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# Roles of ASIC3, TRPV1, and Na<sub>v</sub>1.8 in the transition from acute to chronic pain in a mouse model of fibromyalgia

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## Abstract

**Background:** Tissue acidosis is effective in causing chronic muscle pain. However, how muscle nociceptors contribute to the transition from acute to chronic pain is largely unknown.

**Results:** Here we showed that a single intramuscular acid injection induced a priming effect on muscle nociceptors of mice. The primed muscle nociceptors were plastic and permitted the development of long-lasting chronic hyperalgesia induced by a second acid insult. The plastic changes of muscle nociceptors were modality-specific and required the activation of acid-sensing ion channel 3 (ASIC3) or transient receptor potential cation channel V1 (TRPV1). Activation of ASIC3 was associated with increased activity of tetrodotoxin (TTX)-sensitive voltage-gated sodium channels but not protein kinase C $\epsilon$  (PKC $\epsilon$ ) in isolectin B4 (IB4)-negative muscle nociceptors. In contrast, increased activity of TTX-resistant voltage-gated sodium channels with ASIC3 or TRPV1 activation in Na<sub>v</sub>1.8-positive muscle nociceptors was required for the development of chronic hyperalgesia. Accordingly, compared to wild type mice, Na<sub>v</sub>1.8-null mice showed briefer acid-induced hyperalgesia (5 days vs. >27 days).

**Conclusion:** ASIC3 activation may manifest a new type of nociceptor priming in IB4-negative muscle nociceptors. The activation of ASIC3 and TRPV1 as well as enhanced Na<sub>v</sub>1.8 activity are essential for the development of long-lasting hyperalgesia in acid-induced, chronic, widespread muscle pain.

**Keywords:** Acidosis, APETx2, Hyperalgesic priming, IB4, PKC $\epsilon$

## Background

Chronic muscle pain is a significant clinical problem affecting many people [1]. Although both peripheral and central sensitizations are believed involved in the transition from acute to chronic muscle pain, the underlying mechanism is not well understood [2-4]. An emerging hypothesis of hyperalgesic priming proposed by Jon Levine is that the transition might involve neural plasticity in primary afferent nociceptors, whereby an acute noxious stimulation can trigger long-lasting hypersensitivity of nociceptors to subsequent insults [5]. The hyperalgesic priming phenomenon occurs in a specific subset of nociceptors

that bind isolectin B4 (IB4) and requires increased activity of tetrodotoxin (TTX)-resistant sodium channels and a switch in intracellular signaling pathways from protein kinase A to the epsilon isoform of protein kinase C (PKC $\epsilon$ ) in response to the same stimulus [6-8].

Tissue acidosis in muscles related to ischemia and inflammation has a profound effect on the initiation and development of chronic muscle pain [9,10]. Proton-sensing ion channels, such as acid-sensing ion channel 3 (ASIC3) and transient receptor potential cation channel V1 (TRPV1), are involved in activating muscle nociceptors and inducing the central sensitization that occurs in animal models of chronic muscle pain [11-13]. To probe how acid triggers chronic muscle pain, Sluka and colleagues developed a rodent model of chronic muscle pain induced by acid saline injected twice, 5 days apart, to the gastrocnemius muscle (GM) on one side of mice or rats [14,15].

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The first acid injection triggers a transient referred hyperalgesia in both hind paws that diminishes in 24 h. The second acid injection 5 days later to the same side induces long-lasting referred hyperalgesia. Furthermore, although the dual acid injections are unilateral in the same site, the hyperalgesic effects are bilateral and the induction of chronic widespread pain shifts the autonomic balance to sympathetic predominance and decreases baroreflex sensitivity, which are related to findings in humans with chronic widespread pain or fibromyalgia [16].

The requirement of dual acid injections to evoke chronic muscle pain implies a hyperalgesic priming of muscle nociceptors after the first acid injection. Although proton-sensing ion channels are implicated in the initiation of hyperalgesia induced by intramuscular injections, only ASIC3 was confirmed to play an essential and sufficient role in triggering the acid-induced chronic muscle pain [15,17]. However, how ASIC3 induces the hyperalgesic priming is not known and whether TRPV1 is involved in the acid-induced, chronic, widespread pain is not clear.

In this study, we aimed to reveal the molecular mechanism underlying the transition from acute to chronic muscle pain in the acid-induced, chronic, widespread pain model. We tested how ASIC3 and/or TRPV1 activation affects the duration of the hyperalgesic priming state and the chronic pain state induced by dual intramuscular acid insults.

## Results

### Neural subgroups of acid-sensitive muscle afferent DRG neurons

Although the expression of ASIC3 and its functional implications have been characterized in muscle nociceptors, the response of TRPV1-expressing muscle nociceptors to acid is poorly addressed. We first analyzed acid-induced currents in muscle afferent dorsal root ganglion (DRG) neurons and determined the neural populations of ASIC3- and TRPV1-expressing muscle nociceptors by inhibition with salicylic acid (SA, ASIC3 antagonist) and capsazepine (TRPV1 antagonist) [18,19]. Whole-cell patch clamp recording revealed that most of the small- to medium-sized (20–40  $\mu\text{m}$  in diameter) muscle afferent DRG neurons expressed acid-induced inward currents (34/40), including 17.5% (7/40) ASIC3-like currents, 10% (4/40) TRPV1-like currents, and 7.5% (3/40) ASIC3-/TRPV1-like currents (Figure 1).

### Involvement of TRPV1 and ASIC3 in the establishment of hyperalgesic priming

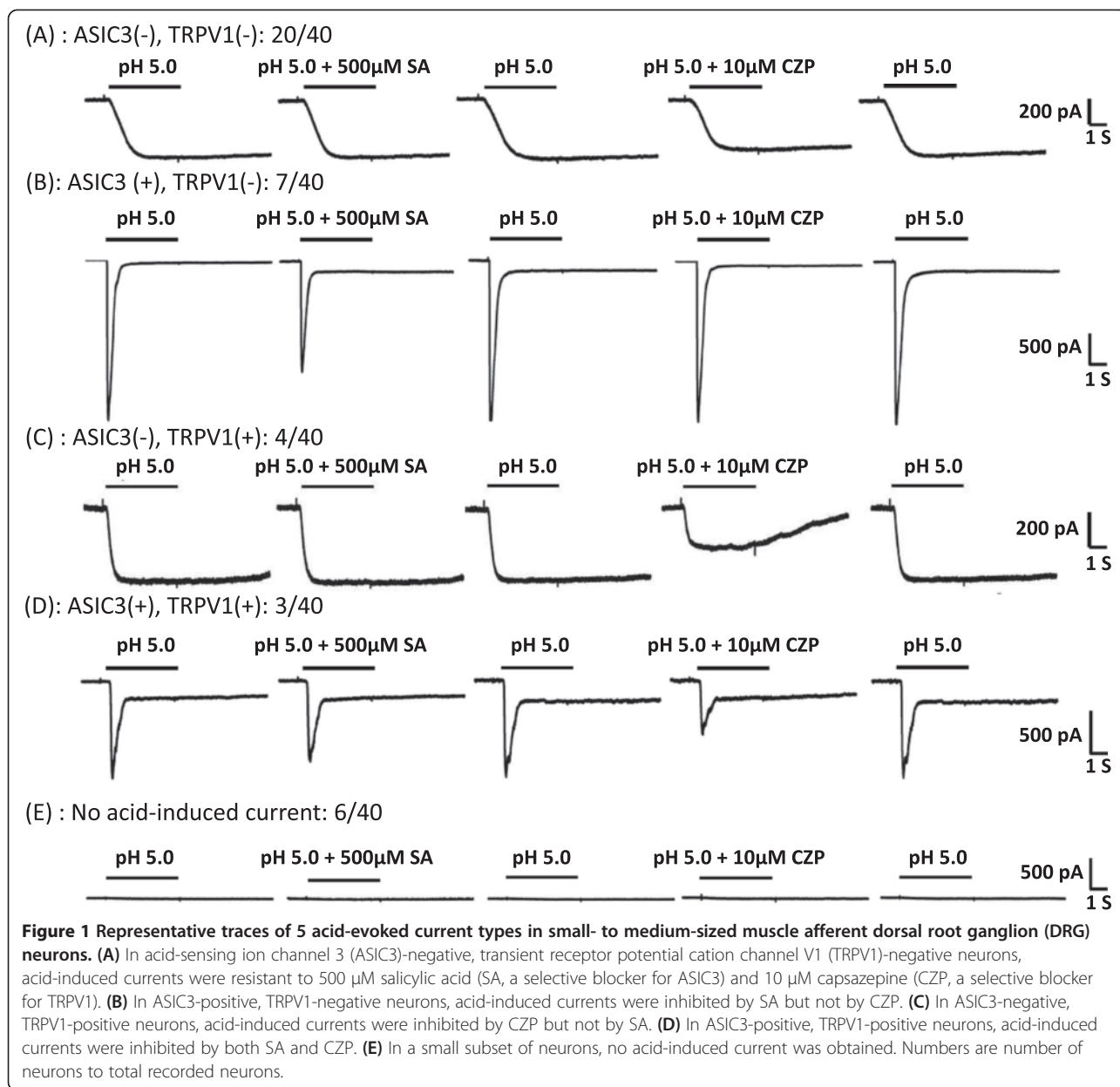
We next examined whether deletion or inhibition of TRPV1 would affect the intramuscular-acid-induced hyperalgesia. *Trpv1*<sup>-/-</sup> mice showed transient hyperalgesia after the first and second acid injections spaced 5 days apart but failed to show long-lasting hyperalgesia after the second acid injection as did *Trpv1*<sup>+/+</sup> mice (Figure 2A

and B). Interestingly, inhibiting TRPV1 by co-injection of acid and capsazepine at the first injection or at the second injection did not affect the development of long-lasting hyperalgesia (Figure 2C and D). Only co-injection of acid with capsazepine in both injections abolished the development of long-lasting hyperalgesia and produced an exact phenocopy of the *Trpv1* gene deletion (Figure 2E). These results suggest a role for TRPV1 in mediating the hyperalgesic priming and hypersensitivity of primed nociceptors. Although TRPV1 inhibition at the first acid injection did not abolish the hyperalgesic priming, it shortened the duration of the long-lasting hyperalgesia induced by the second acid-alone injection (Figure 2F and G). TRPV1 activation at the first acid injection may be required for establishing nociceptor priming, which is important for maintaining long-lasting hyperalgesia induced by a second acid insult.

