line).

and pH × flow rate (Wald's $\chi^2 = 26.558$, degrees of freedom = 7, and $P < 0.001$) significantly affected the VAS of sng. In contrast, only flow rate (Wald's $\chi^2 = 19.973$, degrees of freedom = 7, and $P = 0.03$) significantly affected the VAS of pain, whereas the pH (Wald's $\chi^2 = 0.662$, degrees of freedom = 1, and $P = 0.416$) and pH × flow rate (Wald's $\chi^2 = 12.004$, degrees of freedom = 7, and $P = 0.062$) did not. Thus, intramuscular acidosis did induce an acid-like (sng) perception that was distinguishable from pain in human volunteers.

A patient with ventral thoracic spinal cord herniation senses sng and proprioception, but no pain, in affected leg

To further address whether the somatosensory sensation of sng can be segregated from nociception, a patient who had prominent sensory loss of nociception and pain due to a spinal cord injury at the level of T2-3 after a car accident was assessed. The patient provided written informed consent in accordance with the Helsinki Declaration before participation, as approved by the Local Institutional Review Board (TMU-JIRB:N20190413).

A 34-year-old male, a native Mandarin and Taiwanese speaker, was found to sense only sng and joint position, but not pain, in his right leg (Fig. 5C and table S2). He presented with severe upper back pain found on a magnetic resonance imaging (MRI) scan to be due to a ventral spinal cord herniation (Fig. 5D). Two years earlier, his right femur bone was fractured in a traffic accident but he recovered well following an operation. He could walk normally and was aware of joint position in his right leg, but he felt no sensation at all on the skin of this right leg. During the recovery period, he did not feel bone pain from the right femur bone fracture. In addition, 1 year after the traffic accident, he was unaware of a right distal phalanx fracture until noticing the toe was swollen.

An MRI of the thoracic spine showed a leftward anterior displacement of the spinal cord at the T2–3 level (Fig. 5D). The left anterolateral column of the spinal cord was out of the confinement of the dura matter at the level of T2–3. The posterior column of the spinal cord was still located within the dura matter without signal changes. On the basis of the clinical presentation and the imaging findings, the diagnosis of thoracic ventral cord herniation was established. A neurological examination revealed that joint position and vibration sensation of knee and hip were preserved bilaterally. He had no muscle weakness or bulk loss of muscle in the right lower limb. The muscle strength of the right leg was in good agreement with that of his left leg, and there was no increase in the deep tendon reflex. Plain radiographies were unremarkable. Notably, there was unilateral total cutaneous sensory loss below the level of the right T5 dermatome, but with full preservation of motor function, joint position sensation, and vibration sensation of knee and hip (table S2). He had total loss of tactile, pinprick, and temperature sensation below the right T5 dermatome, including the right lower limb. The muscle pressure induced sng response in the TA muscle was preserved bilaterally, but the sng response was absent from other muscles of the right leg. It should be noted that the patient was an air conditioning system technician so would likely often put weigh on his TA muscle during normal daily activity.

To further understand whether this patient could still detect sng in the condition of acidosis, we designed a study protocol to conduct intramuscular injection of acidic saline at a fixed flow rate of 40 ml/hour. When the acidic PBS was injected into the patient's right TA muscle, the patient reported neither sng nor pain, with VAS scores of zero for both sng and pain during and after the infusion. In contrast, during the acidic PBS infusion into the left TA muscle, the patient reported a sng VAS of 7 and a pain VAS of 4 of 10. Immediately following the infusion, the participant reported a sng VAS of 5 and a pain VAS of 2. Four minutes after infusion, the participant felt a sng VAS of 3 and a pain VAS of 1 (fig. S12). Following the acidic PBS injection into the right TA muscle, a reduction in the muscle pain pressure threshold (PPT) was observed solely in the ipsilateral leg. However, the acidic PBS injection into the left TA muscle resulted in decreased PPTs in both legs (fig. S13 and table S3). Before the trial, the patient experienced a pressure sensation in the right leg and a sng sensation in the left leg during the muscle PPT test. At the postinfusion stage, the patient reported both pressure and sng sensations in the right leg and a consistent sng sensation in the left leg during the muscle PPT test (table S3). These results suggest the patient's right leg preserved the pressure-induced sng response in acid-sensitized muscle but lost the acid-induced sng response. Moreover, it also lost the pathway to trigger the normal mirror-image muscle pain seen in healthy individuals.

