Simultaneous Disruption of Mouse ASIC1a, ASIC2 and ASIC3 Genes Enhances Cutaneous Mechnosensitivity

Sinyoung Kang¹, Jun Ho Jang¹, Margaret P. Price², Mamta Gautam², Christopher J. Benson²,³, Huiyu Gong², Michael J. Welsh²,³,⁵, Timothy J. Brennan¹,⁴*

¹ Department of Anesthesia, Roy J. and Lucille A. Carver College of Medicine, University of Iowa, Iowa City, Iowa, United States of America, ² Department of Internal Medicine, Roy J. and Lucille A. Carver College of Medicine, University of Iowa, Iowa City, Iowa, United States of America, ³ Department of Molecular Physiology and Biophysics, Roy J. and Lucille A. Carver College of Medicine, University of Iowa, Iowa City, Iowa, United States of America, ⁴ Department of Pharmacology, Roy J. and Lucille A. Carver College of Medicine, University of Iowa, Iowa City, Iowa, United States of America, ⁵ Howard Hughes Medical Institute, Howard Hughes Medical Institute, Chevy Chase, Maryland, United States of America

Abstract

Three observations have suggested that acid-sensing ion channels (ASICs) might be mammalian cutaneous mechanoreceptors; they are structurally related to Caenorhabditis elegans mechanoreceptors, they are localized in specialized cutaneous mechanosensory structures, and mechanical displacement generates an ASIC-dependent depolarization in some neurons. However, previous studies of mice bearing a single disrupted ASIC gene showed only subtle or no alterations in cutaneous mechanosensitivity. Because functional redundancy of ASIC subunits might explain limited phenotypic alterations, we hypothesized that disrupting multiple ASIC genes would markedly impair cutaneous mechanosensation. We found the opposite. In behavioral studies, mice with simultaneous disruptions of ASIC1a, -2, and -3 genes (triple-knockouts, TKOs) showed increased paw withdrawal frequencies when mechanically stimulated with von Frey filaments. Moreover, in single-fiber nerve recordings of cutaneous afferents, mechanical stimulation generated enhanced activity in A-mechanonocceptors of ASIC TKOs compared to wild-type mice. Responses of all other fiber types did not differ between the two genotypes. These data indicate that ASIC subunits influence cutaneous mechanosensitivity. However, it is unlikely that ASICs directly transduce mechanical stimuli. We speculate that physical and/or functional association of ASICs with other components of the mechanosensory transduction apparatus contributes to normal cutaneous mechanosensation.

Introduction

Acid-sensing ion channels (ASICs) represent an H⁺-gated subgroup of the degenerin/epithelial Na⁺ channel (DEG/ENaC) superfamily, which are Na⁺-selective or Na⁺-preferring cation channels sensitive to amiloride [1,2]. Structural similarity between ASICs and the degenerin mechanoreceptors (Mec-4, Mec-10 and Deg-1) from nematode Caenorhabditis elegans [3-5] raised the possibility that ASICs might also mediate mammalian mechanosensation. Almost all ASIC subunits are expressed in primary afferent sensory neurons [6,7], and ASIC2 and ASIC3 immunoreactivities were detectable in specialized cutaneous mechanosensory structures such as Meissner corpuscles, Merkel cell neurite complexes and lanceolate nerve endings surrounding the hair shaft, supporting a possible role of ASICs in cutaneous mechanosensation [8-10].

In previous studies, the involvement of ASICs in cutaneous mechanosensation was evaluated by generating mice deficient in specific ASIC subunits and assessing the behavioral and electrophysiological phenotypes in these null mice [9-12]. However, in contrast to the striking impairment of touch sensitivity observed in C. elegans with null mutations in either Mec-4 or Mec-10 [4,13], only subtle or no alterations in normal cutaneous mechanosensitivity were observed in mice with a single ASIC gene disruption. Ablation of genes for single ASIC subunits had no effect on the behavioral responses to mechanical stimuli [10,11,14]. Single fiber responses were affected in a variable manner by selective gene deletion. Reduced mechanosensitivity of the rapidly adapting (RAs) and slowly adapting low-threshold mechanoreceptors (SAs) was reported in ASIC2 null mice [9]. In ASIC2 null mice, RAs showed an enhanced mechanosensitivity, whereas Aδ-mechanonocceptors (Aδ-AMs) had a decreased sensitivity to mechanical stimuli [10]. No difference in the mechanosensitivity of afferents was found in ASIC1 null mice [11]. In a study using cultured mouse dorsal root ganglia (DRG) neurons, no differences in amplitude or kinetics of mechanically activated currents were found between ASIC2 or ASIC3 single-null mice and wild-type (WT) mice [15].

