Anesthesiology 2010; 113:647–54

A Role for Acid-sensing Ion Channel 3, but Not Acid-sensing Ion Channel 2, in Sensing Dynamic Mechanical Stimuli

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ABSTRACT

Background: Acid-sensing ion channels 2 and 3 (ASIC2 and ASIC3, respectively) have been implicated as putative mechanotransducers. Because mechanical hyperalgesia is a prominent consequence of nerve injury, we tested whether male and female ASIC2 or ASIC3 knockout mice have altered responses to mechanical and heat stimuli at baseline and during the 5 weeks after spinal nerve ligation.

Methods: Age-matched, adult male and female ASIC2 knockout (n = 21) and wild-type (WT; n = 24) mice or ASIC3 knockout (n = 20) and WT (n = 19) mice were tested for sensitivity to natural stimuli before and after spinal nerve ligation surgery. All animals were first tested for baseline sensitivity to mechanical and heat stimuli and in a novel dynamic mechanical stimulation test. The same testing procedures were then repeated weekly after spinal nerve injury.

Results: Compared with their respective WT counterparts, ASIC2 and ASIC3 knockout mice had normal baseline sensitivity to standard mechanical and heat stimuli. However, when exposed to a novel stroking stimulus to test sensitivity to dynamic mechanical stimulation, ASIC3 knockout mice were significantly more sensitive than were WT mice. After spinal nerve ligation, ASIC2 and ASIC3 knockout mice developed mechanical and heat hyperalgesia comparable with that of their respective WT controls. In addition, in both experiments, female mice were more sensitive than male mice to heat at baseline and after the nerve injury.

Conclusions: We conclude that ASIC2 and ASIC3 channels are not directly involved in the development or maintenance of neuropathic pain after spinal nerve ligation. However, the ASIC3 channel significantly modulates the sensing of dynamic mechanical stimuli in physiologic condition.

What We Already Know about This Topic

- Acid-sensing ion channels (ASICs) are proteins on sensory neurons thought to be involved in mechanosensation.
- Increased mechanosensitivity is a prominent feature in preclinical models of neuropathic pain.

What This Article Tells Us That Is New

- Mice lacking ASIC 2 or 3 proteins still exhibited mechanical sensitivity after nerve injury, suggesting that strategies to block these channels may not reduce allodynia in patients with peripheral neuropathic pain.

THE degenerin/epithelial sodium family of ion channels is a major group of proton-gated, sodium channels believed to participate in transducing mechanical stimuli into neuronal action potentials. Whereas these channels have been demonstrated as one of the essential mechanotransducers in the nematode *Caenorhabditis elegans*, their putative role as mechanoreceptors in mammals has been more controversial.[1–3]

Acid-sensing ion channel 2 (ASIC2; also known as BNaC1, BNC1, and MDEG), a member of the degenerin/epithelial sodium family, is expressed in the mammalian pe-

Supplemental digital content is available for this article. Direct URL citations appear in the printed text and are available in both the HTML and PDF versions of this article. Links to the digital files are provided in the HTML text of this article on the Journal's Web site (www.anesthesiology.org).

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Received from the Department of Anesthesiology and Critical Care Medicine and the Department of Neurosurgery, The Johns Hopkins University, Baltimore, Maryland. Submitted for publication December 16, 2009. Accepted for publication April 30, 2010. Supported in part by grant NS26363 from the National Institute of Neurological Disorders and Stroke (Washington, D.C.). This work has previously been presented in part at the Society for Neuroscience, Washington, D.C., November 12–16, 2005, and The International Association for the Study of Pain, Sydney, Australia, August 21–26, 2005.

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ripheral nervous system, predominantly in large dorsal root ganglion cells and cutaneous mechanosensory terminals, and in the brain.[4,5] ASIC2 expression, distribution, and activation properties are suggestive of a role in tissue acidosis and mechanotransduction. In fact, both rapidly adapting (RA) and slowly adapting low-threshold mechanoreceptors in ASIC2 knockout mice showed decreased excitability to mechanical stimulation compared with those in wild-type (WT) mice when studied in an *in vitro* skin-nerve preparation.[6] However, two other studies in knockout mice concluded that ASIC2 has a very limited role in mechanosensation.[1,3] The only study that tested a behavioral phenotype in ASIC2 knockout animals found that knockout mice show increased pain responses in the formalin test and greater mechanical hyperalgesia after inflammation with complete Freund's adjuvant.[7] To date, no studies have reported on the role of ASIC2 channels in neuropathic pain.

Acid-sensing ion channel 3 (ASIC3; also known as dorsal root acid-sensing ion channel or TNaC) is almost exclusively expressed in the sensory neurons, more specifically in small- and large-diameter dorsal root ganglion neurons and in the peripheral sensory nerve terminals.[8] Therefore, ASIC3 may be involved in mechanotransduction and nociceptive signaling.[8] Indeed, deletion of ASIC3 in mice alters electrophysiological properties of mechanoreceptors and behavioral responses to noxious stimuli.[8,9] Compared with those in WT mice, large-fiber, RA mechanoreceptors are more responsive in ASIC3 knockout mice, whereas Aδ fiber mechanonociceptors show attenuated responses to mechanical stimuli.[8] The behavior of ASIC3 knockout or transgenic (dominant-negative ASIC3 subunit) mice has been studied by several groups with mixed results.[8–11] In response to acidic saline injection into gastrocnemius muscle, Sluka *et al.*[11] reported reduced inflammatory hyperalgesia in the knockout mice, whereas Mogil *et al.*[10]found that the transgenic mice had greater mechanical hyperalgesia compared with the WT controls. Other inflammatory stimuli tested (acetic acid, carrageenan, zymosan, formalin, complete Freund's adjuvant) resulted in a more sensitive phenotype in the knockout or transgenic mice or produced a similar effect in mutated and WT mice.[8–10]