In summary, in his right leg the patient felt neither deep pain due to bone fracture nor touch or temperature sensation. Despite this, he maintained full motor function and proprioception. However, the patient maintained sng response to muscle pressure, especially after acidosis challenge. Anecdotally, he also reported he felt soreness (sng) in the injured leg after a long-distance walk. We conclude that the sensation of muscle soreness is clearly segregated from nociception and other somatosensory sensations in this special case. Thus, this nonpain soreness (sng) sensation could be detected by a spared subcategory of neurons. Alternatively, proprioceptors could be responsible for this somatosensory sensation of sng, a separate sensation, which we propose should be named as sngception.

DISCUSSION

Here, we reveal a specific function of proprioceptors in acid-induced sngception that is required for acid-induced hyperalgesic priming in a mouse model of chronic widespread pain. Further, we report that acidosis perception from muscle is sng (muscle soreness) but not pain. The involvement of proprioceptors in the acid-induced pain chronicity in the chronic widespread pain model with repeated intramuscular acid injections was unexpected. Previous studies have shown that ASIC1b, ASIC3, and TRPV1 are essential for the acid-induced pain chronicity (*5*, *16*, *17*), but not the exact mechanism or neurons responsible. Through a series of genetic, chemo-optogenetic, and pharmacological manipulations in the present study, we provide compelling evidence that it is the ASIC3 of proprioceptors, but not *Nav1.8*-expressing nociceptors, that is required for the acid-induced priming and chronic hyperalgesia. Moreover, that the antinociceptive signaling of substance P acted on proprioceptors rather than on nociceptors. We propose that this proprioceptor-mediated priming is achieved by an axon reflex mechanism of the group Ia terminal, in which acidosis induces exocytosis of glutamate from synaptic-like vesicles (*2*). Anatomically, the encapsulated sensory region of a muscle spindle contains not only the annulo-spiral endings of the group Ia proprioceptor neuron but also often free-ending terminals of group III or IV nociceptive neurons (*41*), which offers a confined region for local signal integration between these two types of nerve endings (fig. S11). Overall, therefore, we propose that it is time to consider a model where acid-induced "nociception" and "pain" should now be classified as a separate sensation of sngception and sng, respectively, in both animal models and human studies.

Previous psychophysical studies of acidosis perception have revealed that ASICs and TRPV1 are potential acid sensors involved in the acid-induced pain. However, the roles of ASICs and TRPV1 are inconsistently reported in different studies. This is possibly due to the different regimes of acidosis delivery and to the different tissues targeted, via intraepidermal, transdermal, and intramuscular routes. For instance, the pain due to intraepidermal pH 6.0 is TRPV1 dependent, whereas the pain due to transdermal pH 6.0 iontophoresis is ASIC-dependent (*8*, *10*). Acid signaling could be pro-nociceptive and pruriceptive in skin afferents and involving TRPV1 and the proton-sensing G protein–coupled receptor, TDAG8 (*14*), whereas intramuscular acid signaling could be either pro-nociceptive, anti-nociceptive, or proprioceptive (or sngceptive), being mediated via ASIC1b/ASIC3/TRPV1, or ASIC1a, or ASIC3, respectively, in these different muscle afferents (*4*, *5*, *16*, *17*, *19*). Although we were unable to apply pharmacological agents in humans to probe the molecular determinants involved in the intramuscular acidosis-induced sng, evidence from our animal studies suggests that it is ASIC3 in proprioceptors that is the sng transducer. This is further supported by the fact that single-cell transcriptomic analyses reveal that *Asic1*, *Asic2*, and *Asic3* are all expressed in human DRG proprioceptors (*40*, *42*). Future microneurography studies are needed to determine whether intramuscular acid injection in humans directly activates A fibers.