In contrast, *Asic3*<sup>-/-</sup> mice showed neither transient nor long-lasting hyperalgesia with dual intramuscular acid injections spaced 5 days apart (Figure 3A, B). We next used a pharmacological approach to probe the role of ASIC3 in the hyperalgesic priming. With co-injection of acid with APETx2 (2 or 20 pmole), a selective ASIC3 antagonist [20], at the first injection, the transient hyperalgesia was not evoked; a second acid injection on day 5 evoked only transient hyperalgesia, which suggests that the nociceptors were in an unprimed state (Figure 3C). Co-injection of acid with APETx2 (20 pmole) at the second injection induced transient but not long-lasting hyperalgesia (Figure 3D). Therefore, the primed nociceptors require ASIC3 activation for developing chronic hyperalgesia in the dual acid-injection scheme.

### The duration of hyperalgesic priming

Although we did not observe nociceptor priming on day 5 when APETx2 inhibited ASIC3 at the first acid injection, we cannot exclude that a shorter duration of hyperalgesic priming was evoked with TRPV1. Thus, we tested whether activating TRPV1 only (by inhibiting ASIC3 with APETx2) could still contribute to short-term hyperalgesic priming in muscle nociceptors, if the dual acid injections were administered less than 5 days apart. With the dual acid injections administered 1 day apart, the second acid injection produced a robust long-lasting hyperalgesia for more than 12 days as compared with the basal responses before the second injection; however, the hyperalgesia lasted for 7 days with co-injection of acid with APETx2 (20 pmole) at the first injection (Figure 3E and F). The next-day acid-injection-induced hyperalgesia could still last for 3 days even with a higher dose of APETx2 (200 pmole) (Figure 3G). Interestingly, with co-injection of acid and APETx2 (20 pmole) and capsazepine, the response was totally blunted to the next-day acid injection (Figure 3H). Thus, TRPV1 and ASIC3 are the major proton-sensing



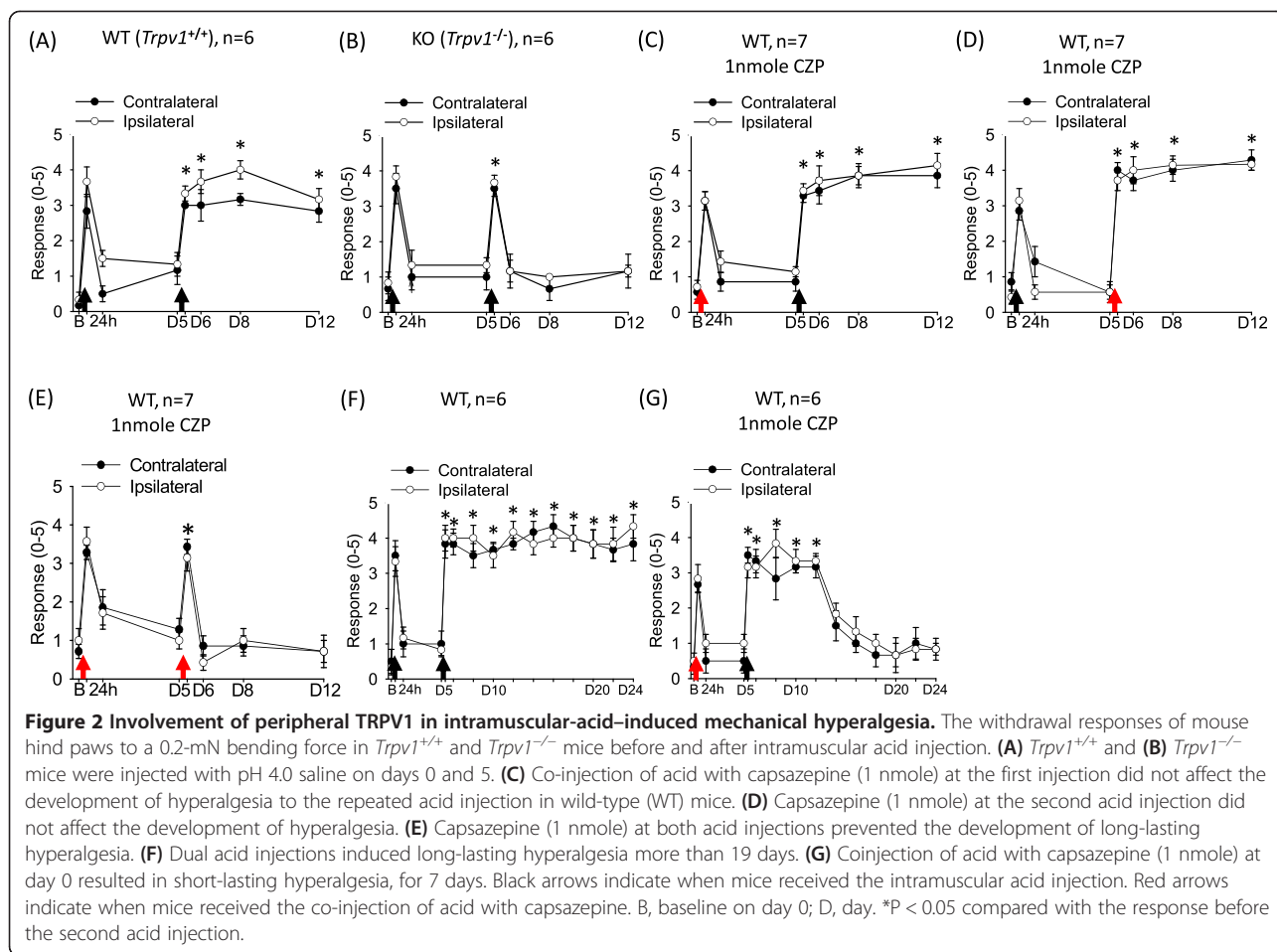
ion channels in muscle nociceptors responsible for acid-induced hyperalgesic priming and hyperalgesia. TRPV1 could play a central role together with ASIC3 in the acid-induced hyperalgesic priming. We further validated this concept by finding that co-injection of acid with capsazepine shortened the duration of the long-lasting hyperalgesia induced by the second acid injection (Figure 3I).

We further tested the contribution of activating only TRPV1 or ASIC3 in the dual acid injections spaced 2 days apart (Figure 3J-L), and the results were very similar to the dual acid injections spaced 1 day apart. Thus, both ASIC3 and TRPV1 channels may contribute to the acid-induced hyperalgesic priming of muscle nociceptors, but both have a different contribution to the duration of the first acid

injection-induced nociceptor priming and the maintenance of long-lasting hyperalgesia induced by the second acid injection (Table 1).

#### ASIC3 and TRPV1 activation enhanced TTX-sensitive (TTXs) and TTX-resistant (TTXr) voltage-gated sodium current ( $I_{NaV}$ ) in muscle nociceptors