Peripheral neuropathies are characterized by abnormal sensitivity to mechanical and thermal stimuli.[12–17] Pain in reaction to stroking stimuli is a common clinical complaint of patients.[18–20] Given that ASIC2 and ASIC3 channels seem to influence properties of low-threshold RA neurons as well as a subpopulation of nociceptors, we postulated that these channels might be involved in the development of allodynia and/or hyperalgesia after spinal nerve ligation (SNL). In addition, because sex was previously a significant factor in the sensitivity of ASIC3 transgenic mice to mechanical stimuli after inflammation, we included both male and female mice in our studies.[21] This is the first set of *in vivo* experiments designed to examine whether deletion of ASIC2 or ASIC3 channels plays a role in neuropathic pain.

Materials and Methods

Subjects and Surgical Procedures

For the first experiment, age-matched, adult WT (n = 24) and ASIC2 knockout (n = 21) male and female mice were used. Age-matched WT (n = 19) and ASIC3 knockout (n = 20) male and female mice were used in the second experiment. Mice were maintained on a 14/10-h light/dark cycle and were provided with food and water *ad libitum*. All testing was performed during the animals' day cycle. To minimize stress and equalize handling time with male mice, female mice were not evaluated for the stage of estrous cycle. However, baseline response values for all outcomes were averaged from measurements obtained on three different days and therefore are likely representative of values across different stages of the estrous cycle. Breeding pairs for both colonies were obtained by a generous donation from Margaret Price, Ph.D., and Michael Welsh, M.D.[6,8] The mice of both colonies have a C57BL6/129 background and were backcrossed to pure C57BL/6 strain for at least two generations. All mice were bred in-house and were derived from their respective colonies. ASIC2 mice were 4–6 months old at the onset of experiments, average female and male weights being 24.4 ± 0.7 and 30.3 ± 0.5 g, respectively. ASIC3 mice were approximately 6 months old at the onset of experiments, average female and male weights being 25.0 ± 0.5 and 36.7 ± 0.9 g, respectively.

After acclimation and baseline test periods, animals underwent ligation/transection of the left middle spinal nerve that forms the sciatic nerve (most likely L4).[22,23] Mice were anesthetized with sodium pentobarbital (75 mg/kg) and placed in a prone position. The L5 transverse process was visualized through a microscope and cut to expose the L3 and L4 spinal nerves. The L4 spinal nerve was then tightly ligated with 7-0 silk sutures and cut just distal to the ligation site. Great care was taken not to damage the L3 spinal nerve during the procedure. The wound was then cleaned with 10% povidone-iodine antiseptic, the muscle layer closed with 4-0 sutures, and the skin closed with metal clips. At the conclusion of experiments, the ligation site was always verified as the middle spinal nerve that contributes to the sciatic nerve, although the lumbar levels were not counted from the ribs.[23] In the ASIC2 experiment, six female and nine male mice had to be excluded from the analyses after the SNL because the autopsy revealed ligation of the L3 nerve. In addition, one female mouse died 2 weeks after the ligation and was not included in subsequent analyses. The Institutional Animal Care and Use Committee at The Johns Hopkins University (Baltimore, Maryland) approved the experimental protocol, and the studies were performed according to the guidelines of the Committee for Research and Ethical Issues of the International Association for the Study of Pain.[24]

Behavioral Testing

Experimenters were blinded to the mouse genotype in all experiments. Mice were acclimated to the testing equipment for at least 3 days before baseline testing. Room temperature of the behavioral testing facility was maintained at 22 ±

0.5°C. All baseline values represent average responses from the left and right hind paws.

Sensitivity to punctate mechanical stimuli was tested by using an ascending series of von Frey hairs (0.05, 0.25, 0.74, 2.1 g) that were applied to the middle of the plantar paw for 3–4 s. The intertrial interval was at least 1 min. The number of paw withdrawals over five trials was recorded and converted to a percentage response rate for each animal. In addition, the average response across all von Frey probes was computed for each animal. In experiments with ASIC3 knockout animals, we also tested their sensitivity to pressure with a Randall-Selitto device[25] applied to the hind foot (Ugo Basile Biologic Research Apparatus, Comerio-Varese, Italy). Animals were acclimated to the Randall-Selitto testing procedure at least 2 days before data collection. Median of three trials on each paw with at least 15 min between trials was taken as a measure of sensitivity to pressure.

To test responsiveness to a dynamic mechanical stimulus, a 0.74-g von Frey hair was gently stroked across the midplantar surface of each paw. The von Frey probe was applied at a 30–45° angle so that one edge of the probe tip was against the skin and bent slightly to achieve a relatively constant force. The average force exerted by this motion was 0.016 g. The tip was moved across the skin at a rate of approximately 10 mm/s. This stroking stimulus was applied five times at least 1 min apart, and the number of times the animal withdrew its paw was converted to a percentage response rate.