Accumulating evidence has shown that ASIC3 is a possible major drug target for chronic pain associated with tissue acidosis. In rodent models, activation of ASIC3 is involved in the development of pain associated with osteoarthritis, rheumatic arthritis, and muscle inflammation (*43*–*47*). In addition, inhibiting ASIC3 promotes neural repair and shortens the duration of long-lasting pain in a mouse model of neuropathic pain induced by a chronic constriction injury of the sciatic nerve (*48*). Because these studies, which used pharmacological inhibition of ASIC3 or a conventional knockout, could not define the cell type–specific roles of ASIC3 in inflammatory pain and neuropathic pain, it would be interesting to determine whether proprioceptor ASIC3 plays important roles in these experimental models. If this reveals that ASIC3 is indeed a/the sng transducer, it will be necessary to then re-examine the clinical pain symptoms of myositis, arthritis, and peripheral neuropathy in line with what we have demonstrated for fibromyalgia and degenerative spine diseases, in which sng and pain are two distinguishable symptoms (*21*, *24*).

The segregation between sng and pain in the spine-injured patient with a spinal cord injury was important for the current study to lead us to reversely interrogate the concept in mouse models, allowing us to determine the causal relationship between proprioceptor activation and sngception. This sngceptive role of proprioceptors echoes previous findings that proprioceptors or large-fiber muscle mechanoreceptors contribute to the development of DOMS after eccentric exercise (*49*, *50*). We propose that this DOMS could refer to the perception of sng. It is notable that intramuscular injection of the ASIC3 antagonist APETx2 was able to inhibit the hypersensitivity of thin-fiber (A-δ and C) afferents in a rat model of DOMS (*51*). Therefore, we cannot exclude the possibility that other somatosensory neurons are also involved in sngception. The segregation of sng and pain is a new concept in biology and requires further investigation in clinical and preclinical studies. We propose that this concept of sngception may open a new frontier of pain biology and may initiate an era of re-examination of pain as we have regarded it in recent decades.

MATERIALS AND METHODS

Animals

All animal care and procedures were followed in accordance with the Guide for the Use of Laboratory Animals (National Academy Press) and approved by the Institutional Animal Care and Use Committee of Academia Sinica. The approval numbers of animal protocols are 19-03-1297, 20-10-1530, and 23-02-1981. All behavioral studies used 8- to 16-week-old female mice. *Asic3*$^{f/f}$ mice were generated as previously described (*4*). *Asic1a*$^{-/-}$, *Asic1b*$^{-/-}$, and *Asic2a*$^{-/-}$ mice were bred from *Asic1a*$^{+/-}$, *Asic1b*$^{+/-}$, and *Asic2a*$^{+/-}$ mice, respectively, as previously described (*16*, *25*, *52*). *Pv*-Cre mice (stock no. 008069) were purchased from Jackson lab and the *Nav1.8*-Cre mice were a gift from J. Wood of University College London, UK (*53*). The eGFP reporter mice (CAG-CAT-EGFP Tg, CARD ID. 509) were purchased from CARD-R-base, Kumamoto University, Japan. Mice were housed in groups of 3 to 5 with ad lib access to chow diet and water in a specific pathogen–free animal facility with a 12-hour light/dark cycle at the Institute of Biomedical Sciences, Academia Sinica. All possible efforts were made to minimize the number of mice used and their suffering without compromising the quality of the experiments.

Generation of *Asic2a*$^{-/-}$ mice

ASIC2a is encoded by the Accn1 gene. We generated the *Asic2a*$^{-/-}$ mouse line by direct application in mouse zygotes of CRISPR-Cas9 technology (*25*). In brief, we injected Cas9 RNA with two single-guide RNAs (sgRNAs) into the nucleus of mouse zygotes. One sgRNA targeted the 5′-upstream of exon 1 and the other one the 3′-downstream of exon 1. After oviduct embryo transfer, we screened the mouse with fully deletion of exon1-a of Accn1 by polymerase chain reaction (PCR) with the following primers: ASIC2a-WT forward, 5′-GAGAGCTCGGAGAGAGTATCCTAC-3′; ASIC2a reverse, 5′-TGACAAGATGTTTCTGTCACACG-3′; and ASIC2a-KO forward, 5′-TCTACTGTAGGGTCTGCACACATG-3′. The PCR product sizes of wild-type and knockout mice are 731 base pairs (bp) and 438 bp, respectively.

