

## Acid-Sensing Ion Channel 3 Expression in Mouse Knee Joint Afferents and Effects of Carrageenan-Induced Arthritis

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**Abstract:** Arthritis is associated with decreases in local pH. Of the acid-sensing ion channels (ASIC), ASIC3 is most sensitive to such a pH change, abundantly expressed in dorsal root ganglion (DRG), and critical for the development of secondary hyperalgesia. The purpose of this study was to investigate the upregulation of ASIC3, using an acute arthritic pain model in mice. We examined ASIC3 expression in DRG neurons innervating the knee joint with and without carrageenan-induced arthritis by means of retrograde labeling and immunohistochemistry. We also examined the difference of DRG phenotype between ASIC3<sup>+/+</sup> and ASIC3<sup>-/-</sup> mice. ASIC3 immunoreactivity was present in 31% of knee joint afferents and dominantly in small cells. After joint inflammation, ASIC3-immunoreactive neurons significantly increased in number by 50%. Calcitonin gene-related peptide (CGRP) increased similarly in both ASIC3<sup>+/+</sup> and ASIC3<sup>-/-</sup> mice. Soma size distribution of ASIC3-immunoreactive neurons without CGRP expression was shifted to smaller-diameter neurons. Our results suggest that ASIC3 plays an important role in acute arthritic pain. Specifically, we propose that ASIC3 upregulation along with CGRP and phenotypic change in ASIC3-immunoreactive neurons without CGRP are responsible for the development of secondary hyperalgesia after carrageenan-induced arthritis.

**Perspective:** This article shows that ASIC3 is upregulated along with CGRP in knee joint afferents and that there is a phenotypic change in ASIC3-immunoreactive nonpeptidergic neurons in an animal model of acute arthritis. Understanding the basic neurobiology after acute arthritis could lead to future new pharmacological management of arthritis.

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**Key words:** Acid-sensing ion channels, joint, pain, dorsal root ganglion, inflammation, acid, calcitonin gene-related peptide.

Arthritis is currently 1 of the most frequent chronic health problems, particularly osteoarthritis. Clinical symptoms are dominated by chronic joint pain, which leads to disability, psychological distress, and impaired quality of life. Despite its frequency and impact, the mechanisms of joint pain are largely unknown.<sup>3,19</sup> Joint pain is uniquely different from cutaneous pain and is characterized as diffuse, longer-lasting, and more unpleasant.<sup>27,32</sup> It is often accompanied by referred pain and secondary hyperalgesia,<sup>27,37</sup> that is, increased nociceptive response to noxious stimuli outside the joint. This may relate to differences in different biochemical mediators or

central anatomical pathways. Dorsal root ganglion (DRG) neurons innervating joint have more calcitonin gene-related peptide (CGRP) and substance P and less isolectin B4 (IB4) and somatostatin when compared with DRG neurons innervating the skin.<sup>16,24</sup> The central projections from nociceptors innervating joint tissue are predominantly to laminae I and deeper dorsal horn, whereas those from cutaneous tissue project to laminae II.<sup>29</sup> Although some important knowledge about joint pain is accumulating, it is not sufficient to develop more effective treatment for arthritic pain. In fact, nonsteroidal anti-inflammatory drugs and acetaminophen are still the class of medication most commonly used for arthritic pain, and their effects are limited.<sup>9,41</sup>

At the site of arthritis, a number of inflammatory substances are released into the joint from nerves, immune cells, synoviocytes, platelets, vascular endothelium, and interstitial fluid. Many of these substances are capable of activating free nerve endings of joint afferents, ultimately resulting in the perception of joint pain.<sup>2,19</sup> Protons are 1

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of these inflammatory substances and can directly activate nociceptors by opening proton-gated cation channels, such as acid-sensing ion channels (ASICs) and capsaicin-sensitive TRPV1.<sup>2,5</sup> Synovial fluid in inflamed knee joint shows a drop in pH to levels around 6.6.<sup>8</sup> Among the proton-gated cation channels, ASIC3 is the most sensitive to such a pH change,<sup>17,40</sup> abundantly expressed in DRGs,<sup>38</sup> and strongly correlated with pain.<sup>25,33,34,36</sup> Although ASIC3 is considered to be 1 of the future targets to control joint pain,<sup>5,30</sup> there have been no studies about ASIC3 in joint afferents except our previous report.<sup>13</sup> Our laboratory recently showed that secondary hyperalgesia after joint inflammation (response to von Frey filaments applied to the paw) does not develop in ASIC3 knockout (*ASIC3*<sup>-/-</sup>) mice, whereas the primary mechanical hyperalgesia (response to tweezer applied to the inflamed knee joint) develops similarly between *ASIC3*<sup>-/-</sup> mice and wild-type mice (*ASIC3*<sup>+/+</sup>).<sup>13</sup> Therefore, we concluded that ASIC3 is critical for the development of secondary hyperalgesia. In addition, we observed that ASIC3 immunoreactive (IR) peripheral nerves were present in inflamed, not in uninflamed, synovium of the knee joint, and that these ASIC3 positive fibers colocalized with CGRP.<sup>13</sup> We hypothesized that ASIC3 was present only in a small proportion of DRG neurons innervating the uninflamed knee joint and that carrageenan-induced arthritis resulted in ASIC3 upregulation that was responsible for the development of secondary hyperalgesia.

In this study, we evaluated the ASIC3 expression of DRG neurons innervating the knee joint and the effects of carrageenan-induced arthritis with particular reference to CGRP coexpression. We also examined the difference of DRG phenotype between *ASIC3*<sup>+/+</sup> and *ASIC3*<sup>-/-</sup> mice in terms of soma size distribution and CGRP expression. Use of *ASIC3*<sup>-/-</sup> mice allows us to confirm specificity of the ASIC3 staining and examine the role of ASIC3 in DRG phenotype.