Heat sensitivity was assessed by measuring the paw withdrawal latency to a radiant heat stimulus (IITC Life Science Inc, Woodland Hills, CA) applied to the plantar surface of the foot.[26] Animals were placed on a heated glass surface (29°C) and allowed to habituate for 30 min before testing. A cutoff of 30 s was imposed to prevent tissue damage. The median of five trials was used as a measure of heat-induced paw withdrawal threshold. Animals were tested at baseline, 2 days after surgery, and then once a week for 5 weeks after surgery. Baseline median responses from two test sessions were averaged for each animal.

Punctate and stroking mechanical testing as well as radiant heat testing was always done on the same test day, starting with punctate mechanical, followed by stroking, and then heat testing. Randall-Selitto testing was performed on days separate from any other behavioral testing. Sham-operated animals were not included in our experiments because we wanted to minimize the number of animals that had to be used in the experiments. We used the contralateral paw responses as an alternate control and an indication of changes that may occur as a result of repeated testing.

Statistics

All statistical analyses were performed using Statistica 6.1 software (StatSoft, Inc., Tulsa, OK). All tests were run as two-tailed tests with a significance level of P less than 0.05. All results are presented as means ± SE. Data from the ASIC2 experiment were analyzed separately from data testing animals from the ASIC3 colony. Mechanical paw withdrawal frequencies were analyzed using nonparametric analyses. Baseline responses were evaluated using Friedman ANOVA followed by Wilcoxon matched-pairs tests to compare significant effects of force, and Kruskal-Wallis followed by Mann–Whitney U tests to evaluate effects of sex and genotype. The significance value was adjusted for the number of multiple comparisons made. The same nonparametric tests were used after the nerve injury to evaluate the effects of time, force, paw, sex, and genotype. Average latencies to respond to pressure using Randall-Selitto device were evaluated using a 2 × 2 factorial ANOVA with between-subjects factors sex and genotype. Baseline paw withdrawal latencies to heat were compared using a 2 × 2 factorial ANOVA with between-subjects factors sex and genotype. Significant interaction effects were further analyzed using Bonferroni *post hoc* tests. Responses to heat after SNL were analyzed using a 2 × 2 × 2 × 7 mixed-model ANOVA with between-subjects factors sex (female *vs.* male) and genotype (WT *vs.* knockout), and within-subject factors paw (ipsilateral *vs.* contralateral) and time (baseline and 5 weeks of testing after SNL). Significant interactions were analyzed using Bonferroni *post hoc* tests.

Results

The Role of ASIC2 Channel in Baseline Responses to Mechanical and Heat Stimuli

ASIC2 WT (12 male, 12 female) and knockout (11 male, 10 female) mice were not different in their responses to punctate von Frey fibers regardless of force (fig. 1A). Dynamic stroking stimulation with the 0.74-g von Frey hair did not result in a different phenotype between WT and knockout mice (fig. 1B), and there were no significant differences based on sex.

As expected, male mice had longer latencies to heat stimulation (11.7 ± 0.4 s) than did female mice [9.0 ± 0.4 s; $F(1,41) = 23.23$, P less than 0.0001]. However, this effect was not influenced by genotype; *i.e.*, ASIC2 knockout and WT mice had similar paw withdrawal latencies regardless of sex (fig. 1C).

The Role of ASIC3 Channel in Baseline Responses to Mechanical and Heat Stimuli

Three types of mechanical stimulation were used during baseline testing: punctate mechanical testing with von Frey hairs, dynamic mechanical testing by stroking, and pressure stimulation with a Randall-Selitto device. Overall, 9 WT female mice, 10 WT male mice, 5 ASIC3 knockout female mice, and 15 ASIC3 knockout male mice were used in all behavioral tests. During punctate von Frey testing, male and female mice responded comparably except at the highest force (2.1 g), at which males showed greater sensitivity (67.7 ± 2.6%) than did females (57.7 ± 1.9%; Mann–Whitney U test, $z = -2.56$, P less than 0.04 after correcting for multiple comparisons). However, knockout mice did not differ from WT animals at any force tested (fig. 2A). It is noteworthy that, in response to dynamic mechanical stimulation by stroking with the 0.74-g von Frey fiber, ASIC3