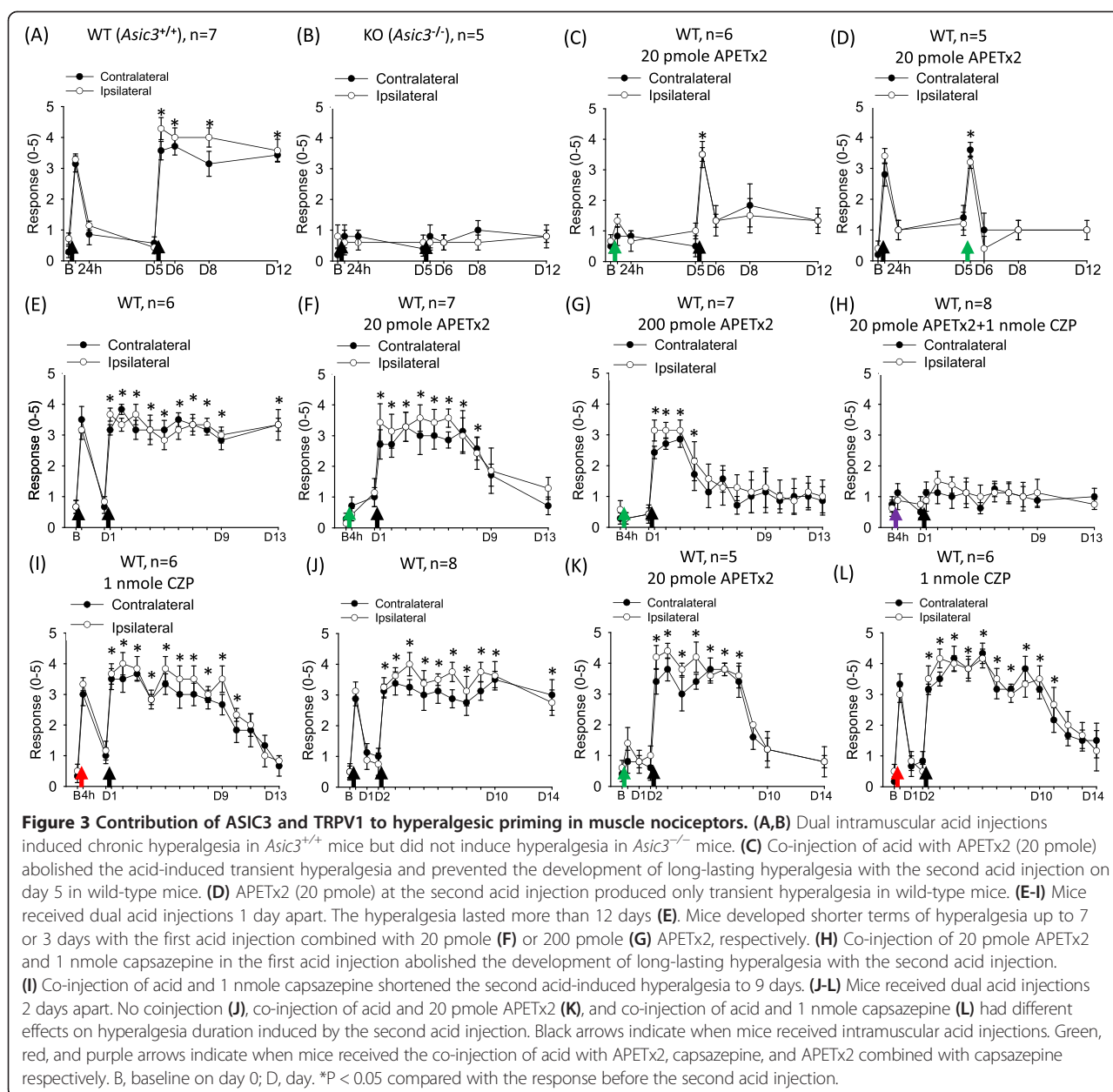
We next probed whether the ASIC3- and TRPV1-mediated hyperalgesic priming in muscle nociceptors resulted from  $I_{NaV}$  as seen in inflammation-induced hyperalgesic priming [7]. We analyzed the peak amplitudes of  $I_{NaV}$  in 2 types of muscle afferent DRG neurons, TTXr and non-TTXr neurons, on the basis of existence of a TTXr current (Figure 4A). We thus compared the  $I_{NaV}$  in muscle afferent



DRG neurons at 2 or 5 days after an intramuscular injection of pH 7.4 saline or acid (pH 4.0 saline) with or without APETx2 or capsazepine (Figure 4B). As compared with pH 7.4 saline injection, at 2 days after acid injection, non-TTXr GM DRG neurons showed significantly enhanced TTXs  $I_{NaV}$ . However, co-injection of acid and APETx2 or capsazepine did not reverse the acid-induced increase in TTXs  $I_{NaV}$  (Figure 4C). In contrast, although TTXr GM DRG neurons did not differ in the TTXs component of  $I_{NaV}$  with treatment, TTXr  $I_{NaV}$  was significantly increased with acid injection, and inhibition of ASIC3 or TRPV1 effectively reversed the acid-induced effect (Figure 4D). In non-TTXr GM DRG neurons, enhanced TTXs  $I_{NaV}$  was maintained with acid only or co-injection with capsazepine but not co-injection with APETx2 5 days after acid injection (Figure 4E), which suggests that inhibition of ASIC3 would shorten the duration of hyperalgesic priming. In TTXr GM DRG neurons, the enhanced TTXr  $I_{NaV}$  was still maintained with acid-only treatment 5 days after acid injection (Figure 4F). Taken together, the acid-induced plastic changes of nociceptors occurred in both non-TTXr and TTXr GM DRG neurons, with a significant increase of  $I_{NaV}$  in both muscle nociceptor populations.

### Subtypes of ASIC3-expressing muscle nociceptors

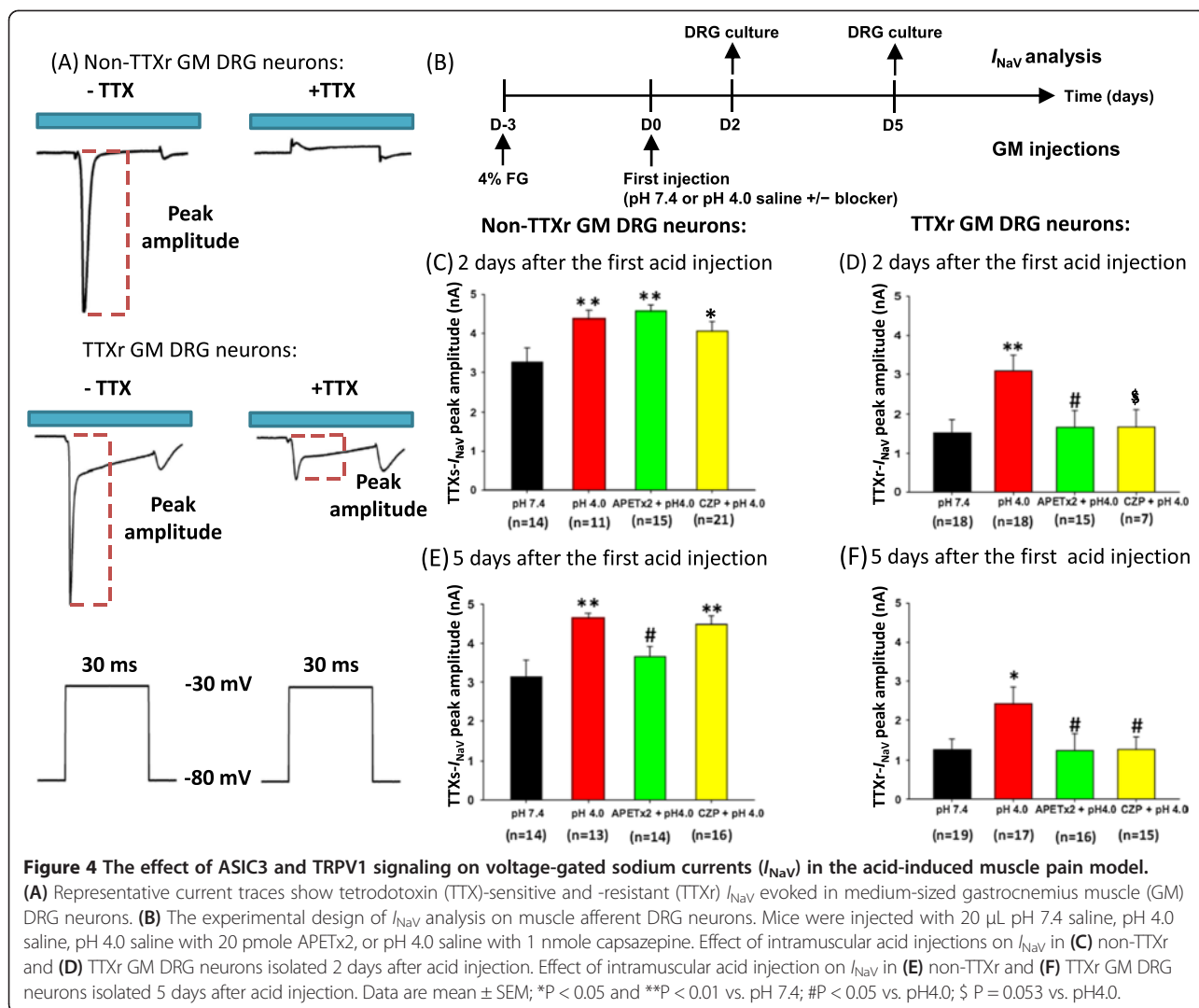
To better understand the cell-type-specific effects of ASIC3 and TRPV1 activation on TTXr  $I_{NaV}$ , we used genetic tools to analyze how these proton-sensing ion channels related to  $Na_v1.8$  expression and whether they were expressed in IB4-positive neurons that are essential for hyperalgesic priming. We first examined the co-expression of  $Na_v1.8$  and IB4 in muscle afferent DRG neurons from mice that carry a  $Na_v1.8$ -Cre allele to drive the GFP reporter allele (Figure 5). Among 1,004 muscle afferent DRG neurons (from 3 mice), 317 (31.6%) were  $Na_v1.8$ -positive. Among the  $Na_v1.8$ -positive muscle afferent DRG neurons, 64% (203/317) were co-localized with IB4, and the other 36% (114/317) were not. Because  $Na_v1.8$  contributes the most to TTXr  $I_{NaV}$  in DRG neurons, we next analyzed the acid-induced currents in  $Na_v1.8$ -expressing GM DRG neurons. Before electrophysiological recordings, cultured DRG neurons were stained with IB4-DyLight to determine whether the  $Na_v1.8$ -expressing GM DRG neurons were IB4-positive or -negative. We categorized the muscle afferent DRG neurons into 2 cell-sized groups, small-sized (20–30  $\mu$ m in diameter) and medium-sized (30–40  $\mu$ m in diameter),