## Methods

### Animals

We used congenic *ASIC3*<sup>-/-</sup> mice and *ASIC3*<sup>+/+</sup> (C57/BL/6 J) mice (age, 2 to 3 months) (Jackson Laboratories, Bar Harbor, Maine). The generation of the *ASIC3*<sup>-/-</sup> mouse is described in detail elsewhere.<sup>26,39</sup> All experiments were approved by the Animal Care and Use Committee at the University of Iowa.

### Retrograde Labeling of Knee Joint Afferents

Animals were deeply anesthetized with 2.5% to 5% isoflurane. After shaving, a 5- to 6-mm-long skin incision was made at the left knee joint and 0.1 mg Fast Blue (FB) (Polysciences, Inc, Warrington, PA) diluted in 10  $\mu$ L of saline was injected into the joint cavity for retrograde labeling. The wound was closed with 5-0 silk. FB-containing neurons were identified *in vitro* by blue fluorescence emission on brief exposure of the cells to ultraviolet light.

### Induction of Joint Inflammation

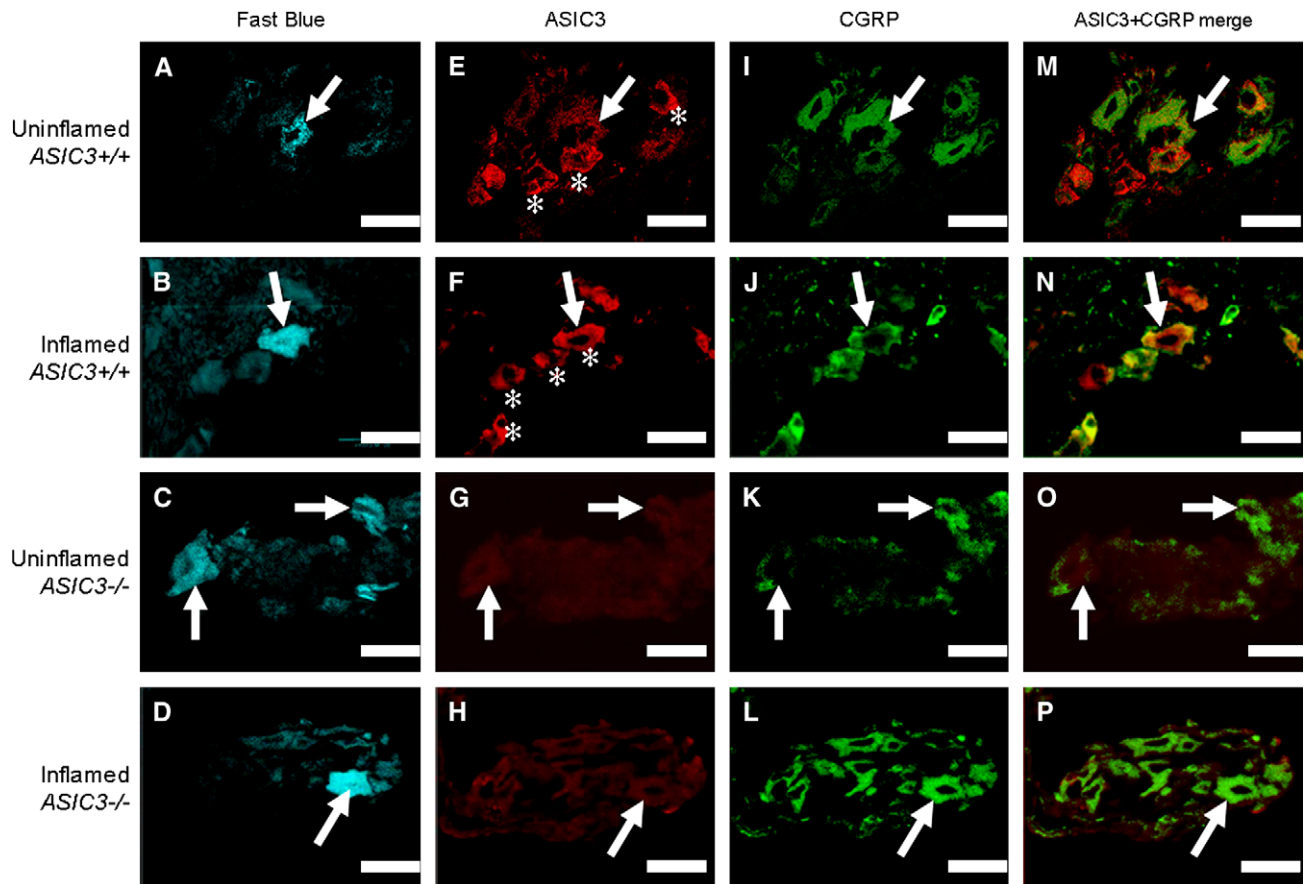
At 6 days after FB injection, an injection of 20  $\mu$ L of 3% carrageenan (20  $\mu$ L of saline for uninflamed control animals) was given into the left knee joint percutaneously while the mice were briefly anesthetized with 3% isoflurane. The carrageenan arthritis model has been extensively studied and it produces primary and secondary hyperalgesia to mechanical and heat stimuli, which develops rapidly within hours and persists for weeks after injection.<sup>13,27,29</sup>

### Immunohistochemistry

At 7 days after FB injection, that is, 24 hours after carrageenan injection, animals were euthanized with an overdose of sodium pentobarbital (150 mg/kg *i.p.*), and the ipsilateral L3-L5 DRG were removed. The contralateral L3-L5 DRG from 3 mice were also removed to examine for systemic spread of FB. The DRGs were placed in 2% paraformaldehyde and 15% sucrose overnight, embedded in OCT compound (Sakura Finetek, Torrance, California), frozen on dry ice, and kept in  $-70^{\circ}\text{C}$  until sectioning. Ten-micrometer frozen sections were then cut using a cryostat.