Generation of LMO reporter mice

The construct of LMO3 was generated by U. Hochgeschwender as previously described (*30*). We added a loxP-stop cassette between human syntexin promoter and LMO3-YFP by Kpn1. For homologous recombination, the construct was insert between 3K of 5′ upstream and 1K of 3′ downstream of hipp11. Germ-line transmission was screened by the following primers: LMO3-Ben-F, 5′-GATCAGGGCAGTCTGGTACTTC-3′; LMO3-Ben-R, 5′-CCCACCAGCCTTGTCCTAATAAA-3′; and LMO3-Ben-RR, 5′-GTTTGACACATCCTGCCCTTAC-3′. The wild-type and transgender amplicons are 180 and 550 bp, respectively.

Reagents

All compounds were prepared in stocks of high concentration. The water-soluble luminescent enzyme substrate, CTZ (#3031, Nanolight Technologies, AZ, USA), was prepared in normal saline (stock, 4 mg/ml) and stored at −80°C. PCCG-13 is a selective antagonist of the PLD-coupled mGluR, a metabotropic GluK2 receptor, made by R. Pellicciari and M. Marinozzi in Università degli Studi di Perugia, Italy (*33*). The stock of PCCG-13 (10 mM) was dissolved in dimethyl sulfoxide and stored at −20°C. The membrane impermeable blocker of voltage-gated sodium channel, QX314, was dissolved in distilled water (stock, 100 mM; #552233, Sigma-Aldridge, MO, USA). The

recognized protein of Toll-like receptor 5, Flagellin, was dissolved in distilled water (stock, 0.5 mg/ml; #tlrl-pbsfla, InvivoGen, CA, USA). APETx2, an ASIC3-selecive antagonist, was dissolved in distilled water (stock: 200 μM, #STA-160, Alomone Labs, Jerusalem, Israel). RPRFamide, an ASIC3 potentiator, was prepared in stock (200 μM in distilled water) as previously described (*38*). The neurokinin 1 receptor (NK1R)–selective agonist SM-SP (#S3672, Sigma-Aldridge, MO, USA) was prepared in stock (200 μM in distilled water) and stored at −20°C.

Behavioral assays

All behavioral studies were performed between 9 a.m. and 3 p.m. A mouse model of chronic widespread pain, also considered a model of fibromyalgia, and named the Sluka model, was used, in which chronic widespread muscle pain was induced by dual intramuscular injections of pH 4.0 acidic saline (*5*). The acidic saline was prepared in 10 mM 2-[*N*-morpholino]ethanesulfonic acid and adjusted to pH 4.0 with 1 N NaOH. Two injections of 20 μl of pH 4.0 acidic saline were administrated to unilateral gastrocnemius muscle, spaced at 1 or 5 days, by which the first acid injection normally induces transient mechanical hypersensitivity of bilateral hind paws and muscle lasting less than 24 hours, whereas a second acid injection induces a long-lasting hyperalgesia lasting for more than 3 weeks. For CTZ-acid experiments, CTZ was diluted in 20 mM HEPES-buffered normal saline in pH 7.4. Intramuscular injection of CTZ was followed by injection of pH 4.0 saline 1 day later. For CTZ-CTZ experiments, dual intramuscular injection of CTZ 1 day apart was performed. A second mouse model of fibromyalgia was induced by ICS as previously described (*7*, *27*).

Pain behavioral assays were conducted in a double-blinded manner, by which the experimenters did not have the information of genotypes and drug treatments. Before the testing, mice were acclimated in an acrylic cubicle for 60 min. Withdrawal responses of hind paws were assessed by applying a 0.2 mN von Frey filament to the plantar surface of both hind paws. Mice received 5 von Frey stimuli in each paw at 30-s intervals. A positive response was defined as foot lifting in each von Frey filament stimulation. Muscle withdrawal thresholds were assessed by using the Pressure Application Measurement system (Ugo Basile, VA, Italy) with a modified cone-shape rounded probe (2.23 mm diameter) to the ipsilateral gastrocnemius muscle.