ASICs exist in heteromultimers in sensory neurons [7], and different ASIC subunits might function cooperatively in mechanosensation [16]. Considering the functional redundancy of ASIC subunits and the likelihood of functional compensation by remaining subunits that might occur during the development of

Citation: Kang S, Jang JH, Price MP, Gautam M, Benson CJ, et al. (2012) Simultaneous Disruption of Mouse ASIC1a, ASIC2 and ASIC3 Genes Enhances Cutaneous Mechanosensitivity. PLoS ONE 7(4): e35225. doi:10.1371/journal.pone.0035225

Editor: Hiroaki Matsumani, Duke University, United States of America

Received November 28, 2011; Accepted March 12, 2012; Published April 10, 2012

Copyright: © 2012 Kang et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Funding: MW is an Investigator of the HHMI (Howard Hughes Medical Institute). TJB was supported by NIH GM067762. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

* E-mail: tim-brennan@uiowa.edu
*ASIC* single-knockout mice, analysis of animals missing multiple subunits should be of value in addressing the role of *ASICs* in cutaneous mechanosensation. In this study, we therefore generated triple-knockout (TKO) mice with simultaneous disruptions of *ASIC1a*, -2 and -3 genes, and the cutaneous mechanosensitivity was evaluated in these animals using behavioral tests and single-fiber nerve recordings. Chemical responsiveness was evaluated using a combination of protons, lactate and ATP [17], and the effect of this chemical combination on the mechanical responses of cutaneous afferents was also evaluated in *ASIC TKO* and WT mice.

**Materials and Methods**

**Generation of *ASIC* 1a/2/3 Null Mice**

The generation of *ASIC1a*, -2, and -3 null mice has been described [9,10,18]. Each of these lines was backcrossed for ten generations onto a C57BL/6J background to generate congenic lines of each. Once obtained, these congenic lines were crossed to one another to generate a congenic C57BL/6J line with the simultaneous disruption of *ASIC1a*, -2, and -3 (*ASIC1a/2/3* null mice).

**Animal Care and Use**

All experimental procedures were approved by The University of Iowa Animal Care and Use Committee, Iowa City, Iowa. The animals were treated in accordance with the Ethical Guidelines for Investigations of Experimental Pain in Conscious Animals issued by the International Association for the Study of Pain [19]. Combined behavioral and electrophysiological experiments were performed on 53 *ASIC* TKO mice (27 females and 26 males) and 59 age-matched WT C57BL/6J mice control group (30 females and 29 males, Jackson Laboratory, Bar Harbor, ME). The weight of the mouse was 20–30 gm, and the age was 6–12 weeks. One to five animals were housed in plastic cages in a climate-controlled room under a 12-h light/dark cycle.

**Electrophysiological Recordings in Isolated DRG Neurons**

Lumbar (L4–6) DRG neurons from WT or *ASIC TKO* mice were collected and dissociated as described previously [7]. Briefly DRGs were treated with papain followed by collagenase/dispase, then gently triturated to isolated neurons, and then plated on poly-d-lysine and laminin-coated dishes. Culture medium contained F12 supplemented with heat inactivated serum (10%), penicillin/streptomycin and NGF (50ng/ml). Whole cell patch clamp recordings of DRG neurons were carried out at -70mV 18–24 h after isolation. Currents were filtered at 1 kHz and sampled at 2 kHz using the Axopatch 200B amplifier, Digidata 1200 and Clampex 8.2 (Axon Instruments, Union City, CA). Micropipettes (3–5 MΩ) were filled with internal solution (mM): 100 KCl, 10 EGTA, 40 Hepes, 5 MgCl2, pH 7.4 with KOH. External solutions contained (mM) 120 NaCl, 5 KCl, 1 MgCl2, 2 CaCl2, 10 HEPES, and 10 MES; pH was adjusted with tetramethylammonium hydroxide, and osmolarity was adjusted with tetramethylammonium chloride.

**Behavioral Study**

Mice were acclimated to the testing environment for at least 5 days before any testing was performed. Withdrawal responses to mechanical and heat stimuli were tested in two separate groups of animals. Testing was performed at approximately the same time of day for each subject. The person performing the behavioral experiments was blinded to the genotype of animals.