For simultaneous visualization of ASIC3 with CGRP, a double-immunofluorescence method was used. The primary antisera used in this study were rabbit anti-ASIC3 serum (1:500; Alomone Labs, Jerusalem, Israel) and rabbit anti-CGRP serum (1:1000; Peninsula Laboratories, San Carlos, CA). For visualization of ASIC3 antibody, avidin-biotin complex method was used. Because anti-ASIC3 and anti-CGRP serum were raised in a same species (rabbit), nonspecific rabbit IgG and goat anti-rabbit monovalent Fab fragment were used between ASIC3 and CGRP staining to avoid cross-reactivity between the detection systems. Absence of cross-reactivity was confirmed by omitting either of the primary antibodies.

The sections were blocked in 3% normal goat serum for 30 minutes, then incubated in rabbit anti-ASIC3 serum overnight in a humid atmosphere. The next day, the sections were incubated with goat anti-rabbit biotinylated IgG (1:250; Vector, Burlingame, CA) for 2 hours followed by streptavidin Alexa 568 (1:500; Invitrogen, Carlsbad, CA) for 2 hours. Subsequently, the sections were incubated with nonspecific rabbit IgG (1:1000; Jackson ImmunoResearch Laboratories, West Grove, PA) for 1 hour to absorb the excess goat anti-rabbit biotinylated IgG, followed by monovalent Fab fragment (final concentration 0.025 mg/mL; Jackson ImmunoResearch Laboratories) for 1 hour to absorb the excess nonspecific rabbit IgG. Afterward, the sections were incubated with rabbit anti-CGRP serum overnight in a humid atmosphere. On the third day, the sections were incubated with goat anti-rabbit Alexa 647 (1:500; Invitrogen) for 2 hours. All antisera used were diluted in PBS containing 1% normal goat serum and 0.05% Triton X-100. Before, between, and after each incubation step, the sections were washed with 5 times for 5 minutes in PBS. Finally, all sections were mounted with Vectashield (Vector, Burlingame, CA).



**Figure 1.** Fast Blue labeling (A, B, C, and D) and immunohistochemistry staining for ASIC3 (E, F, G, and H) and calcitonin gene-related peptide (CGRP) (I, J, K, and L) showing dorsal root ganglion (DRG) from *ASIC3*<sup>+/+</sup> (first and second rows) and *ASIC3*<sup>-/-</sup> (third and fourth rows) mice without joint inflammation (first and third rows) and those 24 hours after joint inflammation (second and fourth rows). Photos in each row are the same DRG. Arrows indicate Fast Blue-labeled DRG neurons. Asterisks indicate ASIC3-immunoreactive (IR) neurons. No immunoreactivity for ASIC3 was observed in *ASIC3*<sup>-/-</sup> mice (G and H), whereas CGRP was observed in all groups. Some DRG neurons showing both ASIC3 and CGRP immunoreactivity were observed as yellow in *ASIC3*<sup>+/+</sup> merged images (M and N). No double-labeled neurons were found in the merged images in *ASIC3*<sup>-/-</sup> mice (O and P).

### Microscopic Observation

Sections were viewed with Olympus BX-51 microscope (Olympus, Tokyo, Japan). Representative photos were taken with Bio-Rad Radiance 2100MP Multiphoton/Confocal Microscope (Bio-Rad, Richmond, CA). We counted FB-labeled neurons with visible nuclei from every fifth section to eliminate the possibility of double counting. More than 100 FB-labeled neurons were analyzed from 4 mice in each group. For each FB-labeled neuron, ASIC3 and CGRP expression was examined and quantified as the percentage of total FB-labeled neurons. Data were presented as the mean (%)  $\pm$  SEM. Soma size of FB-labeled neurons was measured using imaging software as the area of the cell in  $\mu\text{m}^2$  (Image J; National Institutes of Health, available at: <http://rsb.info.nih.gov/ij/>). Soma size distribution was calculated as the total for each population, retrogradely labeled knee joint afferents, CGRP-IR afferents, and ASIC3-IR afferents and so on, and divided into 6 different categories: <250, <500, <750, <1000, <1250, and <1500, as previously described.<sup>28</sup>

### Statistical Analysis

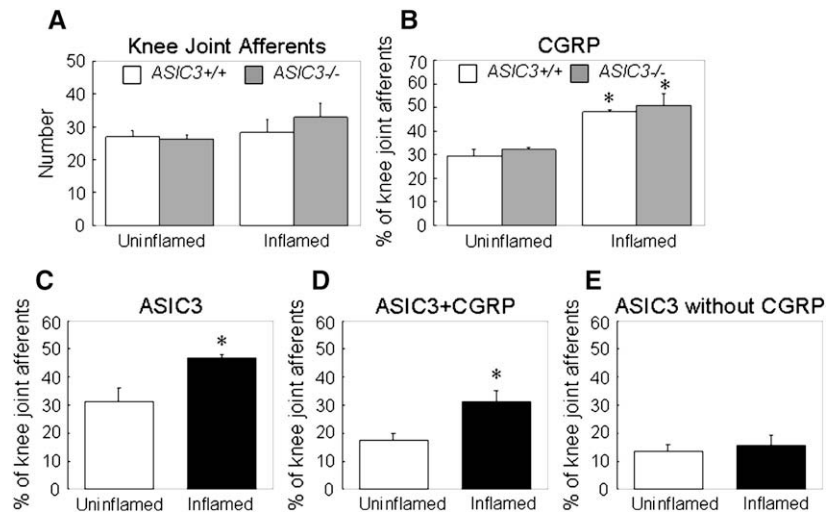
The Student *t* test was used for between-group (uninflamed versus inflamed) comparisons in *ASIC3*<sup>-/-</sup> mice.