Chemo-optogenetics

Optogenetic techniques have been developed as an ideal tool to selectively activate or inhibit specific neuron populations and/or cell types in the central nervous system. However, the application of optic stimuli to peripheral nerves is challenging and limited, because peripheral nerves are difficult to target in free-moving animals. A solution is to combine the optogenetic and chemogenetic approaches to allow the use of both modes of interrogation via the same transducer molecules in the same neurons. A chemo-optogenetic tool was thus developed by fusing a luciferase gene to a channelrhodopsin gene (named lumiopsin or LMO3), by which the channelrhodopsin moiety can be activated directly by light and activated chemically by the luciferase substrate, CTZ (*30*).

Immunohistochemistry

We used an ERK phosphorylation response to monitor neural activity of the DRG. Mice received 1 to 2% isofluorane for about 30 min to stabilize body physiological functions. After 1, 3, and 5 min of a bolus injection into gastrocnemius muscle, mice were immediately fixed with 4% paraformaldehyde. Lumbar L4 DRGs were collected, dehydrated with 30% sucrose for 1 day and embedded in tissue-freezing medium for frozen sections. Twelve-micrometer-thick sections were collected every other 72 μm, then incubated with blocking solution (50 mM tris-buffered saline containing 5% bovine serum albumin, 0.1% Triton X-100, and 0.02% sodium azide) for 2 hours, in room temperature. The primary antibody, rabbit-anti pERK (1:200, #9101, Cell Signaling Technology, MA, US), was diluted in blocking solution and incubated with sections overnight at 4°C. The secondary antibodies, Alexa Fluor 594(ab′)2 fragments of goat anti-rabbit (1:250, A11072, Thermo Fisher Scientific, MA, US) were also diluted in blocking solution and incubated with sections for 1 hour at room temperature.

DRG primary culture

Lumbar DRGs of *Pv-Cre::TdTomato* or *Nav1.8-Cre::TdTomato* mice were collected in Ca^{2+}/Mg^{2+} free Hank's balanced salt solution (Thermo Fisher Scientific, MA) and the DRG samples were digested with 0.125% (w/v) collagenase IA (Sigma-Aldrich, MO) for 70 min and 0.125% (v/v) trypsin (Thermo Fisher Scientific, MA) for 15 min at 37°C. The digested DRG samples were triturated and plated on poly-L-lysine–coated coverslips. After 1 hour, the DRG-seeded coverslips were cultured in 3.5-cm petri dish with Dulbecco's minimum essential medium plus 10% (v/v) fetal bovine serum (Thermo Fisher Scientific, MA), 1% (v/v) penicillin/streptomycin (Thermo Fisher Scientific, MA), and were maintained in a 5% CO_2 to 95% O_2 (v/v) incubator at 37°C.

Whole-cell patch-clamp recording

All recordings were performed within 30 hours after seeding of DRG neurons. Whole-cell patch clamp recordings of DRG neurons were made with an Axopatch MultiClamp 700B (Axon Instruments, CA) at room temperature (22° to 25°C). Only neurons with a membrane potential more negative than −40 mV were assessed. The bridge was balanced in current-clamp recording mode, and the series resistance was compensated 70% in voltage-clamp recording mode with Axopatch 700B compensation circuitry. The patch pipettes had a resistance of 6 to 10 megohms and were filled with an internal solution containing 100 mM KCl, 2 mM Na_2–adenosine 5′-triphosphate, 0.3 mM Na_3–guanosine 5′-triphosphate, 10 mM EGTA, 5 mM $MgCl_2$, and 40 mM HEPES, adjusted to pH 7.4 with KOH, with an osmolarity of 290 to 300 mOsm/liter. Neurons were superfused with artificial cerebrospinal fluid (ACSF) with flow controlled by gravitational force. The ACSF contained 130 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 10 mM glucose, and 20 mM HEPES adjusted to pH 7.4 with 5 N NaOH, with an osmolarity of ~300 mOsm/liter. The SM-SP–induced currents were recorded in the voltage-clamp mode and neurons were held at −70 mV. SM-SP in ACSF solution (3 μM) was puffed for 4 s at 30-s intervals through a theta pipette 100 μm away from the recorded neuron and controlled by a VC-6 six-channel valve controller (Warner Instruments, Hamden, CT) via gravity, with the fast change of the solution enabled by using a fast-stepper (SF-77C, Warner Instruments).