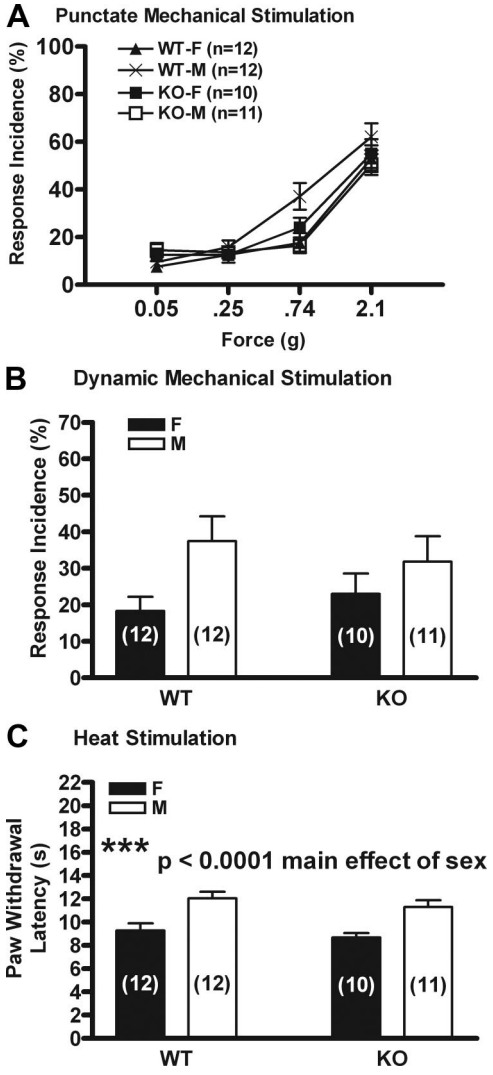

Fig. 1. Baseline responses of acid-sensing ion channel 2 (ASIC2) knockout (KO) and wild-type (WT) mice did not differ. Data are shown as mean ± SE in all figures. (*A*) ASIC2 KO (n = 20) and WT (n = 24) mice showed a similar stimulus response function to application of a punctate mechanical stimulus. The average percentage of trials that elicited a withdrawal response after application of a von Frey probe is plotted as a function of force. (*B*) ASIC2 KO mice and WT mice showed a similar response to stroking of the foot with a von Frey filament. The average percentage of trials in which the animals responded to the stroking stimulus is plotted. Values in parentheses indicate sample sizes. (*C*) ASIC2 KO mice and WT mice showed a similar response to radiant heat stimuli. The average withdrawal latency is plotted. For a given mouse, the baseline responses obtained after stimulation of the left and right paw were averaged together. Values in parentheses indicate sample sizes. *** $P <$ 0.0001 main effect of sex. F = female; M = male.

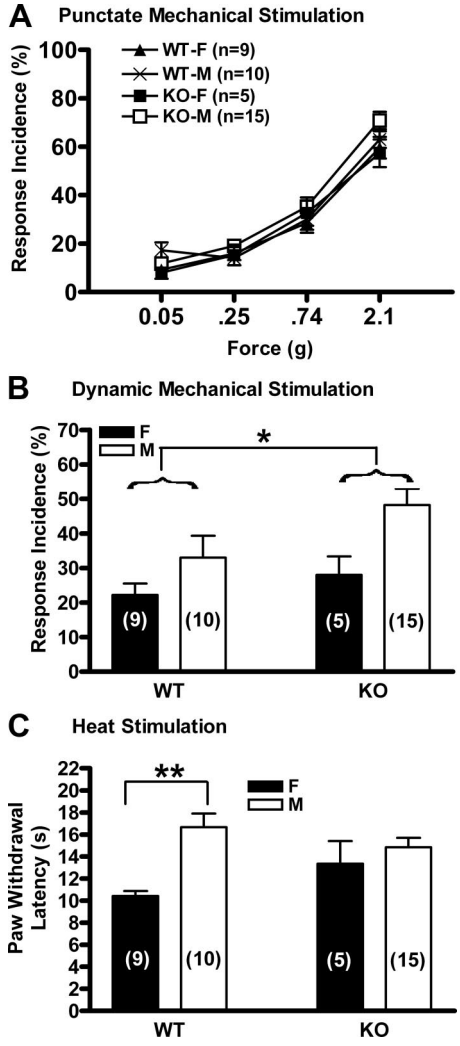

Fig. 2. Acid-sensing ion channel 3 (ASIC3) knockout (KO) mice differed from wild-type (WT) mice in their baseline responses to a stroking mechanical stimulus. Data are shown as mean ± SE in all figures. (*A*) In response to punctate von Frey stimulation, ASIC3 KO (n = 20) and WT (n = 19) mice displayed similar stimulus response functions. The average percentage of trials that resulted in paw withdrawal is plotted as a function of force. (*B*) ASIC3 KO mice had greater sensitivity to stroking with a 0.74-g von Frey hair than did ASIC3 WT mice (* $P <$ 0.02). The percentage response was calculated and plotted as the average percentage of stroking trials that resulted in a withdrawal response. Values in parentheses indicate sample sizes. (*C*) ASIC3 WT and KO mice responded similarly to radiant heat stimuli. The average withdrawal latency was calculated and plotted by averaging baseline left and right paw median responses from five trials. Values in parentheses indicate sample sizes. ** $P <$ 0.002 WT male *versus* WT female mice. F = female; M = male.

knockout animals showed significantly greater response frequency than their WT counterparts (fig. 2B, Mann–Whitney U test, *P* less than 0.02 after correction). Overall, males were more sensitive to dynamic stroking stimulation than were females (42.1 ± 4 *vs.* 24.3 ± 2.8%; Mann–Whitney U test, $z = -2.88$, $P = 0.016$ after correction). There were no differences in mechanical threshold between the groups based on genotype or sex when tested with a Randall-Selitto device (data not shown).

Analysis of variance on baseline paw withdrawal latency values showed no difference between ASIC3 knockout and WT mice (fig. 2C) but indicated a significant effect of sex