For comparisons in the number of knee joint afferents and CGRP-IR cells, 2-way-analysis of variance (ANOVA) followed by Tukey test was used. For the evaluation of soma size distribution, the Kolmogorov-Smirnov test was used to determine whether the distribution of soma size differed between each population. The level of significance was set at  $P < .05$ .

## Results

### Knee Joint Afferents in *ASIC3*<sup>+/+</sup> and *ASIC3*<sup>-/-</sup> Mice

Intense FB labeling was found in DRG somata without leakage of fluorescence into the surrounding tissue (Fig 1, A, B, C, and D). Twenty to 38 FB-labeled neurons were observed from L3-L5 DRG in each animal. L4 DRG usually contained most FB-labeled neurons among L3-L5 DRG. No significant difference was seen among the number of FB-labeled neurons of each of the 4 groups: *ASIC3*<sup>+/+</sup> and *ASIC3*<sup>-/-</sup> with and without inflammation (Fig 2A). Fig 3A shows a histogram of the soma size distribution of FB-labeled neurons. There was a unimodal distribution with a broad range of sizes. The median soma



**Figure 2.** The number of knee joint afferents (A) and the percentage of knee joint afferents immunoreactive for calcitonin gene-related peptide (CGRP) (B), ASIC3 (C), ASIC3+CGRP (D), and ASIC3 without CGRP (E). Values are represented as mean ± SEM. Knee joint afferents immunoreactive for CGRP, ASIC3, and ASIC3+CGRP were significantly increased 24 hours after carrageenan-induced arthritis. \* $P < .05$  vs uninflamed mice.

size of FB-labeled neurons was  $425 \mu\text{m}^2$  in *ASIC3*<sup>+/+</sup> and  $413 \mu\text{m}^2$  in *ASIC3*<sup>-/-</sup> mice. No significant difference was observed among the soma size distribution of FB-labeled neurons of each group. There was no nonspecific systemic spread of FB because the contralateral DRGs had no FB staining.

### ASIC3 and CGRP Expression in Uninflamed Knee Joint Afferents

DRGs contained many ASIC3-IR and CGRP-IR neurons (Fig 1). No immunoreactivity for ASIC3 was observed in *ASIC3*<sup>-/-</sup> mice, whereas CGRP was observed in all groups. Colocalization of ASIC3 with CGRP was frequently observed in DRG. In normal uninflamed animals, counts revealed that  $31\% \pm 5\%$  of knee joint afferents were ASIC3-IR in *ASIC3*<sup>+/+</sup> mice. Similar amounts of CGRP were observed in *ASIC3*<sup>+/+</sup> and *ASIC3*<sup>-/-</sup> mice without inflammation:  $29\% \pm 3\%$  (*ASIC3*<sup>+/+</sup>) and  $32\% \pm 1\%$  (*ASIC3*<sup>-/-</sup>). The percentage of FB-labeled neurons that expressed both ASIC3 and CGRP was  $18\% \pm 2\%$ , and the percentage of FB-labeled neurons that expressed ASIC3 without CGRP was  $14\% \pm 3\%$  (Fig 2). CGRP was found in  $62\% \pm 11\%$  of ASIC3-IR neurons retrogradely labeled from the knee joint, and  $94\% \pm 5\%$  of ASIC3-IR small neurons ( $<500 \mu\text{m}^2$ ). Although more than half (55 %) of ASIC3-IR neurons were smaller than  $500 \mu\text{m}^2$ , their soma size distribution was unimodal with a broad range of size (median,  $419 \mu\text{m}^2$ ). CGRP-IR neurons consisted of small to medium cells with most cells smaller than  $500 \mu\text{m}^2$  (median,  $296 \mu\text{m}^2$  in *ASIC3*<sup>+/+</sup> and  $395 \mu\text{m}^2$  in *ASIC3*<sup>-/-</sup>). Neurons that showed colocalization of ASIC3 with CGRP were small to medium size (median  $259 \mu\text{m}^2$ ), whereas ASIC3 neurons that did not colocalize with CGRP were larger (median  $725 \mu\text{m}^2$ ). There was no significant difference in number and soma size distribution of knee joint afferents and CGRP-IR neurons between *ASIC3*<sup>+/+</sup> and *ASIC3*<sup>-/-</sup> mice (Fig 3).

### Effects of Carrageenan-Induced Arthritis

All mice injected with carrageenan showed swelling of the knee joint and abnormal gait posture with holding up the affected leg 24 hours after carrageenan injection. Counts revealed that knee joint inflammation significantly increased the number of CGRP-IR neurons retrogradely labeled from the knee joint from  $29\% \pm 3\%$  to  $48\% \pm 1\%$  in *ASIC3*<sup>+/+</sup> and from  $32\% \pm 1\%$  to  $51\% \pm 5\%$  in *ASIC3*<sup>-/-</sup> mice ( $F_{1,15}=40.7$ ,  $P=.0001$ ). ASIC3-IR neurons retrogradely labeled from the knee joint significantly increased in number from  $31\% \pm 5\%$  to  $47\% \pm 2\%$  ( $P=.019$ ). Further, there was a significant increase, from  $18\% \pm 2\%$  to  $31\% \pm 4\%$ , in the number of neurons that showed colocalization of ASIC3 with CGRP in knee joint afferents 24 hours after induction of inflammation ( $P=.034$ ) (Fig 2). The increase in the number of CGRP-IR neurons after inflammation was not significantly different between *ASIC3*<sup>+/+</sup> and *ASIC3*<sup>-/-</sup> mice, and there was no significant difference in the number of ASIC3-IR neurons without CGRP expression between uninflamed and inflamed mice.