**Table 1 Effect of acid-sensing ion channel 3 (ASIC3) and transient receptor potential cation channel V1 (TRPV1) inhibition on maintenance of hyperalgesia induced by dual acid injections**

First injection	Maintenance of hyperalgesia induced by the second acid injection on		
	Day 1	Day 2	Day 5
Acid with APETx2	7 days (20 pmole) 3 days (200 pmole)	6 days (20 pmole)	4 hr (20 pmole)
Acid with capsazepine	9 days	9 days	7 days
Acid with vehicle	>12 days	>12 days	>12 days

because we found all IB4-positive GM DRG neurons were smaller than 30  $\mu\text{m}$  in diameter. Whole-cell patch clamp recording revealed that ASIC3 was expressed in 30% (9/30) of Na<sub>v</sub>1.8-positive and IB4-negative medium-sized GM DRG neurons but not in IB4-positive small-sized GM DRG neurons (Table 2). This result echoes our previous finding of acid-induced enhanced TTXr-I<sub>NaV</sub> found only in medium-sized GM DRG neurons but not in small-sized GM DRG neurons [21]. We thus further examined the Na<sub>v</sub>1.8-negative medium-sized GM DRG neurons and found that 23% (6/26) expressed an ASIC3-like current. Interestingly, a high frequency of ASIC3-expressing neurons also expressed TRPV1 (Table 2). The expression of



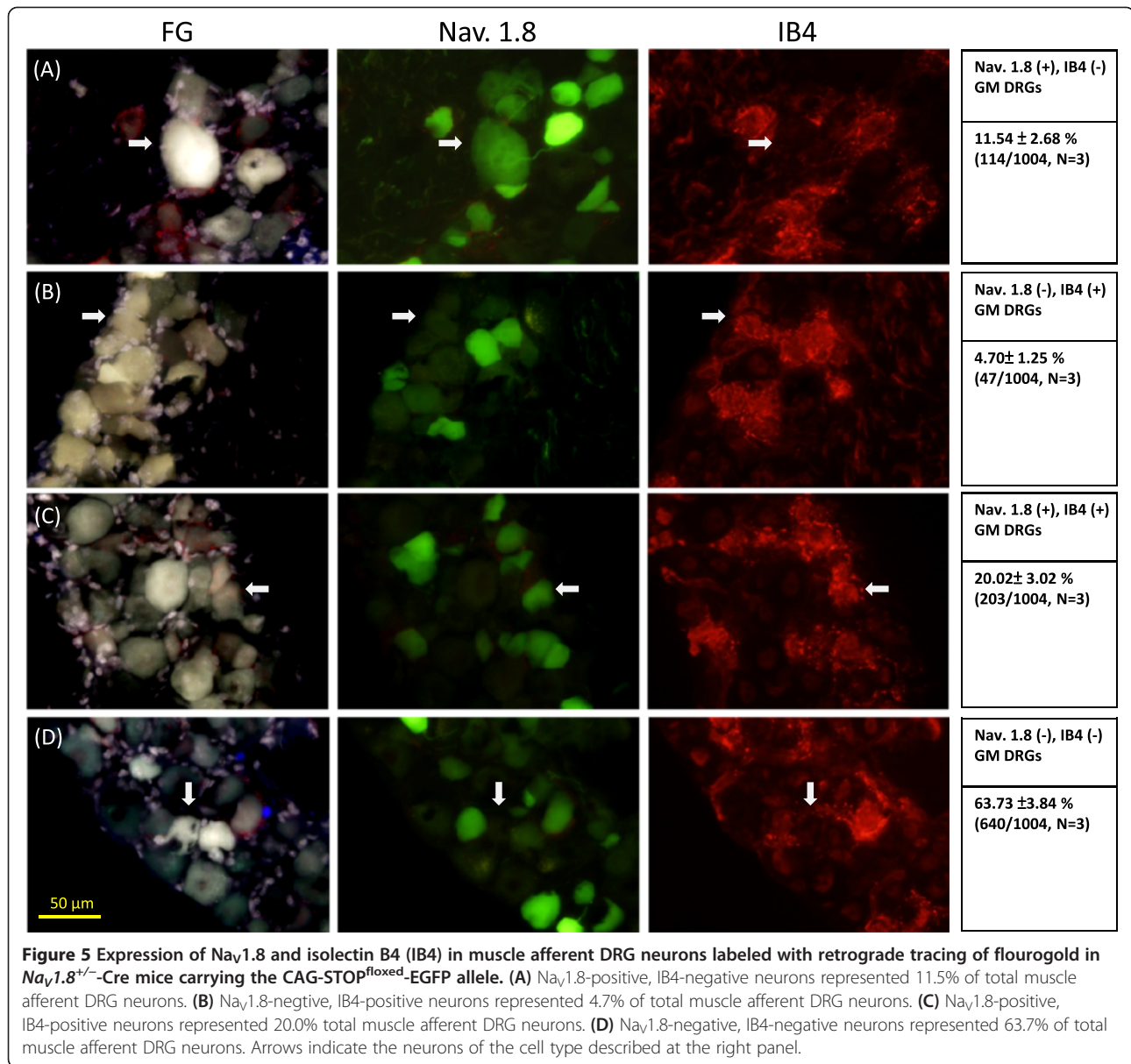
ASIC3 in both  $Na_v1.8$ -positive and -negative medium-sized GM DRG neurons supports that ASIC3 activation could contribute to the acid-enhanced  $I_{NaV}$  in both TTXr and non-TTXr GM DRG neurons and that APETx2 could significantly attenuate the acid-enhanced  $I_{NaV}$ .

#### Roles of $Na_v1.8$ and PKC $\epsilon$ in acid-induced chronic hyperalgesia

Although enhanced TTXr  $I_{NaV}$  was observed in PKC $\epsilon$ -dependent nociceptor priming [7], we found no association of acid-induced nociceptor priming and ASIC3- or TRPV1-enhanced TTXr  $I_{NaV}$  (Figures 3 and 4). To determine the biological meaning of the acid-enhanced TTXr  $I_{NaV}$ , we probed the effect of  $Na_v1.8$  deletion on the intramuscular acid-induced hyperalgesia. With dual acid injections spaced 2 or 5 days apart,  $Na_v1.8^{-/-}$  mice showed transient hyperalgesia after the first acid injection but not long-lasting hyperalgesia after the second acid injection (Figure 6A, B). The second acid injection induced hyperalgesia that lasted

for only 2 to 4 days in  $Na_v1.8^{-/-}$  mice. These data suggest a role for  $Na_v1.8$  in establishing nociceptor priming, which is important for maintaining the long-lasting hyperalgesia induced by repeat acid injections, whereas the plastic changes of  $Na_v1.8$  did not contribute to setting the duration of priming. Furthermore, an  $Na_v1.8$ -selective blocker, A-803467, had analgesic effects on wild-type mice that had developed chronic hyperalgesia induced by second acid injection, which suggests that  $Na_v1.8$  is involved in maintaining the acid-induced chronic hyperalgesia (Figure 6C, D).

Finally, we examined whether the acid-induced hyperalgesic priming depends on the activation of PKC $\epsilon$  as seen in inflammatory pain models [5]. With the dual acid-injection model, we intramuscularly injected mice with the cell-permeable PKC $\epsilon$  inhibitor peptide (TAT-PKC $\epsilon$ I) at 5 h after the first acid injection (Figure 7A, B) or 3 min before the second acid injection (Figure 7C, D). In both cases, the inhibition of PKC $\epsilon$  activity did not affect the acid-induced long-lasting hyperalgesia. To further validate that PKC $\epsilon$



(or other PKC isoforms) is not involved in the acid-induced hyperalgesic priming, we intramuscularly injected mice with a general PKC inhibitor, BIM, at 5 h after the first acid injection or 3 min before the second acid injection. Again, BIM had no effect on the acid-induced long-lasting hyperalgesia (Figure 7E-H). Therefore, activation of PKCε is not required in the acid-induced hyperalgesic priming.