In situ hybridization

The RNA probes for *Pv* (catalog no. 421931), *Nav1.8* (catalog no. 426011), and *Asic3* (catalog no. 480541) were purchased from Advance Cell Diagnostics and used according to the manufacturer's instructions

of the RNAscope fluorescent multiplex reagent kit (Advanced Cell Diagnostics, US). In brief, 4% paraformaldehyde-fixed L4 DRGs were sectioned in12 μm thickness and stored at −80°C for further use. Before in situ hybridization, sections were pretreated with antigen retrieval and proteinase III digestion. Probes were hybridized at ~40°C for 2 hours in HybEZ hybridization system (catalog no. 321711). Fluorescent images of sections were acquired using a confocal microscope (LSM 700 stage, Zeiss, Germany). Three to four images of each genotype that lacked evidence of folding or breaks were randomly chosen and quantified.

The causal connection of sng and muscle acidosis: A randomized double-blinded control trial

Participant selection

We designed a randomized double-blinded control trial to establish the causal connection of sng perception and muscle acidosis. All participants provided written informed consent in accordance with the Helsinki Declaration before participation, as approved by the Local Institutional Review Board (TMU-JIRB: N201909038). The subjects were evaluated by RAND-36 (*54*), ODI (*55*), and the HADS (*56*). The subjects were selected according to the following inclusion and exclusion criteria.

Inclusion criteria

1) The subject age ranges from 20 to 40 years old.

2) The subject has no chronic pain symptoms or complaint in last 6 months.

3) The subject is subjectively able to discriminate sng and pain.

4) The subject has no history of major diseases that required treatment or were currently under treatment.

Exclusion criteria

1) The subject is unable to reliably delineate or assess his or her own pain by anatomical location/distribution.

2) The subject has undergone lumbar spine surgery within the last 6 months or has received treatment with epidural injections, nerve blocks, or acupuncture within 4 weeks before screening.

3) The subject had a history of lower extremity injury or neuropathic pain or malignancy or alcohol or narcotic substance abuse according to his/her report.

4) The subject is pregnant or breastfeeding at the time of the screening visit or plans to become pregnant during the study period.

Randomization and double-blinding procedures

Stratified randomization based to gender was processed by using Excel (Microsoft) and participants were randomly assigned to the acid (pH 5.2 PBS) or control (pH 7.4 PBS) group. The acid and neutral PBS were prepared in a vial form by the pharmacy of Taipei Medical University Hospital. In total, 20 vials were prepared with one half containing acid PBS and the other half containing normal PBS. These vials were arranged in a sequence determined by the randomization process mentioned above. Only the pharmacy knew the sequence of these 20 vials. The label on the vial showed its sequential number (first, second, third, fourth, ….) without any other information about its content. The subject received the solution injection following the order of the sequential number shown on the vials. The principal investigator or the study nurse who did the injection or the subject or the film reader did not know the content of the vial injected.

Acid and normal PBS preparation

The sterile PBS was prepared by the Clinic Pharmacy of Taipei Medical University Hospital and stored as sterile solutions in 30-ml syringes. Two types of PBS were prepared: acidic (pH 5.2, 140 mM) following the method described by Law *et al.* (*11*), and neutral (pH 7.4, 140 mM) using 0.367 g of monobasic sodium phosphate monohydrate and 3.033 g of dibasic sodium phosphate heptahydrate diluted to 100 ml with sterile water. Both PBS solutions had an osmolality of 300 ± 20 mOsm/kg H_2O.