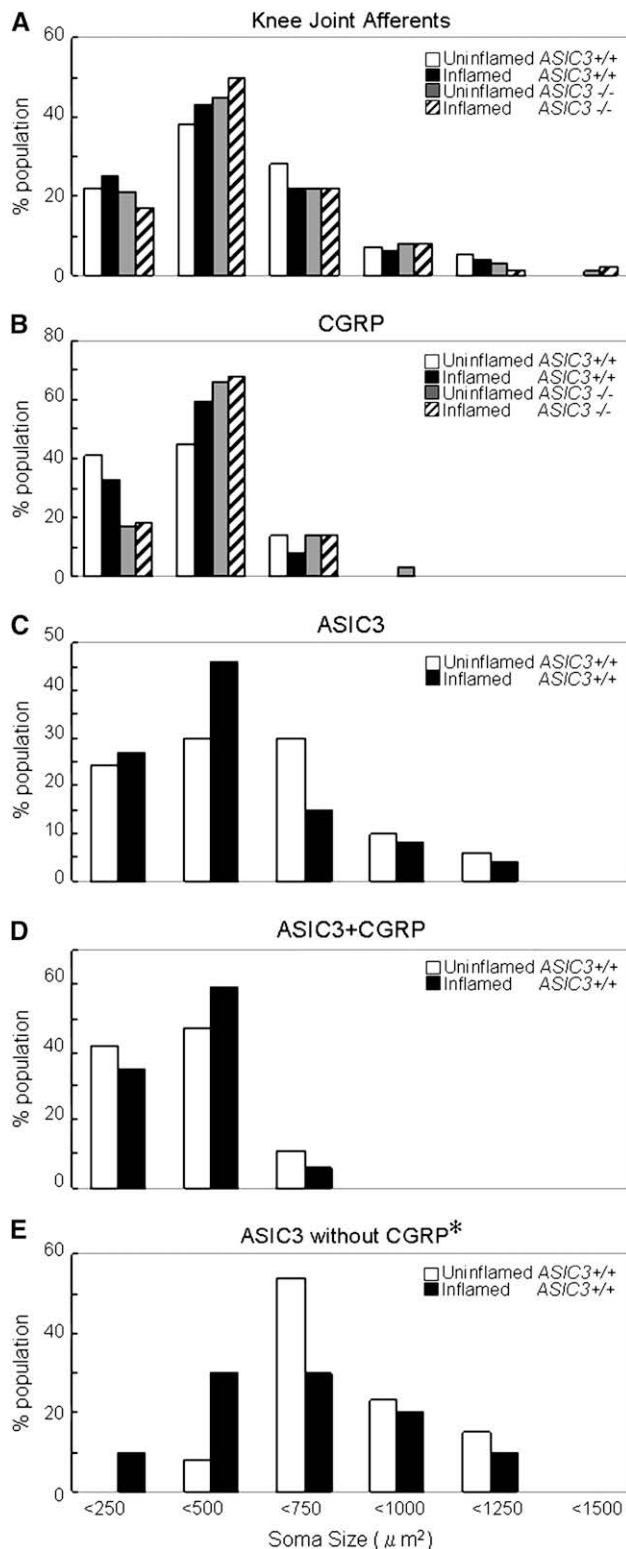
Soma size distribution after inflammation was similar to that of uninflamed mice in knee joint afferents for CGRP, ASIC3, and colocalization of ASIC3 with CGRP. However, there was a significant difference in the soma size distribution between uninflamed and inflamed mice in the ASIC3-IR neurons that did not express CGRP ( $P=.027$ ) (Fig 3). The median soma size of ASIC3-IR neurons without CGRP expression decreased from  $725 \mu\text{m}^2$  in uninflamed to  $572 \mu\text{m}^2$  in inflamed mice, suggesting there was an up-regulation of ASIC3 in smaller nonpeptidergic neurons.

### Discussion

#### ASIC3 Subpopulation in Uninflamed Knee Joint Afferents

Our study showed that ASIC3 expression was observed in 31% of uninflamed knee joint afferents in *ASIC3*<sup>+/+</sup>





**Figure 3.** Soma size distribution of knee joint afferents (A) was unimodal with a broad range of size and similar to that of ASIC3-immunoreactive (IR) knee joint afferents (C). Calcitonin gene-related peptide (CGRP) knee joint afferents consisted of small to medium cells with most cells smaller than 500  $\mu\text{m}^2$  (B). ASIC3 and CGRP double-labeled knee joint afferents were small to medium as well (D), whereas ASIC3 without CGRP-IR knee joint afferents were larger (E). There were no significant differences in soma size distribution among each group in knee joint afferents, CGRP, ASIC3, and ASIC3+CGRP. Only significant difference in soma size distribution was observed in ASIC3 without

mice. There was no ASIC3 expression in *ASIC3*<sup>-/-</sup> mice confirming the specificity of the antibody to ASIC3. In our previous study, however, we did not see ASIC3 expression in peripheral nerve fibers in uninflamed synovium of the mice knee joint.<sup>13</sup> One possible reason for the difference in ASIC3 expression between DRG and peripheral terminals could be a greater localization of ASIC3 in the cell bodies of DRG neurons. Garcia-Anoveros et al<sup>7</sup> reported that ASIC2a is actively transported from soma to peripheral terminals. It is possible, however, that ASIC3 may not be transported to peripheral terminals in the synovium of the knee joint sufficient quantity to be detected by means of immunohistochemistry, especially in the uninflamed knee joint. In other words, ASIC3 transport to the peripheral terminals innervating the knee joint may occur only after inflammation. Further studies involving nerve transection are necessary to confirm changes in ASIC3 transport in the axonal flow during acute arthritis. Another possible reason is the difference in background staining between joint tissue and DRG. The synovium is immediately adjacent to fat which results in a greater background fluorescence using the avidin-biotin amplification technique.