To assess mechanosensitivity, mice were placed on a stainless steel mesh floor covered with a clear plastic cage top and allowed to acclimate. Calibrated von Frey filaments were applied to the plantar surface of the left hind paw near the medial heel, from least to greatest forces (0.6, 1.5, 3.4, 5.7, 13.7 and 26.0 mN). Each filament was applied five times at each time point, with 30 s between applications. Data were expressed as percent of paw withdrawal for each filament.

To assess heat withdrawal response, mice were placed on a heat-tempered glass floor (3 mm thick) covered with clear plastic cage top and allowed to acclimate. Withdrawal latencies to heat were assessed by applying a focused radiant heat source underneath a glass floor onto the proximal half of the plantar surface of the left hind paw. The heat stimulus was a light from a 50-W projector lamp, with an aperture diameter of 6 mm. Paw withdrawal latencies were measured to the nearest of 0.1 s. The latency time to evoke a withdrawal was determined with a cutoff value of 30 s. Before data collection, the intensity of the heat was adjusted to produce withdrawal latencies in WT mice of 23–25 s. Three trials 10 min apart were used to obtain the average paw withdrawal latency.

**Single-fiber Electrophysiology in the Mouse Skin-Nerve Preparation**

Single-fiber recordings using the isolated skin-nerve preparation were performed in 16 *ASIC TKO* and 16 WT mice. The experimenters performing the recordings were blinded to the genotype.

The mouse saphenous skin-nerve *in vitro* preparation [20] was used to evaluate the receptive characteristics of cutaneous primary afferent fibers. Mice were euthanized in a carbon dioxide chamber, and the hair on the leg was clipped. The saphenous nerve and its innervated territory on the hairy skin of the hind paw were dissected free from muscles or tendons. To ensure a sufficient length of axons for recording, the nerve was dissected up to the lumbar plexus. The skin was then placed epidermal side down in the *in vitro* perfusion chamber, and was continuously superfused with modified Krebs-Hensleit (K-H) solution (in mM: 110.9 NaCl, 4.8 KCl, 2.5 CaCl2, 1.2 MgSO4, 1.2 KH2PO4, 24.4 NaHCO3 and 20.0 glucose, pH 7.4), which was saturated with a mixture of 95% oxygen and 5% carbon dioxide. The temperature of the bath solution was maintained at 32 ± 0.5°C. The nerve attached to the skin was then drawn through a small hole into the recording chamber containing a superficial layer of mineral oil and a bottom layer of modified K-H solution. The nerve was desheathed on a mirror stage, and small filaments were repeatedly split with sharpened forceps to allow single fiber recording to be made using extracellular gold-wire recording electrodes. Neural activity was amplified (DAM50, Harvard Apparatus, Holliston, MA), filtered, and displayed using standard techniques. Amplified signals were fed to a digital oscilloscope and an audiometer and fed into a PC computer via a data acquisition system (spike2/CED1401 program, Cambridge Electronic Design Ltd., Cambridge, UK). Action potentials collected on a computer were analyzed off-line with a template-matching function of Spike 2 software (Cambridge Electronic Design Ltd.). If more than one fiber was present in a recording, data were analyzed only if the unit’s action potential shape and amplitude could be easily discriminated from the other unit’s action potential.

The mechanoreceptive fields of afferent units were identified by probing the dermis side of the skin with a blunt glass rod; thus, mechanosensitive afferents were recorded. Only units with a clearly distinguished signal to noise ratio (greater than 2:1) were further studied. Once the receptive field was identified, a 1 mN von Frey...
Table 1. Fiber classification of mouse cutaneous mechanosensitive afferents.

| Classification                  | Conduction velocity | Adaptation characteristics | Mechanical threshold |
|---------------------------------|---------------------|----------------------------|----------------------|
| Mechanosensitive C-nociceptor   | < 1.2 m/s           | Slow adaptation            | > 1 mN               |
| A-mechanoneceptor (AM)          | ≥ 1.2 m/s           | Rapid adaption             | ≤ 1 mN               |
| D-hair receptor (DH)            | 1.2 – 8 m/s         | Slow adaptation            | ≥ 1 mN               |
| Rapidly adapting low-threshold mechanoreceptor (RA) | > 8 m/s     | Rapid adaptation            | ≤ 1 mN               |
| Slowly adapting low-threshold mechanoreceptor (SA) | > 8 m/s     | Slow adaptation            | ≥ 1 mN               |

doi:10.1371/journal.pone.0035225.t001

Fiber classification of mouse cutaneous mechanosensitive afferents. Units responding to a 1 mN von Frey filament were classified as low-threshold mechanoreceptors. Those not responding to a 1 mN von Frey filament were classified as mechanonociceptors.