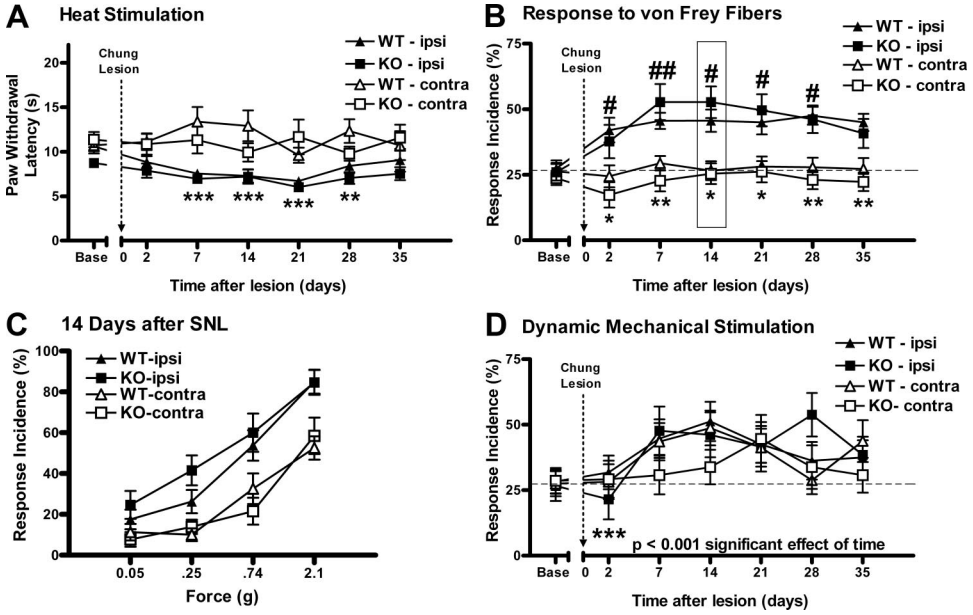

Fig. 3. The responses of acid-sensing ion channel 2 (ASIC2) knockout (KO; n = 13) and wild-type (WT; n = 17) mice did not differ after spinal nerve ligation (SNL). Data are shown as mean ± SE in all figures. (*A*) SNL resulted in significant heat hyperalgesia in the ipsilateral (ipsi) paw, but no response difference was apparent based on genotype of the animals. ASIC2 KO and WT mice had comparable decreases in response latency (relative to baseline levels) after the nerve injury. The average withdrawal latency to a radiant heat stimulus is plotted as a function of time after the lesion. ** $P < 0.01$; *** $P < 0.001$ ipsilateral *versus* contralateral (contra) paw. (*B*) SNL resulted in significant hyperalgesia to punctate mechanical stimuli in the ipsilateral paw, but mouse genotype did not influence the level of response. The ASIC2 KO and WT mice did not differ in their responses to mechanical stimulation across 5 weeks after the SNL. Both KO and WT animals displayed increased responses to mechanical stimulation after the nerve injury compared with their responses at baseline. The average response to the four von Frey forces is plotted as a function of time after the SNL. * $P < 0.05$; ** $P < 0.01$ ipsilateral *versus* contralateral paw in WT animals; # $P < 0.05$; ## $P < 0.01$ ipsilateral *versus* contralateral paw in KO mice. (*C*) An example of response frequencies to each von Frey stimulus (14 days after the SNL, marked with a *rectangle* in *B*). ASIC2 WT and KO mice did not differ in their responses to any force applied. See also the figure in Supplemental Digital Content 1, http://links.lww.com/ALN/A608. (*D*) SNL resulted in significantly enhanced responses to the mechanical stimulus of stroking in both the ASIC2 KO and WT mice. The average percentage of trials in which the animal responded to stroking of the skin with a von Frey filament is plotted as a function of time after the lesion. *** $P < 0.001$ significant effect of time by Friedman analysis of variance.

[$F(1,35) = 11.1$, $P = 0.002$] and a significant genotype by sex interaction [$F(1,35) = 4.2$, P less than 0.05]. The only significant comparison obtained with the Bonferroni *post hoc* test was that WT female mice were more sensitive than WT male mice to heat stimulation (P less than 0.002).

The Role of ASIC2 and ASIC3 Channels in Neuropathic Pain

After SNL, both ASIC2 WT and knockout animals developed punctate and stroking mechanical hyperalgesia as well as heat hyperalgesia in the ipsilateral paw (fig. 3). There was no effect of genotype on any of the outcome measures. Sex of the animals affected the paw withdrawal latency so that female mice had greater heat hyperalgesia than did male mice after injury (main effect of sex; [$F(1,25) = 8.15$, P less than 0.01]). However, this effect was not dependent on the animals' genotype (no significant interaction). Mixed model ANOVA on paw withdrawal latencies also indicated a main effect of paw and a significant paw × time interaction. When comparing ipsilateral and contralateral paws over time in the Bonferroni *post hoc* comparisons, the results showed a significant effect on days 7, 14, 21, and 28 after the lesion (fig. 3A). Friedman ANOVA analysis on responses to punctate mechanical stimuli indicated a significant effect of time (P less than 0.0001). Wilcoxon matched-pairs comparisons of ipsilateral and contralateral paw were significantly different on all days except 35 days after the lesion after correcting for multiple comparisons (fig. 3B). However, knockout and WT mice did not differ in their responses on any given day (fig. 3B) or in their sensitivity to different forces of von Frey hairs (fig. 3C and the figure in Supplemental Digital Content 1, http://links.lww.com/ALN/A608). Friedman ANOVA analysis on stroking stimulation also showed a significant effect of time (P less than 0.001). After correcting for multiple comparisons using Wilcoxon matched-pairs tests, there were no significant differences between ipsilateral and contralateral paw responses (fig. 3D).