ASIC3 was present in diverse DRG neurons in terms of soma size. Although the histogram showed small cells (<500  $\mu\text{m}^2$ ) were dominant in ASIC3-IR population (55%), there was considerable number of ASIC3-IR middle to large cells (>500  $\mu\text{m}^2$ ). This finding is consistent with previous reports about ASIC3 population in rat sensory neurons.<sup>12,20</sup> Because nociceptors generally belong to small to medium size neurons,<sup>11</sup> it seems reasonable to suppose that ASIC3 is present in both nociceptors and non-nociceptors among knee joint afferents.

ASIC3 expression is different among specific tissue afferents.<sup>12,20</sup> Ichikawa et al<sup>12</sup> reported that there was more ASIC3 expression in tooth pulp afferents than skin afferents in rats (33% vs 13%), and also that colocalization of ASIC3 with CGRP in tooth pulp afferents was more frequent than that in skin (100% vs 81%). Molliver et al<sup>20</sup> reported similar results comparing muscle afferents with skin afferents in rats. There was more ASIC3 expression (50% vs 28%) and colocalization of ASIC3 with CGRP in muscle afferents than skin afferents (83% vs 69%). In the current study, ASIC3 was present predominantly in peptidergic neurons among small cell population because most of ASIC3-IR small cells (94%) coexpressed CGRP. This is consistent with prior reports<sup>4,18</sup> showing IB4-negative small neurons, that is, peptidergic neurons, are the primary responders to protons with a higher prevalence and greater amplitude of ASIC currents compared with IB4-positive small neurons, that is, nonpeptidergic neurons. An increased response to protons through ASIC3 in peptidergic neurons may well be due to unique characteristics of tooth pulp afferents and deep tissue afferents.

←  
CGRP (E). Carrageenan-induced arthritis resulted in an overall shift in soma size distribution of ASIC3 without CGRP-IR knee joint afferents to the left (E). \* $P < .05$  between uninflamed versus inflamed mice.

## Effects of Joint Inflammation

Our study showed that there was an upregulation of both ASIC3 (50%) and CGRP (64% in *ASIC3*<sup>+/+</sup> and 57% in *ASIC3*<sup>-/-</sup>) by carrageenan-induced arthritis. Although there are a few reports of ASIC3 upregulation by inflammation in rat DRG at the mRNA<sup>38</sup> and protein level,<sup>25</sup> this is, to our knowledge, the first report about ASIC3 upregulation by inflammation specifically in knee joint afferents. The upregulation of CGRP observed in the current study after inflammation in mice is consistent with previous reports specific to knee joint afferents in cat<sup>10</sup> and rat.<sup>6</sup> In terms of soma size distribution, not only CGRP but also ASIC3 was present predominantly in small to medium cells, that is, putative nociceptors, after joint inflammation. Therefore, ASIC3 upregulation presumably contributes to development of arthritic pain, as CGRP does.<sup>6,10,23,35</sup>

We found a significant change of soma size distribution for ASIC3 in neurons that do not express CGRP after joint inflammation. Carrageenan-induced arthritis resulted in an overall shift in its distribution to the left (smaller). Meanwhile, there was no significant change in soma size distribution for ASIC3 in peptidergic neurons. Although the immunohistochemical absence of CGRP does not necessarily mean non-peptidergic, these data presumably suggest a relatively increased expression of ASIC3 in nonpeptidergic small neurons.<sup>21</sup>

## Role of ASIC3 in Phenotype of Knee Joint Afferents

This study was the first to examine ASIC3 distribution in DRG neurons innervating the knee joint in mice and to confirm this distribution using *ASIC3*<sup>-/-</sup> mice. We also examined the phenotype of DRG neurons using both *ASIC3*<sup>+/+</sup> and *ASIC3*<sup>-/-</sup> mice. CGRP was used to label peptidergic neurons to examine distribution between peptidergic and nonpeptidergic neurons. Although IB4 is a commonly used marker for nonpeptidergic neurons, IB4 was not used because there are few IB4 binding neurons in joint afferents.<sup>14,22</sup>

Interestingly, there was no difference between *ASIC3*<sup>+/+</sup> and *ASIC3*<sup>-/-</sup> mice in every comparison except

ASIC3 expression, including the number, soma size distribution, and effects of inflammation. CGRP upregulation was the only change similarly observed in both *ASIC3*<sup>+/+</sup> and *ASIC3*<sup>-/-</sup> mice after joint inflammation. Meanwhile, significant differences between *ASIC3*<sup>+/+</sup> and *ASIC3*<sup>-/-</sup> mice with arthritis showed an upregulation of ASIC3 and ASIC3 in CGRP positive neurons, as well as a phenotypic change in ASIC3 neurons without CGRP.