## Discussion

### Acid-induced hyperalgesic priming is PKCε-independent in muscle nociceptors

Accumulating evidence has suggested that the hyperalgesic priming of nociceptors is essential for the transition from acute to chronic pain states in many perplexing chronic

pain conditions that are stress-related or neuropathic [5,22,23]. In inflammatory pain models, the hyperalgesic priming occurs exclusively in IB4-positive primary afferent nociceptors and depends on a switch in intracellular signaling pathways from PKA to PKCε [6,8,24]. The PKCε-dependent hyperalgesic priming is also present in vibration-induced muscle pain and chemotherapy-induced neuropathic pain and thus may constitute a general cellular basis for nociceptor plasticity in chronic pain [22,23]. Here we systematically examined the effect of the modality of the noxious acid insult on the duration of the hyperalgesia priming and the development of chronic hyperalgesia with a fixed, second acid injection. ASIC3 and TRPV1 were the major proton sensors responsible for the acid-induced

**Table 2 Electrophysiological characterization of ASIC3 and TRPV1 expression in subsets of muscle afferent dorsal root ganglion neurons**

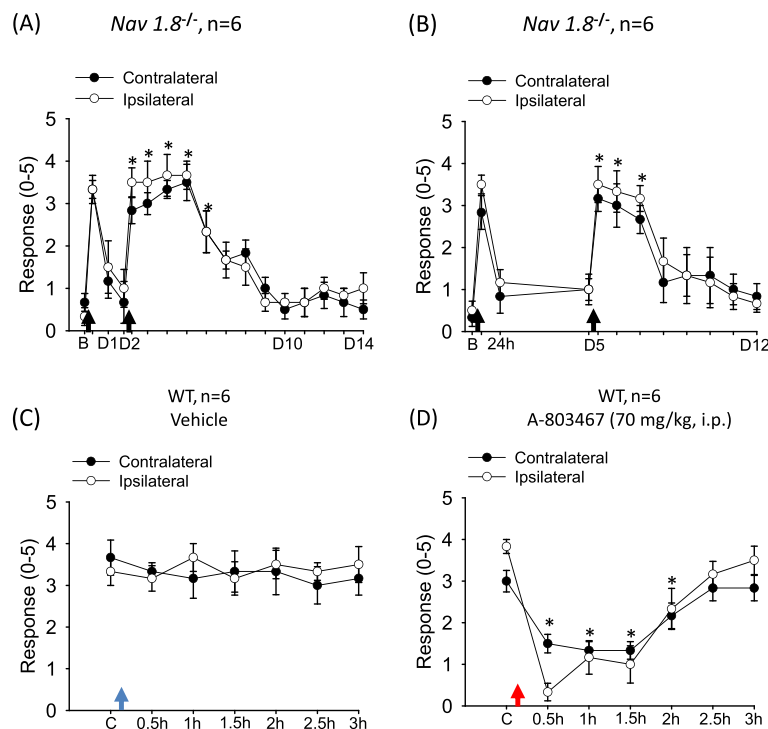
Type of acid-induced currents	No. of acid-sensitive neurons in subsets of muscle afferent DRG neurons		
	Nav1.8(-), IB4(-)	Nav1.8(+), IB4(-)	Nav1.8(+), IB4(+)
	(30–40 μm)	(30–40 μm)	(<30 μm)
ASIC3(+), TRPV1(-)	1 (4%)	4 (13%)	0
ASIC3(+), TRPV1(+)	5 (19%)	5 (17%)	0
ASIC3(-), TRPV1(+)	5 (19%)	2 (7%)	10 (40%)
ASIC3(-), TRPV1(-)	13 (50%)	14 (47%)	6 (24%)
No current	2 (8%)	5 (17%)	9 (36%)
<b>Total</b>	<b>26</b>	<b>30</b>	<b>25</b>

hyperalgesic priming in non-IB4 muscle nociceptors, which manifests a new type of hyperalgesic priming mediated by ion channels (but not by PKCε) in the non-inflammatory model of chronic muscle pain (Figure 8).

**Acid-induced muscle nociceptor priming is modality-dependent mediated via ASIC3 and/or TRPV1**

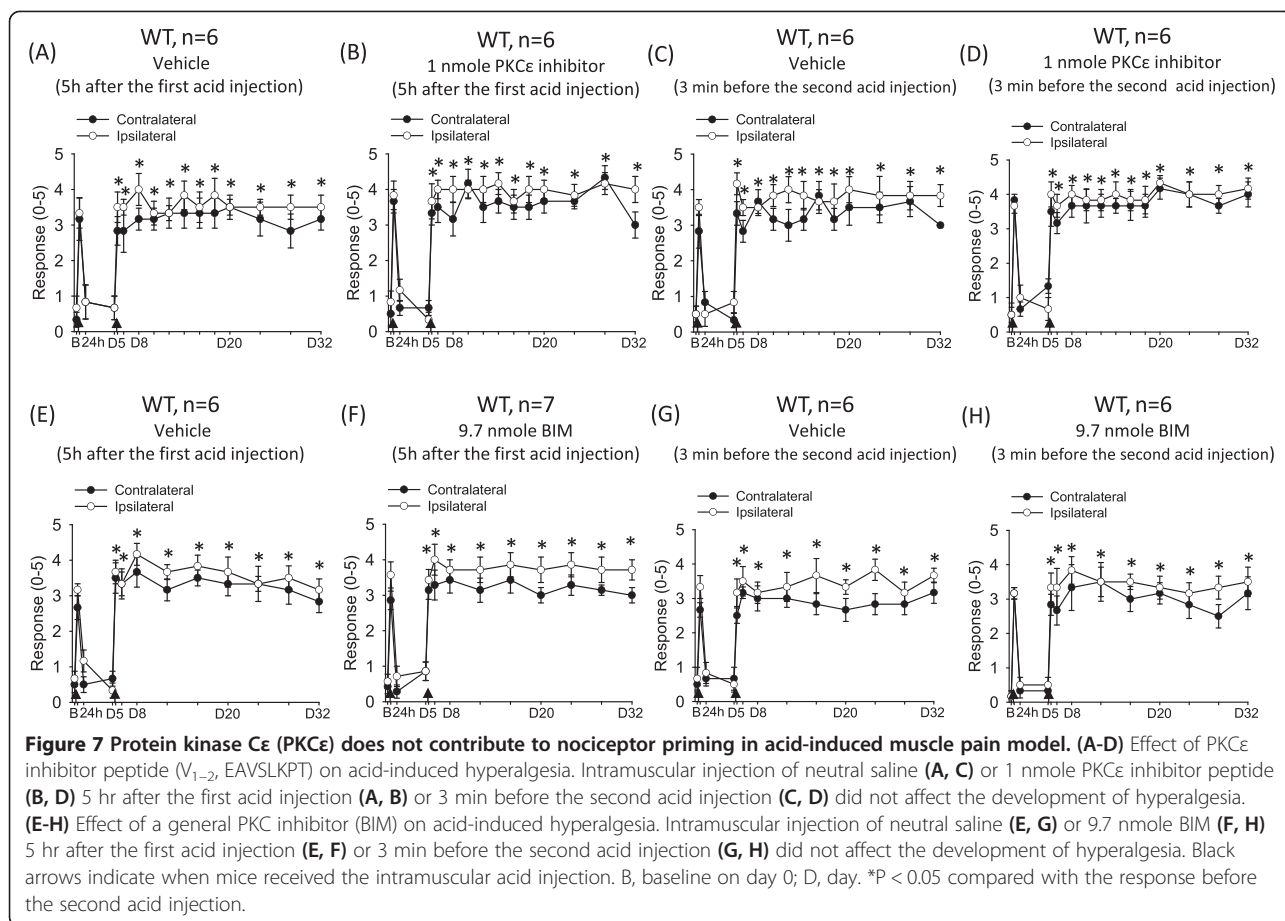
ASIC3 is responsible for the acid-induced transient hyperalgesia and hyperalgesic priming, and the development

of chronic hyperalgesia to repeat acid injections. TRPV1 plays a minor but important role in the acid-induced hyperalgesic priming and the development of chronic hyperalgesia (Figure 8). The inhibitory effects of APETx2 and capsazepine on  $I_{NaV}$  enhancement were consistent with their effects on the duration of hyperalgesic priming and the maintenance of the chronic hyperalgesia induced by the second acid injection in the dual acid-injection model. Of note, inhibiting ASIC3 abolished the enhanced  $I_{NaV}$  in non-TTXr GM DRG neurons on day 5 but not day 2, so the lack of  $I_{NaV}$  enhancement was associated with no chronic hyperalgesia induced by the second acid injection in mice receiving APETx2 5 days previous. In contrast, the TTXr GM DRG neurons seemed to have little role in setting the priming state for future acid insult. Instead, the enhanced TTXr  $I_{NaV}$  in GM DRG neurons was inhibited by both APETx2 and capsazepine, which was associated with the effects of both drugs on shortening the hyperalgesia phase induced by the second acid injection (Table 1). Taken together, acid-induced hyperalgesic priming seems to be modality-dependent. ASIC3- and TRPV1-mediated nociceptor priming has differential effects on the development and maintenance of chronic hyperalgesia induced by a repeated acid insult.



**Figure 6 Involvement of Nav1.8 in acid-induced chronic muscle pain.** (A, B) Dual acid injections spaced 2 (A) or 5 (B) days apart induced short-term hyperalgesia (2–4 days) in *Nav1.8<sup>-/-</sup>* mice. (C, D) Analgesic effect of Nav1.8-selective blocker A-803467 was tested at 3 days after mice have developed chronic hyperalgesia induced by dual intramuscular acid injection spaced 5 days apart. The A-803467 (70 mg/kg, i.p.) or vehicle was injected immediately after the baseline response (control) had been obtained. B, baseline on day 0; D, day. Blue and red arrows indicate the time mice receive intraperitoneal injection of vehicle or A-803467 respectively. \*P < 0.05 compared with the response before the second acid injection or control.