ASIC3 knockout also did not influence the development or maintenance of neuropathic pain after the nerve injury. Figure 4 and the figure in Supplemental Digital Content 2, http://links.lww.com/ALN/A609, show that animals developed heat and mechanical hyperalgesia after ligation; again,

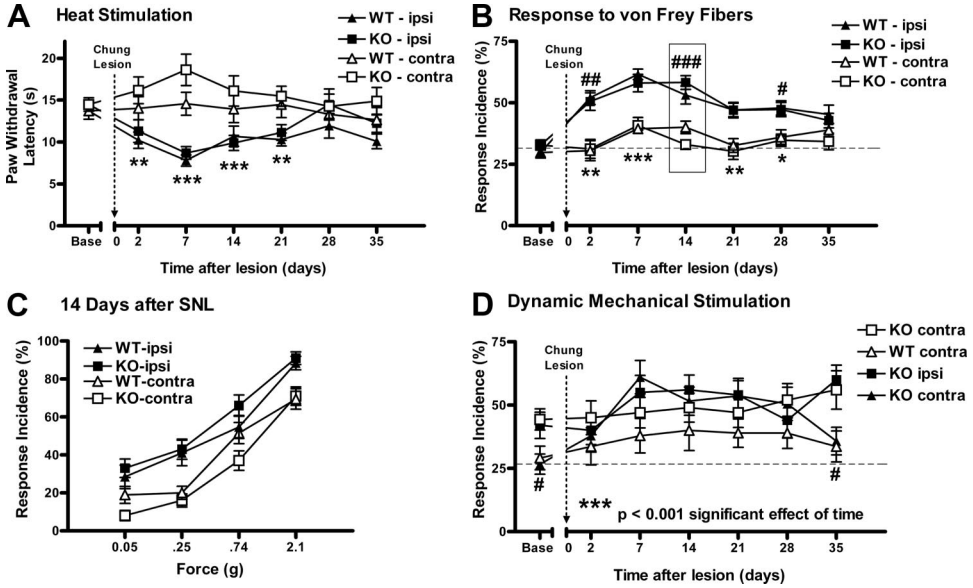

Fig. 4. The responses of acid-sensing ion channel 3 (ASIC3) knockout (KO; n = 20) and wild-type (WT; n = 19) mice did not differ after spinal nerve ligation (SNL). Data are shown as mean ± SE in all figures. (*A*) SNL resulted in significant heat hyperalgesia in both ASIC3 KO and WT mice. The average withdrawal latency to a radiant heat stimulus is plotted as a function of time after the lesion. ** $P < 0.01$; *** $P < 0.001$ ipsilateral (ipsi) *versus* contralateral (contra) paw. (*B*) SNL resulted in significant and comparable hyperalgesia to punctate mechanical stimuli in ASIC3 KO and WT mice. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ ipsilateral *versus* contralateral paw in WT animals; # $P < 0.05$; ## $P < 0.01$; ### $P < 0.001$ ipsilateral *versus* contralateral paw in KO mice. (*C*) Detailed representation of the response frequency to different von Frey forces 14 days after the SNL (marked with a rectangle in *B*). WT and KO animals did not significantly differ in their sensitivity to any force tested. See also the figure in Supplemental Digital Content 2, http://links.lww.com/ALN/A609. (*D*) SNL resulted in significantly enhanced responses to the mechanical stimulus of stroking in both the ASIC3 KO and WT mice. The average percentage of trials in which the animal responded to stroking of the skin with a von Frey filament is plotted as a function of time after the lesion. Although ASIC3 KO animals were more responsive than WT mice to stroking stimulation at baseline, the level of hyperalgesia that developed after nerve injury was comparable in the two genotypes. *** $P < 0.001$ significant effect of time by Friedman analysis of variance. # $P < 0.05$ knockout *versus* WT mice.

this was not dependent on genotype except with the stroking stimulation (fig. 4D). Even with stroking stimulation, the differences between knockout and WT mice were significant only at baseline and again at 35 days after the injury when there are signs of recovery. The level of heat hyperalgesia was again dependent on sex but not genotype, female mice being more sensitive than male mice to heat stimulation [$F(1,35) = 20.76$, P less than 0.0001].

Discussion

The putative mechanoreceptor channels ASIC2 and ASIC3 seem to have a limited role in neuropathic pain. Our results suggest two important conclusions. First, under baseline conditions, ASIC3 channels are important for signaling the response to dynamic mechanical stimuli. Otherwise, ASIC2 and ASIC3 channels have a very limited contribution to the behavioral responses to mechanical or heat stimuli under normal physiologic conditions. Second, ASIC2 and ASIC3 channels do not alter the development or maintenance of neuropathic pain after nerve lesion. Although we cannot rule out potential compensatory effects of specific ASIC gene deletion, our findings suggest that ASIC channel contribution to low threshold mechanoreceptors is not critical in signaling mechanical hyperalgesia after SNL.

In agreement with other published reports, specific deletion of ASIC2 or ASIC3 channels did not alter baseline responses to punctate mechanical or heat stimuli when measured as a frequency of response to von Frey probes and by radiant heat, respectively.[10,11] It is noteworthy that our experiments also employed a novel testing paradigm designed to specifically test sensitivity to dynamic mechanical stimuli. Dynamic mechanical stimulation such as stroking is often extremely painful to patients with peripheral nerve injuries.[18–20] Because the average force applied with our stroking stimulus was 0.016 g, this stimulus most likely does not significantly activate nociceptors, but does activate RA and slowly adapting mechanosensors. Although there are nociceptors that may respond to a brush stimulus, electrophysiological data indicate that responsiveness to brush is a trademark of RA and/or slowly adapting mechanosensors.[27,28] Electrophysiological data from both ASIC2 and ASIC3 knockout mice implicate these channels as modulators of tactile stimuli.[6,8] Specifically, RA mechanosensors that respond best to dynamic mechanical stimulation showed increased responsiveness in ASIC3 knockout animals. However, reports are conflicting regarding the ASIC2 contribution to RA fiber function. The study by Price *et al.*[6] found that the stimulus-response curve of RA mechanore-

ceptors was attenuated in ASIC2 knockout animals compared with WT mice. In contrast, using the same skin-nerve preparation, Roza *et al.*[3] reported no differences in mechanical response patterns of any afferents between ASIC2 knockout and WT mice.