In terms of pain behaviors, our previous study<sup>13</sup> showed that secondary hyperalgesia did not develop in *ASIC3*<sup>-/-</sup> mice, whereas primary hyperalgesia of the inflamed knee joint developed in *ASIC3*<sup>-/-</sup> mice and was similar to *ASIC3*<sup>+/+</sup> mice. Previous data similarly show that secondary mechanical hyperalgesia does not develop after muscle insult.<sup>33,34</sup> Taken together, these data suggest that the upregulation of ASIC3 in CGRP positive neurons, and a phenotypic change in neurons expressing ASIC3 without CGRP are responsible for the secondary hyperalgesia.

It is unknown why ASIC3 plays such a significant role in the development of secondary but not primary hyperalgesia. Secondary hyperalgesia is widely accepted to result from sensitization of dorsal horn neurons<sup>31</sup> and characterized by enhanced nociception to mechanical stimuli.<sup>1</sup> There is rarely a direct monosynaptic pathway from nociceptive primary afferents to dorsal horn neurons.<sup>15</sup> As such, Ziegler et al<sup>42</sup> proposed that central sensitization of nociceptive pathways involves a more complex circuitry than is assumed by a single-neuron model. Further, central sensitization does not occur after muscle insult in *ASIC3*<sup>-/-</sup> mice.<sup>33</sup> If central sensitization is driven mainly by proton-sensitive afferents innervating the inflamed knee joint, ASIC3 upregulation in knee joint afferents could induce central sensitization that is independent of primary mechanical hyperalgesia of the inflamed knee joint.

In conclusion, our results suggest that ASIC3 in knee joint afferents plays an important role in acute arthritic pain. Specifically, ASIC3 upregulation along with CGRP and the phenotypic change of ASIC3-immunoreactive neurons without CGRP are hypothesized to be responsible for the development of secondary hyperalgesia after carrageenan-induced arthritis.

## References

1. Ali Z, Meyer RA, Campbell JN: Secondary hyperalgesia to mechanical but not heat stimuli following a capsaicin injection in hairy skin. *Pain* 68:401-411, 1996
2. Coutaux A, Adam F, Willer JC, Le Bars D: Hyperalgesia and allodynia: Peripheral mechanisms. *Joint Bone Spine* 72:359-371, 2005
3. Dieppe PA, Lohmander LS: Pathogenesis and management of pain in osteoarthritis. *Lancet* 365:965-973, 2005
4. Dirajlal S, Pauers LE, Stucky CL: Differential response properties of IB(4)-positive and -negative unmyelinated sensory neurons to protons and capsaicin. *J Neurophysiol* 89:513-524, 2003
5. Dray A, Read SJ: Arthritis and pain: Future targets to control osteoarthritis pain. *Arthritis Res Ther* 9:212, 2007
6. Fernihough J, Gentry C, Bevan S, Winter J: Regulation of calcitonin gene-related peptide and TRPV1 in a rat model of osteoarthritis. *Neurosci Lett* 388:75-80, 2005
7. Garcia-Anoveros J, Samad TA, Zuvela-Jelaska L, Woolf CJ, Corey DP: Transport and localization of the DEG/ENAC ion channel BNaC1alpha to peripheral mechanosensory terminals of dorsal root ganglia neurons. *J Neurosci* 21:2678-2686, 2001
8. Goldie I, Nachemson A: Synovial pH in rheumatoid knee-joints, I: The effect of synovectomy. *Acta Orthop Scand* 40:634-641, 1969
9. Guidelines for the management of rheumatoid arthritis: 2002 Update. *Arthritis Rheum* 46:328-346, 2002
10. Hanesch U, Heppelmann B, Schmidt RF: Quantification of cat's articular afferents containing calcitonin gene-related

peptide or substance P innervating normal and acutely inflamed knee joints. *Neurosci Lett* 233:105-108, 1997

11. Harper AA, Lawson SN: Conduction velocity is related to morphological cell type in rat dorsal root ganglion neurons. *J Physiol* 359:31-46, 1985

12. Ichikawa H, Sugimoto T: The co-expression of ASIC3 with calcitonin gene-related peptide and parvalbumin in the rat trigeminal ganglion. *Brain Res* 943:287-291, 2002

13. Ikeuchi M, Kolker SJ, Burnes LA, Walder RY, Sluka KA: Role of ASIC3 in the primary and secondary hyperalgesia produced by joint inflammation in mice. *Pain* 137:662-669, 2008

14. Ivanavicius SP, Blake DR, Chessell IP, Mapp PI: Isolectin B4 binding neurons are not present in the rat knee joint. *Neuroscience* 128:555-560, 2004

15. Jasmin L, Carstens E, Basbaum AI: Interneurons presynaptic to rat tail-flick motoneurons as mapped by transneuronal transport of pseudorabies virus: Few have long ascending collaterals. *Neuroscience* 76:859-876, 1997

16. Kitchener PD, Wilson P, Snow PJ: Selective labelling of primary sensory afferent terminals in lamina II of the dorsal horn by injection of *Bandeiraea simplicifolia* isolectin B4 into peripheral nerves. *Neuroscience* 54:545-551, 1993

17. Lingueglia E: Acid-sensing ion channels in sensory perception. *J Biol Chem* 282:17325-17329, 2007

18. Liu M, Willmott NJ, Michael GJ, Priestley JV: Differential pH and capsaicin responses of *Griffonia simplicifolia* IB4 (IB4)-positive and IB4-negative small sensory neurons. *Neuroscience* 127:659-672, 2004

19. McDougall JJ: Arthritis and pain: Neurogenic origin of joint pain. *Arthritis Res Ther* 8:220, 2006

20. Molliver DC, Immke DC, Fierro L, Pare M, Rice FL, McCleskey EW: ASIC3, an acid-sensing ion channel, is expressed in metaboreceptive sensory neurons. *Mol Pain* 1:35, 2005