Intramuscular infusion protocol (I)

The subjects were not allowed to do intensive exercise 1 day before the trial. Before intramuscular infusion, the differences of pressure pain thresholds of TA muscle between both legs were less than 50 mmHg in all participants and none of them reported any pain or sng at their legs. Each subject received 30 min intramuscular infusion into the mid-belly of the TA muscle with a vial containing acidic PBS (pH 5.2) or normal PBS (pH 7.3) at eight different flow rates from 0 to 30 ml/hour. Both the participant and the treatment giver were blinded to the pH of the solution infused. The subject simultaneously expressed their VAS for sng and pain on two verniers during the intramuscular infusion. The sng VAS was previously validated in a cohort of patients receiving spine surgery (*21*). There was 0 to 100 mm on the vernier representing a numeric pain rating scale ranging from 0 to 100. The two verniers allowed the subjects to change the intensity of VAS immediately whenever they felt the intensity of sng or pain changed. We filmed the whole course of VAS changes against different flow rates. The flow rate started from 0, then increased in order of 2.5, 5, 7.5, 10, 15, 20, and 30 ml/hour. After the rate of 30 ml/hour, the flow rate decreased in the order reverse. Each flow rate lasted for 2 min. In total, 2.5 ml of solution was infused in a trial.

Intramuscular infusion protocol (II)

We conducted a functional MRI (fMRI) study approved by Taipei Medical University Hospital (TMUJIRB: N201902030), using an acid injection to investigate the brain areas activated in association with sngception. A patient with a painless right leg participated in this study. The patient received an acid injection in the painless right leg first, and after 1 month, received an acid injection in the healthy left leg. To ensure a robust response for sngception, we used a fixed flow rate of 40 ml/hour instead of a ramp-up flow rate protocol. The trial was conducted in a controlled fMRI room environment and lasted approximately 40 min.

The following protocols were used:

1) Initial assessment: The participant underwent a muscle pressure pain threshold test on the bilateral TA muscles, performed three times on each side.

2) Preparation: The participant lies down on a bed. After recording the initial sng pain VAS score, a soft needle was inserted into the right or left TA muscle. A second sng pain VAS score was then immediately taken to ensure that there was no discomfort due to the needle insertion alone.

3) Baseline measurement: After waiting a further period of about 10 min, a third sng pain VAS score was recorded to confirm the absence of sng and pain responses before the injection. If pain/sng was reported, the procedure was stopped at this point. Only those reporting no pain nor sng were taken to the injection stage (stage 4).

4) Injection protocol: An acidic PBS of pH 5.2 was injected into the participant's TA muscle at a steady rate of 40 ml/hour. Ten seconds after the injection began, the participant reported a fourth sng pain VAS score. The infusion of the acidic PBS lasted for 8 min, totaling

5.3 ml. Midway through the infusion, a fifth sng pain VAS score was recorded. Upon completion of the infusion, the participant reported the sixth sng pain VAS score.

5) Post-infusion assessment: The seventh and eighth sng pain VAS scores were recorded at 4 and 15 min post-infusion, respectively.

6) Final assessment: At the end of the procedure, muscle pressure pain thresholds on the bilateral TA muscles were measured again.

Data presentation and statistical analysis

All data are presented as mean ± SEM. Mouse behavioral data were analyzed by using two-way analysis of variance (ANOVA) with Sidak's or Dunnett's correction, or one-way ANOVA with Dunnett's multiple comparison test correction (table S4). An unpaired t test was used to compare the VAS of sng or pain between the acidic and normal PBS group at different flow rates.

The whole course of trials was filmed and after the end of the trial, the film was reviewed for the VAS for sng and pain, and the relevant flow rates were noted per each 10 s. The film reviewer was also blinded to the pH of solution. The VAS for sng and pain at any particular flow rate was presented as an average. Thereafter, for each subject, there were eight different average VASs for sng and pain at the eight different corresponding flow rates. Descriptive statistics (mean and SEM) were calculated for age, SF36, ODI, BDI, and VAS for sng and pain. A GEE, using a general linear regression model, was used to test whether acidic pH or flow rate affected the VAS of sng and pain.

Supplementary Materials

This PDF file includes:

Figs. S1 to S13
Tables S1 to S4

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