Our stroking stimulus, which used a single von Frey hair, detected a behavioral phenotype in ASIC3 knockout mice. ASIC3 knockout mice were more sensitive to dynamic stimulation than were their WT counterparts. This finding supports the argument that ASIC3 channels, but not ASIC2 channels, are significant modulators of mechanosensation *in vivo* and is in agreement with the results of Roza *et al.*[3] It is also possible that ASIC2 channel contribution to mechanotransduction differs between hairy and glabrous skin. The electrophysiological studies that indicated a role for ASIC2 and ASIC3 channels in RA mechanoreceptors were conducted by stimulating hairy skin, whereas all of our testing was performed on the plantar surface of the paw.[6] However, the most likely conclusion is that ASIC3 channels have a greater role, although limited, in mechanosensory function than do ASIC2 channels.

Our experiments included male and female mice to assess potential interactions between the factors of genotype and sex. Although the literature on baseline sex differences in sensitivity to painful stimuli is not uniform, our results are mostly consistent with previously reported results in the C57BL/6 mouse strain. In both ASIC2 and ASIC3 experiments, female mice were more sensitive to radiant heat stimulation than were male mice, a finding that supports those of other noxious heat test results reported by Kest *et al.*[29] Males from only the ASIC3 colony were more sensitive to punctate von Frey stimulation than their female counterparts, a result that is consistent with the findings of Bourquin *et al.* (2006),[30] but not of Kest *et al.* (1999).[29] However, in our ASIC3 experiment, males were also more sensitive than females to stroking, a finding that is consistent with the direction of our punctate stimulation results. The fact that we did not observe sex differences in mechanical threshold in mice from the ASIC2 colony or in response to a pressure test in the ASIC3 mice adds to the complexity of the interpretation of the role of sex in baseline sensitivity to noxious stimulation and argues for only a modest effect of sex in this modality. After nerve injury, female mice in both colonies developed greater heat hyperalgesia than did male mice, but there were no sex differences in the sensitivity to mechanical stimulation.

We were surprised to find that, after SNL, both ASIC2 knockout and ASIC3 knockout mice developed levels of hyperalgesia that were similar to those of their WT counterparts in all of the behavioral assays. Studies in humans with peripheral nerve injuries have shown that pain to light touch is signaled predominantly by large myelinated afferents, either sensitized Aβ nociceptors or low-threshold mechanoreceptors.[31] More recently, it has been suggested that low threshold C-mechanoreceptors that express vesicular glutamate transporter 3 may play an important role in mechanical hypersensitivity after injury.[32] Our results suggest that ASIC2 and ASIC3 channels have a limited contribution to hyperalgesia in peripheral neuropathies.

ASIC channels have an important role in inflammation,[7–11] so perhaps they do play a role in neuropathic states with an inflammatory component, such as complex regional pain syndrome. However, our behavioral data suggest that ASIC channels are not the primary transduction channels responsible for mechanical hyperalgesia in neuropathic pain.

The authors thank Margaret Price, Ph.D. (Associate Research Scientist, Department of Internal Medicine, Roy J. and Lucille A. Carver College of Medicine, University of Iowa, Iowa City, Iowa), and Michael Welsh, M.D. (Investigator, Howard Hughes Medical Institute, Chevy Chase, Maryland, and Roy J Carver Chair in Internal Medicine and Physiology and Biophysics, University of Iowa), for their generous contribution of acid-sensing ion channels 2 and 3 knockout breeding pairs. The authors thank Tim Hartke, B.S. (Technician, Department of Neurosurgery, The Johns Hopkins University, Baltimore, Maryland), for his technical support during the experiments and Claire Levine, M.S., E.L.S. (Scientific Editor, Department of Anesthesiology and Critical Care Medicine, The Johns Hopkins University), for her editorial help.