21. Molliver DC, Snider WD: Nerve growth factor receptor TrkA is down-regulated during postnatal development by a subset of dorsal root ganglion neurons. *J Comp Neurol* 381:428-438, 1997

22. Nakajima T, Ohtori S, Inoue G, Koshi T, Yamamoto S, Nakamura J, Takahashi K, Harada Y: The characteristics of dorsal-root ganglia and sensory innervation of the hip in rats. *J Bone Joint Surg Br* 90:254-257, 2008

23. Neugebauer V, Rumenapp P, Schaible HG: Calcitonin gene-related peptide is involved in the spinal processing of mechanosensory input from the rat's knee joint and in the generation and maintenance of hyperexcitability of dorsal horn-neurons during development of acute inflammation. *Neuroscience* 71:1095-1109, 1996

24. O'Brien C, Woolf CJ, Fitzgerald M, Lindsay RM, Molander C: Differences in the chemical expression of rat primary afferent neurons which innervate skin, muscle or joint. *Neuroscience* 32:493-502, 1989

25. Ohtori S, Inoue G, Koshi T, Ito T, Doya H, Saito T, Moriya H, Takahashi K: Up-regulation of acid-sensing ion channel 3 in dorsal root ganglion neurons following application of nucleus pulposus on nerve root in rats. *Spine* 31:2048-2052, 2006

26. Price MP, McIlwrath SL, Xie J, Cheng C, Qiao J, Tarr DE, Sluka KA, Brennan TJ, Lewin GR, Welsh MJ: The DRASIC cation channel contributes to the detection of cutaneous touch and acid stimuli in mice. *Neuron* 32:1071-1083, 2001

27. Radhakrishnan R, Moore SA, Sluka KA: Unilateral carrageenan injection into muscle or joint induces chronic bilateral hyperalgesia in rats. *Pain* 104:567-577, 2003

28. Rebelo S, Chen ZF, Anderson DJ, Lima D: Involvement of DRG11 in the development of the primary afferent nociceptive system. *Mol Cell Neurosci* 33:236-246, 2006

29. Schaible HG, Grubb BD: Afferent and spinal mechanisms of joint pain. *Pain* 55:5-54, 1993

30. Schaible HG, Schmelz M, Tegeder I: Pathophysiology and treatment of pain in joint disease. *Adv Drug Deliv Rev* 58:323-342, 2006

31. Simone DA, Sorkin LS, Oh U, Chung JM, Owens C, LaMotte RH, Willis WD: Neurogenic hyperalgesia: Central neural correlates in responses of spinothalamic tract neurons. *J Neurophysiol* 66:228-246, 1991

32. Sluka KA: Stimulation of deep somatic tissue with capsaicin produces long-lasting mechanical allodynia and heat hypoalgesia that depends on early activation of the cAMP pathway. *J Neurosci* 22:5687-5693, 2002

33. Sluka KA, Price MP, Breese NM, Stucky CL, Wemmie JA, Welsh MJ: Chronic hyperalgesia induced by repeated acid injections in muscle is abolished by the loss of ASIC3, but not ASIC1. *Pain* 106:229-239, 2003

34. Sluka KA, Radhakrishnan R, Benson CJ, Eshcol JO, Price MP, Babinski K, Audette KM, Yeomans DC, Wilson SP: ASIC3 in muscle mediates mechanical, but not heat, hyperalgesia associated with muscle inflammation. *Pain* 129:102-112, 2007

35. Staton PC, Wilson AW, Bountra C, Chessell IP, Day NC: Changes in dorsal root ganglion CGRP expression in a chronic inflammatory model of the rat knee joint: Differential modulation by rofecoxib and paracetamol. *Eur J Pain* 11:283-289, 2007

36. Ugawa S, Ueda T, Ishida Y, Nishigaki M, Shibata Y, Shimada S: Amiloride-blockable acid-sensing ion channels are leading acid sensors expressed in human nociceptors. *J Clin Invest* 110:1185-1190, 2002

37. Vecchiet L, Vecchiet J, Giamberardino MA: Referred muscle pain: Clinical and pathophysiologic aspects. *Curr Rev Pain* 3:489-498, 1999

38. Voilley N, de Weille J, Mamet J, Lazdunski M: Nonsteroid anti-inflammatory drugs inhibit both the activity and the inflammation-induced expression of acid-sensing ion channels in nociceptors. *J Neurosci* 21:8026-8033, 2001

39. Wemmie JA, Chen J, Askwith CC, Hruska-Hageman AM, Price MP, Nolan BC, Yoder PG, Lamani E, Hoshi T, Freeman JH Jr., Welsh MJ: The acid-activated ion channel ASIC contributes to synaptic plasticity, learning, and memory. *Neuron* 34:463-477, 2002

40. Wemmie JA, Price MP, Welsh MJ: Acid-sensing ion channels: advances, questions and therapeutic opportunities. *Trends Neurosci* 29:578-586, 2006

41. Zhang W, Moskowitz RW, Nuki G, Abramson S, Altman RD, Arden N, Bierma-Zeinstra S, Brandt KD, Croft P, Doherty M, Dougados M, Hochberg M, Hunter DJ, Kwoh K, Lohmander LS, Tugwell P: OARSI recommendations for the management of hip and knee osteoarthritis, II: OARSI evidence-based, expert consensus guidelines. *Osteoarthritis Cartilage* 16:137-162, 2008

42. Ziegler EA, Magerl W, Meyer RA, Treede RD: Secondary hyperalgesia to punctate mechanical stimuli: Central sensitization to A-fibre nociceptor input. *Brain* 122(Pt 12):2245-2257, 1999