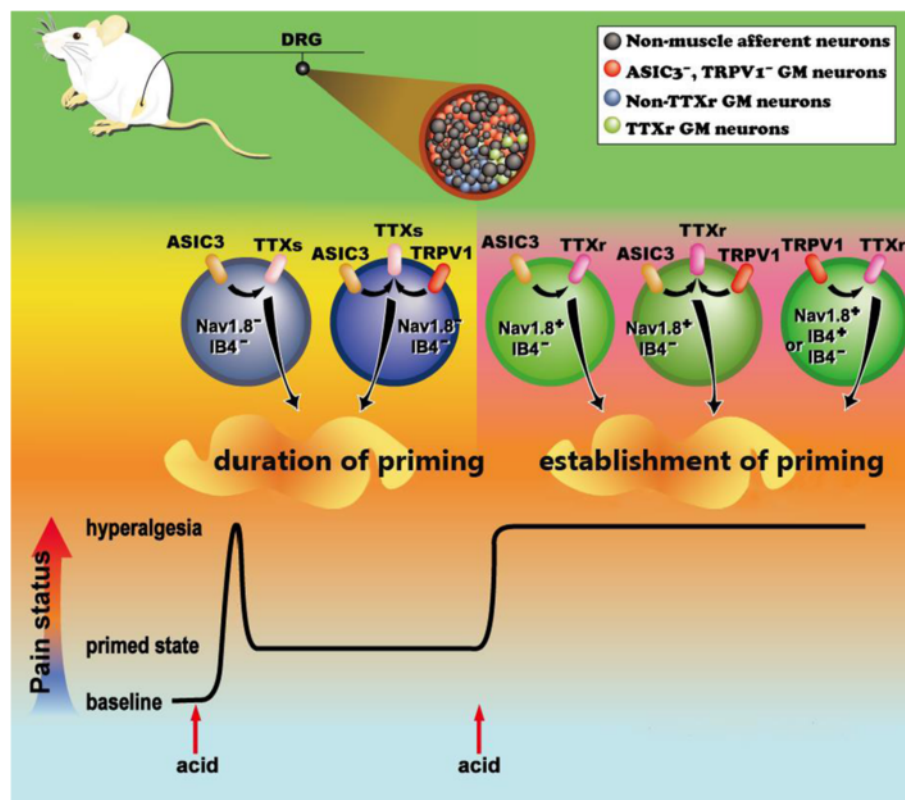
### The duration of priming and the establishment of priming are mechanistically different

Our current study addresses neural subgroups involved in the acid-induced nociceptor priming and also different aspects of priming: (1) the duration of priming that determines how long the primed state can stay and (2) the establishment of priming that determines how long the second acid injection-induced hyperalgesia can be maintained (Figure 8). Mechanistically, in  $Na_v1.8$ -negative muscle nociceptors, the activation of ASIC3 contributes to acid-induced transient hyperalgesia and enhanced TTX-s  $I_{NaV}$  as well as the duration of priming; TRPV1 might play a minor but essential role in setting the duration of priming. In contrast, in  $Na_v1.8$ -positive muscle nociceptors, both ASIC3 and TRPV1 contribute to the acid-enhanced TTXr  $I_{NaV}$ , which is required for the establishment of priming that permits the development and maintenance of long-term hyperalgesia induced by a second acid insult. Accordingly, although mice lacking  $Na_v1.8$  still showed hyperalgesic priming, they could develop hyperalgesia for only 2 to 4 days in the dual acid-injection model (Figure 6).

### ASIC3, TRPV1, and $Na_v1.8$ play different roles in muscle pain associated with acidosis

Accumulating evidence has revealed that most metabonociceptive muscle afferent neurons contain ASIC3 and/or TRPV1, so both channels might be responsible for the muscle pain associated with acidosis [11,12,25]. Our study echoes this finding and further suggests a role for ASIC3 and TRPV1 in acid-induced hyperalgesic priming in muscle nociceptors. Although ASIC3 plays an important role in hyperalgesic priming and triggering chronic hyperalgesia, a recent study showed that ASIC3 is not involved in maintaining hyperalgesia in the dual acid-injection model [26]. Accordingly, the ASIC3-selective antagonists (e.g., APETx2) work in pre-emptive analgesia (a treatment that is initiated before injury or noxious stimulation to reduce sensitization) in rodent models of acid-induced chronic widespread pain, postoperative pain, and inflammatory pain but not in animals with chronic pain [27-30].

However, the role of TRPV1 in maintaining the acid-induced chronic hyperalgesia is still not known. Recent studies revealed that TRPV1 plays a role in the development of heat hypersensitivity after muscle inflammation



**Figure 8** A schematic model of ion channel-mediated hyperalgesic priming in muscle nociceptors. ASIC3 and TRPV1 are expressed in different subsets of muscle nociceptors with or without  $Na_v1.8$  expression. In  $Na_v1.8$ -negative muscle nociceptors, ASIC3 is the major acid sensor responsible for acid-induced transient hyperalgesia and the duration of hyperalgesic priming; TRPV1 may play a minor but essential role in the nociceptor priming. In  $Na_v1.8$ -positive muscle nociceptors, both ASIC3 and TRPV1 contribute to the acid-enhanced TTXr  $I_{Na_v}$ , which is required for the establishment of priming that permits the development and maintenance of long-term hyperalgesia induced by a second acid insult. ASIC3 and TRPV1 are expressed alone or together, but ASIC3 is exclusively expressed in non-IB4 muscle nociceptors.

and contributes to delayed onset muscle soreness downstream of NGF and GDNF [31,32]. TRPV1 is a pronociceptive polymodal receptor sensing for vanilloid compounds (e.g., capsaicin), noxious heat ( $>43^{\circ}C$ ) and low pH ( $<5.9$ ) and could act as the final substrate of multiple inflammatory mediators that operate via distinct intracellular signaling pathways such as PKC [33]. PKC $\epsilon$ -mediated potentiation of TRPV1 in DRG neurons contributes to heat hyperalgesia in rats [34]. However, PKC signaling is probably not involved in acid-induced TRPV1 activation in muscle nociceptors.

In contrast, the involvement of TTXr sodium channels in maintaining chronic hyperalgesia sheds light on the clinical use of the channel blocker.  $Na_v1.8$  is clearly not involved in setting the duration of hyperalgesic priming. Instead, the increased TTXr sodium current after the first acid injection was related to the long-lasting hyperalgesia after the second acid injection. In mice lacking  $Na_v1.8$ , dual acid-injection-induced hyperalgesia was shortened to 2 to 4 days as compared with more than 19 days in wild-

type mice. Accordingly, the  $Na_v1.8$ -selective antagonist (A-803467) and general sodium channel blockers such as mexiletine or lamotrigine (37.5 mg/kg, intraperitoneally) have analgesic effects on the acid-induced, chronic, widespread pain model [35]. Thus, a selective sodium channel blocker (e.g., A-803467) might be a good choice to treat chronic muscle pain associated with recurrent ischemic insults [36]. Since the expression of  $Na_v1.8$  is restricted to the peripheral nervous system, the selective antagonists would reduce the risk of side effects on the central nervous system [37,38].

One concern with the study may be the selectivity of APETx2; a recent study revealed that APETx2 inhibited  $Na_v1.8$  currents of DRG neurons with an  $IC_{50}$  of 2.6  $\mu M$  *in vitro* [39]. In the current study, we used a total of 20 pmole APETx2 (in 20  $\mu L$  acid saline with a concentration of 1  $\mu M$  APETx2) to inhibit the acid-induced nociceptor priming; with this dose, APETx2 should mostly inhibit homomeric ASIC3 channels or partially heteromeric ASIC3 channels and had little inhibitory effect on  $Na_v1.8$

[20,39]. As well, we found that 2 pmole APETx2 (at the first acid injection) was enough to abolish the second acid injection (on day 5) inducing chronic hyperalgesia (data not shown), which further confirmed the involvement of ASIC3 (but not Nav1.8) in the nociceptor priming. Nevertheless, a more selective ASIC3 antagonist without an inhibitory effect on Nav1.8 will be helpful to clearly distinguish the roles of ASIC3 and Nav1.8 in the acid-induced nociceptor priming.

#### **Is other acid-induced signaling involved in the acid-induced hyperalgesic priming?**

Apart from Nav1.8, other cellular signaling might be involved in initiating the hyperalgesia in the first few days after the second acid insult or in regulating the increased activity of Nav1.8. For instance, we previously showed that substance P-mediated antinociceptive signaling in muscle nociceptors is diminished after repeat acid injection [21]. Intramuscular acid stimulation triggers the release of substance P from muscle nociceptors, which acts on NK1 receptors and activates M-type potassium via a G-protein-independent but Src-kinase-dependent manner. As well, proton-sensing G-protein-coupled receptors (e.g., G2A, GPR4, OGR1, TDAG8) and MrgprB4 are abundantly expressed in ASIC3-positive nociceptors and may contribute to the development of the intramuscular acid-induced hyperalgesia [40-42]. Moreover, other acid-induced responses in muscle afferent DRG neurons express neither ASIC3 nor TRPV1 (Figure 1 and Table 2) [43-46]. Future studies of muscle nociceptor-specific acid signaling would bring new insights into the molecular mechanism of chronic muscle pain and new opportunities for effective treatment.