References

1. Drew LJ, Rohrer DK, Price MP, Blaver KE, Cockayne DA, Cesare P, Wood JN: Acid-sensing ion channels ASIC2 and ASIC3 do not contribute to mechanically activated currents in mammalian sensory neurones. J Physiol 2004; 556:691–710
2. O'Hagan R, Chalfie M, Goodman MB: The MEC-4 DEG/ENaC channel of *Caenorhabditis elegans* touch receptor neurons transduces mechanical signals. Nat Neurosci 2005; 8:43–50
3. Roza C, Puel JL, Kress M, Baron A, Diochot S, Lazdunski M, Waldmann R: Knockout of the ASIC2 channel in mice does not impair cutaneous mechanosensation, visceral mechanonociception and hearing. J Physiol 2004; 558:659–69
4. García-Añoveros 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 2001; 21: 2678–86
5. Price MP, Snyder PM, Welsh MJ: Cloning and expression of a novel human brain Na^+ channel. J Biol Chem 1996; 271:7879–82
6. Price MP, Lewin GR, McIlwrath SL, Cheng C, Xie J, Heppenstall PA, Stucky CL, Mannsfeldt AG, Brennan TJ, Drummond HA, Qiao J, Benson CJ, Tarr DE, Hrstka RF, Yang B, Williamson RA, Welsh MJ: The mammalian sodium channel BNC1 is required for normal touch sensation. Nature 2000; 407:1007–11
7. Staniland AA, McMahon SB: Mice lacking acid-sensing ion channels (ASIC) 1 or 2, but not ASIC3, show increased pain behaviour in the formalin test. Eur J Pain 2009; 13:554–63
8. 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 2001; 32:1071–83
9. Chen CC, Zimmer A, Sun WH, Hall J, Brownstein MJ, Zimmer A: A role for ASIC3 in the modulation of high-intensity pain stimuli. Proc Natl Acad Sci USA 2002; 99: 8992–7
10. Mogil JS, Breese NM, Witty MF, Ritchie J, Rainville ML, Ase A, Abbadi N, Stucky CL, Séguéla P: Transgenic expression of a dominant-negative ASIC3 subunit leads to increased sensitivity to mechanical and inflammatory stimuli. J Neurosci 2005; 25:9893–901

11. 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 2003; 106:229–39
12. Ali Z, Meyer RA, Belzberg AJ: Neuropathic pain after C7 spinal nerve transection in man. Pain 2002; 96:41–7
13. Cohen SP, Raja SN: Pathogenesis, diagnosis, and treatment of lumbar zygapophysial (facet) joint pain. ANESTHESIOLOGY 2007; 106:591–614
14. Dworkin RH, Gnann JW Jr, Oaklander AL, Raja SN, Schmader KE, Whitley RJ: Diagnosis and assessment of pain associated with herpes zoster and postherpetic neuralgia. J Pain 2008; 9:837–44
15. Gibbs GF, Drummond PD, Finch PM, Phillips JK: Unravelling the pathophysiology of complex regional pain syndrome: Focus on sympathetically maintained pain. Clin Exp Pharmacol Physiol 2008; 35:717–24
16. Kudel I, Edwards RR, Kozachik S, Block BM, Agarwal S, Heinberg LJ, Haythornthwaite J, Raja SN: Predictors and consequences of multiple persistent postmastectomy pains. J Pain Symptom Manage 2007; 34:619–27
17. Zochodne DW: Diabetic polyneuropathy: An update. Curr Opin Neurol 2008; 21:527–33
18. Martin C, Solders G, Sönnerborg A, Hansson P: Painful and non-painful neuropathy in HIV-infected patients: An analysis of somatosensory nerve function. Eur J Pain 2003; 7:23–31
19. Otto M, Bak S, Bach FW, Jensen TS, Sindrup SH: Pain phenomena and possible mechanisms in patients with painful polyneuropathy. Pain 2003; 101:187–92
20. Samuelsson M, Leffler AS, Hansson P: Dynamic mechanical allodynia: On the relationship between temporo-spatial stimulus parameters and evoked pain in patients with peripheral neuropathy. Pain 2005; 115:264–72
21. Chanda ML, Mogil JS: Sex differences in the effects of amiloride on formalin test nociception in mice. Am J Physiol Regul Integr Comp Physiol 2006; 291:R335–42
22. Kim SH, Chung JM: An experimental model for peripheral neuropathy produced by segmental spinal nerve ligation in the rat. Pain 1992; 50:355–63
23. Rigaud M, Gemes G, Barabas ME, Chernoff DI, Abram SE, Stucky CL, Hogan QH: Species and strain differences in rodent sciatic nerve anatomy: Implications for studies of neuropathic pain. Pain 2008; 136:188–201
24. Zimmermann M: Ethical guidelines for investigations of experimental pain in conscious animals. Pain 1983; 16: 109–10
25. Randall LO, Selitto JJ: A method for measurement of analgesic activity on inflamed tissue. Arch Int Pharmacodyn Ther 1957; 111:409–19
26. Hargreaves K, Dubner R, Brown F, Flores C, Joris J: A new and sensitive method for measuring thermal nociception in cutaneous hyperalgesia. Pain 1988; 32:77–88
27. Edin BB, Essick GK, Trulsson M, Olsson KA: Receptor encoding of moving tactile stimuli in humans. I. Temporal Pattern of discharge of individual low-threshold mechanoreceptors. J Neurosci 1995; 15:830–47
28. Essick GK, Edin BB: Receptor encoding of moving tactile stimuli in humans. II. The mean response of individual low-threshold mechanoreceptors to motion across the receptive-field. J Neurosci 1995; 15:848–64
29. Kest B, Wilson SG, Mogil JS: Sex differences in supraspinal morphine analgesia are dependent on genotype. J Pharmacol Exp Ther 1999; 289:1370–5
30. Bourquin AF, Suveges M, Pertin M, Gilliard N, Sardy S, Davison AC, Spahn DR, Decosterd I: Assessment and analysis of mechanical allodynia-like behavior induced by spared nerve injury (SNI) in the mouse. Pain 2006; 122: 14.e1–14
31. Campbell JN, Raja SN, Meyer RA, Mackinnon SE: Myelinated afferents signal the hyperalgesia associated with nerve injury. Pain 1988; 32:89–94
32. Seal RP, Wang X, Guan Y, Raja SN, Woodbury CJ, Basbaum AI, Edwards RH: Injury-induced mechanical hypersensitivity requires C-low threshold mechanoreceptors. Nature 2009; 462:651–5