Recent studies show that IB4-positive muscle nociceptors are responsible for chronic muscle pain triggered by acute inflammation (e.g., intramuscular carrageenan or glial cell-derived neurotrophic factor) or ergonomic intervention (e.g., eccentric exercise or vibration) [47,48]. Although Nav1.8 is largely expressed in IB4-positive muscle nociceptors, ASIC3 is exclusively expressed in non-IB4 muscle afferent DRG neurons with or without Nav1.8 expression (Table 2). However, we cannot exclude the role of IB4-positive muscle nociceptors in the development of long-lasting hyperalgesia in the dual acid-injection model, because many IB4- and Nav1.8-positive muscle nociceptors express TRPV1, which also contributes to the enhanced TTXr  $I_{NaV}$  and the chronic hyperalgesia induced by the second acid injection. Because TRPV1 channels are expressed in both IB4-positive and -negative muscle nociceptors, further studies should explore the differential roles of these 2 TRPV1-expressing muscle nociceptors in the pathogenesis of acid-induced chronic widespread pain.

#### **Does the acid-induced priming effect occur in the central nervous system?**

The priming hypothesis aims to describe a new mode of neuroplastic change in primary afferent nociceptors, in which basal nociceptive thresholds are still normal but nociceptors are sensitized against exposure to algogens or sensitizing agents [5]. However, whether the priming effect occurs in the central nervous system is not known. In the acid-induced chronic widespread pain model, the first acid injection induces a transient hyperalgesia that declines in 24 h (basal nociceptive threshold is back to normal in von Frey assay), but a priming effect lasts for 5 days, annotated by an increase in  $I_{NaV}$  in muscle nociceptors and a potential to develop chronic hyperalgesia in response to future acid insults. Although we have focused on the plastic changes of nociceptors in the primed state (1–5 days after the first acid injection), we cannot exclude possible plastic changes in the central nervous system after the first intramuscular acid injection.

The bilateral effect from a unilateral intramuscular injection suggests the involvement of central sensitization. Evidence has shown the unilateral dual acid injections induce activation of the cAMP pathway in the spinal cord, ERK activation in the anterior nucleus of paraventricular thalamus (PVA), and plastic changes in capsular central amygdaloid neurons [49-51]. However, no study has addressed whether a single acid injection can induce a plastic change (or the priming effect) in the central nervous system. We have not been able to demonstrate a plastic change in capsular central amygdaloid neurons in the primed state (Cheng SJ and Chen CC, unpublished observation). Interestingly, our previous studies showed that blockade of Ca<sub>v</sub>3.2 T-type Ca<sup>2+</sup> channel signaling or ERK activation in the PVA at 15 min before the second acid injection can prevent the chronic hyperalgesia, which suggests a possible central role of the PVA in the primed state [50]. Furthermore, acid can only induce transient hyperalgesia but not chronic hyperalgesia in mice lacking Ca<sub>v</sub>3.2. Further studies of Ca<sub>v</sub>3.2-dependent synaptic plasticity of PVA circuits in the primed state may shed light on the central control of the transition from acute to chronic pain.

#### **Conclusions**

In conclusion, our data manifest a new type of nociceptor priming mechanism that involves activation of ASIC3 and TRPV1 in muscle nociceptors and requires the development of acid-induced, chronic, widespread muscle pain. We highlight the role of Nav1.8 in developing and maintaining the chronic pain and rule out the involvement of IB4-positive nociceptors and PKCε signaling in the transition from acute to chronic pain. These results will be clinically useful, because we provide a new opportunity

for mechanism-based treatment for chronic, widespread muscle pain resulting from re-current acid insults possibly associated with symptoms of fibromyalgia and myofascial pain syndrome [11,52].

## Methods

### Animals

We used adult (8- to 12-wk-old) male C57/BL6 mice. All procedures followed the US Guide for the Care and Use of Laboratory Animals (National Academy of Sciences, Washington DC) and were approved by the Institutional Animal Care and Use Committee of Academia Sinica. *Asic3*<sup>-/-</sup> and *Nav1.8*<sup>-/-</sup>-*Cre* mice were generated and genotyped as described [53,54]. *Trpv1*<sup>-/-</sup> mice were purchased from the Jackson Lab (Bar Harbor, ME). All null-mutant mice were backcrossed to C57BL6 mice for at least 10 generations to establish a congenic strain. Congenic *Asic3*<sup>-/-</sup>, *Nav1.8*<sup>-/-</sup>-*Cre*, and *Trpv1*<sup>-/-</sup> mice were offspring of *Asic3*<sup>+/-</sup>, *Nav1.8*<sup>+/-</sup>-*Cre*, and *Trpv1*<sup>+/-</sup> intercrosses respectively. To identify Nav1.8-positive dorsal root ganglion (DRG) neurons, *Nav1.8*<sup>-/-</sup>-*Cre* mice were crossed with mice carrying a CAG-CAT-enhanced green fluorescent protein (CAG-CAT-EGFP) reporter allele with a stop-flxed segment inserted upstream of the EGFP (CAG-STOP<sup>flxed</sup>-EGFP) [55].

### Behavioral assays

Mice received 2 injections, spaced 1–5 days apart, into the GM, of 20  $\mu$ L acid saline (pH 4.0) with or without capsazepine (1 nmole), APETx2 (2, 20, or 200 pmole), or capsazepine (1 nmole) with APETx2 (20 pmole) (Figure 9A-C). The acid saline was prepared in 10 mM 2-[N-morpholino]ethanesulfonic acid and adjusted to pH 4.0 with 1 N NaOH. To test the effect of Nav1.8 on the maintenance of chronic hyperalgesia, the selective channel blocker A-803467 (Tocris, Avonmouth, UK) was dosed in 70 mg/kg intraperitoneally at 3 days after the mice have received dual acid injection (Figure 9D). To test the effect of PKC $\epsilon$ , the PKC $\epsilon$  inhibitor peptide (V<sub>1-2</sub>, EAVSLKPT) was conjugated with protein transduction domain of TAT protein (CYGRKKRRRQRRR-CEAVSLKPT, TAT-PKC $\epsilon$ I) and was kindly provided from KAI pharmaceuticals (South San Francisco, CA). We injected 20  $\mu$ L of the cell-permeable TAT-PKC $\epsilon$ I (50  $\mu$ M in pH 7.4 saline) or a general PKC inhibitor BIM (485  $\mu$ M in pH 7.4 saline, purchased from Cayman Chemical, Ann Arbor, Michigan) into the GM 5 h after the first acid injection or 3 min before the second injection (Figure 9E). Mechanical hyperalgesia was assessed as described [21]. Briefly, a 0.2 mN von Frey filament was applied to the plantar surface of both hind paws. A positive response was defined as foot lifting when the von Frey filament was applied. For each paw, the filament was applied 5 times at 30-s intervals. The experimenters

were blinded to the experimental manipulations and/or mouse genotypes.

### DRG primary culture

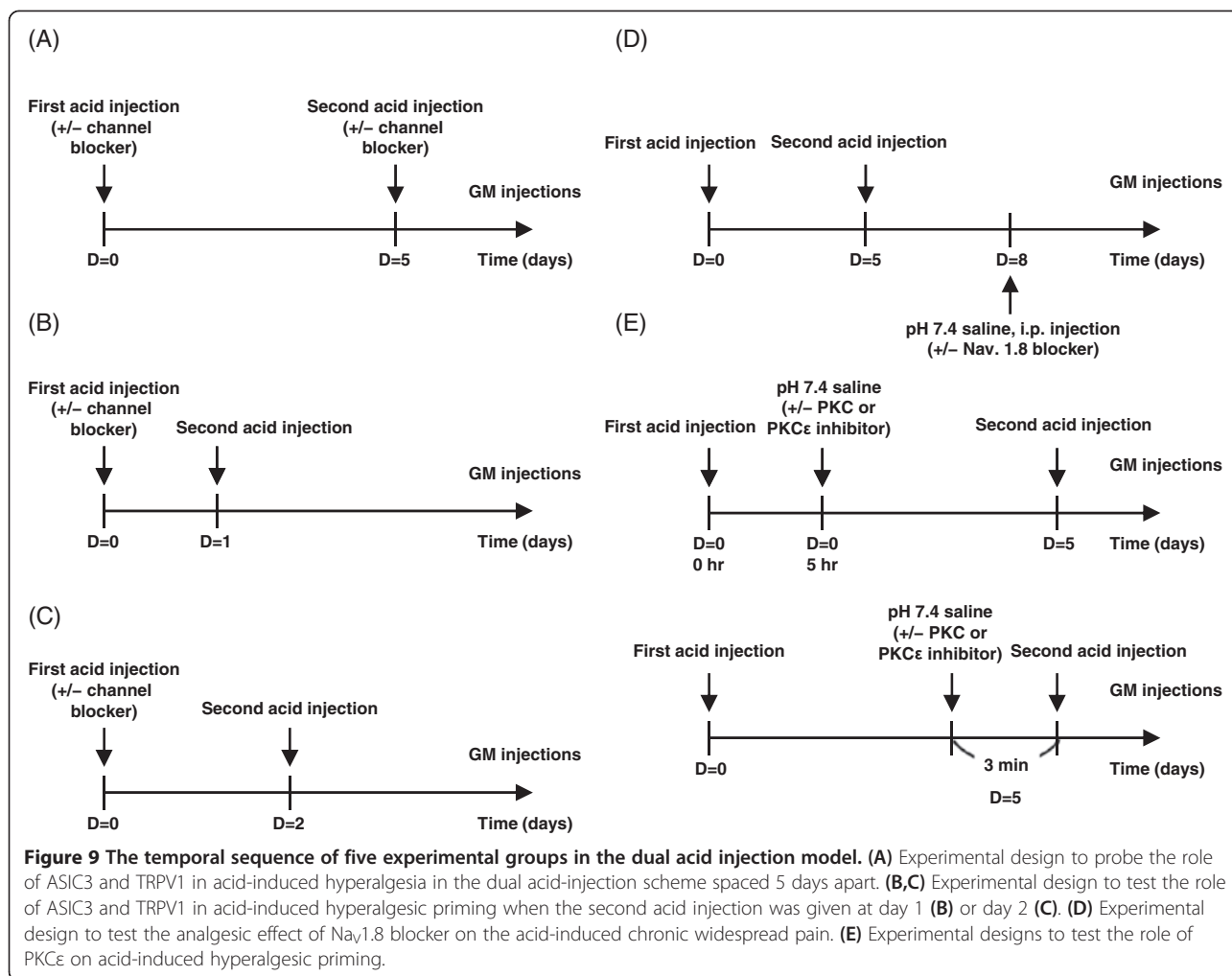
To retrograde-trace muscle afferent DRG neurons, mice were anesthetized with 2% isoflurane and injected with 10  $\mu$ L of 4% (wt/vol) fluorogold into the GM of both legs for 5 to 8 days. Lumbar DRG neurons were isolated, dissociated and cultured as described [21]. After seeding, DRG neurons were maintained in a 5% (vol/vol) CO<sub>2</sub> incubator at 37°C and used for patch-clamp recording within 30 h. IB4-positive neurons were determined by staining with IB4-DyLight 594 (2.5  $\mu$ g/mL in solution containing 0.1 mM MgCl<sub>2</sub>, CaCl<sub>2</sub>, and MnCl<sub>2</sub>; Vector Lab) for 2 min immediately before the recording. To visualize Nav1.8-expressing GM DRG neurons, DRG neurons were isolated from *Nav1.8*<sup>+/-</sup>-*Cre* mice carrying the CAG-STOP<sup>flxed</sup>-EGFP allele.

### Whole-cell patch-clamp recording

Whole-cell patch clamp recordings of muscle afferent DRG neurons involved use of an Axopatch MultiClamp 700B (Axon Instruments). Neurons with membrane potential > -40 mV were not accepted. The bridge was balanced in the current clamp mode and the series resistance was compensated 70% in voltage clamp mode with Axopatch 700B compensation circuitry. All DRG neuron recordings were performed at room temperature and were completed within 30 h after plating. The recording electrodes had a resistance of 1–5 M $\Omega$  when filled with an internal solution containing (in mM) 100 KCl, 2 Na<sub>2</sub>-ATP, 0.3 Na<sub>3</sub>-GTP, 10 EGTA, 5 MgCl<sub>2</sub>, and 40 Hepes, adjusted to pH 7.4 with KOH. Recording cells were superfused in artificial cerebrospinal fluid (ACSF) containing (in mM) 130 NaCl, 5 KCl, 1 MgCl<sub>2</sub>, 2 CaCl<sub>2</sub>, 10 glucose, and 20 Hepes, adjusted to pH 7.4 with NaOH. Osmolarity was adjusted to approximately 300 mOsm. ACSF was controlled by gravitational force. The acidic ACSF was titrated to pH 5.0 by 2-[N-morpholino]ethanesulfonic acid (MES). Salicylic acid (SA) was prepared from a 1-M stock solution (in 100% ethanol) to a final concentration of 500  $\mu$ M in ACSF. Capsazepine was prepared from a 20 mM stock solution (in 100% ethanol) to a final concentration of 10  $\mu$ M in ACSF. TTX was prepared from a 100- $\mu$ M stock solution to a final concentration of 200 nM in ACSF. Capsazepine and TTX were purchased from Tocris (Avonmouth, UK) and APETx2 was from Alomone (Jerusalem, Israel). Otherwise, drugs were from Sigma Chemicals (St. Louis, MO).

### Acid-induced currents

First, a 1-ms 2 nA current step was used to evoke an action potential (AP). An AP with inflected falling phase indicates involvement of a TTXr sodium channel [56]. To determine



**Figure 9** The temporal sequence of five experimental groups in the dual acid injection model. **(A)** Experimental design to probe the role of ASIC3 and TRPV1 in acid-induced hyperalgesia in the dual acid-injection scheme spaced 5 days apart. **(B,C)** Experimental design to test the role of ASIC3 and TRPV1 in acid-induced hyperalgesic priming when the second acid injection was given at day 1 **(B)** or day 2 **(C)**. **(D)** Experimental design to test the analgesic effect of  $Na_v1.8$  blocker on the acid-induced chronic widespread pain. **(E)** Experimental designs to test the role of PKC $\epsilon$  on acid-induced hyperalgesic priming.

whether TTXr sodium channels contributed to the AP configuration, inflections were determined by differentiation of AP. To obtain acid-induced currents, the acidic ACSF was applied through a glass pipette 50  $\mu$ m from the neuron and via gravity controlled by a VC-6 six-channel valve controller (Warner Instruments). Acidic ACSF was applied for 4 s in 30-s intervals. After 3 applications, SA (500  $\mu$ M) was bath-applied to examine whether the acid-induced current was inhibited. SA-containing bath was then replaced with normal ACSF for another 3 min. Next, capsazepine was bath-applied to examine whether the acid-induced current was inhibited. ASIC3-expressing neurons were defined when the acid-induced current was inhibited by SA [18]; TRPV1-expressing neurons were defined when the acid-induced current was inhibited by capsazepine [19].

#### Voltage-gated sodium currents

Mice were injected first with 4% (wt/vol) fluorogold into GM and 3 days later with acidic saline (pH 4.0, 20  $\mu$ L) alone or with 1 nmole capsazepine or 20 pmole APETx2.

Mice were killed 2 or 5 days later, and DRG neurons were isolated and cultured as stated above and used to study voltage-gated sodium currents. Medium-sized DRG neurons with cell diameters 30–40  $\mu$ m were selected for recording. The internal solution contained (in mM) 10 NaCl, 110 CsCl, 20 tetraethylammonium-chloride, 2.5  $MgCl_2$ , 5 EGTA, 3  $Mg^{2+}$ -ATP, and 5 HEPES, adjusted to pH 7.0 with CsOH. The external solution contained (in mM) 100 NaCl, 5 CsCl, 30 tetraethylammonium-chloride, 1.8  $CaCl_2$ , 1  $MgCl_2$ , 0.1  $CdCl_2$ , 25 glucose, 5 4-aminopyridine, and 5 HEPES, adjusted to pH 7.4 with HCl. Osmolarity was adjusted to 300 mOsm with glucose. The  $I_{NaV}$  was evoked by a 30-ms test pulse at  $-30$  mV from a holding potential of  $-80$  mV. For TTXr sodium currents, recordings were performed in external solution containing 200 nM TTX.

#### Immunohistochemistry

To examine the expression of  $Na_v1.8$  and IB4 in muscle afferent DRG neurons,  $Na_v1.8^{+/-}$ -Cre mice carrying the CAG-STOP<sup>flxed</sup>-EGFP allele were injected with 4% (wt/vol)

fluorogold into the GM 5 days before DRG isolation. Lumbar DRGs (L3-5) were isolated and fixed with 4% paraformaldehyde (in pH 7.4 phosphate buffered saline [PBS]) at 4°C for 2 h. Post-fixed tissues were placed in 20% sucrose at 4°C overnight, then embedded in OCT and rapidly frozen with use of dry ice and stored at -80°C. Frozen sections 12-μm thick were cut on a cryostat and mounted on glass slides. Slides were fixed with 4% paraformaldehyde at 4°C for 10 min, then incubated with blocking solution containing 1% bovine serum albumin, 0.1% Triton X-100, 0.02% sodium azide in PBS for 1 h at room temperature. After a PBS wash, the slides were stained with IB4-DyLight 594 for 30 min at room temperature.

#### Data analysis

Data for  $I_{NaV}$  are presented as mean ± SEM and were analyzed by use of Origin 8.0 (OriginLab). One-way ANOVA and then Fisher's least significant difference post-hoc test were used to compare differences between groups. The Mann-Whitney U test was used to compare withdrawal responses to von Frey filament application in mice before and after acid injection.  $P < 0.05$  was considered statistically significant.

#### Abbreviations

ASIC3: Acid-sensing ion channel 3; DRG: Dorsal root ganglion; GM: Gastrocnemius muscle; IB4: Isolectin B4;  $I_{NaV}$ : Voltage-gated sodium current;  $Na_v1.8$ : Voltage-gated sodium channel 1.8; PKCε: Protein kinase Cε; TRPV1: Transient receptor potential V1; TTX: Tetrodotoxin.

#### Competing interest

The authors declare no competing financing interests.

#### Authors' contributions

WN Chen conducted and analyzed electrophysiological experiments. WN Chen, CH Lee, CW Wong performed behavioral experiments. WN Chen, SH Lin, WH Sun, CC Chen designed experiments. JN Wood provided  $Na_v1.8$  mice and interpreted data. CC Chen collected, integrated, and interpreted the results and wrote the manuscript. All authors read and approved the final manuscript.

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