The experimental protocol was as follows: Once a single afferent was identified, the receptive field was isolated with a metal ring (3-mm internal diameter), which could seal by its own weight, was used. In some cases, inert silicone grease was added to ensure a waterproof seal. After a 2-min baseline recording, the mechanical response properties were measured using a servo force-controlled mechanical stimulator. Then, the modified K-H solution inside the ring was replaced with either test solution (15 mM lactic acid plus 5 µM ATP; pH 5.0; 32°C) or control solution (modified K-H solution; pH 7.4; 32°C) without any movement of the mechanical probe or ring. After 2-min application of the chemical solution, the same series of mechanical stimuli was applied to the receptive field in the presence of the applied solution. The conduction velocity (CV) was measured at the end of the experiment. The methods for evaluation of mechanosensitivity and CV are described in more detail below. To avoid sensitization/desensitization of nociceptors, fibers having receptive fields in the previously studied area were avoided for subsequent recording. The person recording the afferents was aware of the content of the solution, pH 5.0 lactic acid/ATP or control K-H solution.

To determine quantitative mechanosensitivity, a feedback-controlled constant-force mechanical stimulator (Series 300B Dual Mode Servo System, Aurora Scientific, Aurora, Ontario, Canada) [21] was used. A flat-ended cylindrical probe (tip diameter of 0.7 mm) attached to the tip of the stimulator arm was placed just close to the most sensitive spot of the receptive field so that no force was generated. After a 2-min baseline recording, a computer-controlled ascending series of square force stimuli (100-ms rise time, 1.9-s duration of sustained force plateau) was applied at 60-s intervals. Force stimuli of 5–40 mN range were used for low-threshold mechanoreceptors and 5–80 mN for mechanonociceptors.

To make the chemical test solution, NaHCO₃ (24.4 mM), normally contained in modified K-H solution, was replaced with L-lactic acid (Sigma, St. Louis, MO; 85% to a final concentration of 15 mM), and the pH of lactic acid was adjusted to pH 5.0 with a few drops of 1N NaOH. The final sodium concentration was approximately 125 mM. Then, a frozen aliquot of the ATP (disodium salt, Sigma, St. Louis, MO) stock solution (1mM in saline, pH 4.0) was added to a final concentration of 5 µM. Lactic acid maintained its pH (pH 5.0) when mixed with the ATP stock solution. Aliquots of the ATP stock solution were stored at −80°C until use. The test solution was equilibrated with room air prior to application to the receptive field.

The CV of each unit was determined by electrical stimulation of the receptive field with bipolar platinum electrodes (0.3 mm in diameter, Surepure Chemetals Inc., Florham Park, NJ). Then the distance between the receptive field and the recording electrode (conduction distance) was divided by the latency of the action potential. Afferent fibers conducting slower than 1.2 m/s were classified as C-fibers; those conducting between 1.2 and 8.0 m/s as Aδ-fibers, and those conducting faster than 8 m/s as Aβ-fibers.

Fibers were further classified according to their mechanical threshold (low-threshold mechanoreceptors versus nociceptors, see above) and adaptation responses to sustained suprathreshold mechanical stimuli (SA fibers versus RA fibers) (Table 1). Aδ- and Aβ-fibers with a threshold of ≥ 1 mN and a slowly adapting response to sustained suprathreshold force were considered as A-mechanoreceptors (AMs). Aδ-fibers with a threshold of ≤ 1 mN and a rapidly adapting response at the onset and offset of the force stimulus were classified as D-hair receptors (DHs). Aβ, low-threshold mechanoreceptors were further categorized as SAs if they responded throughout a sustained suprathreshold force stimulus or as RAs if they responded only at the onset and offset of the force stimulus.

Action potentials collected on a computer were analyzed off-line with a template-matching function of Spike 2 software. If a unit discharged at a rate of 0.1 imp/s or more without any intentional stimulus, it was categorized as spontaneously active. Background activity of spontaneously active units was subtracted from any evoked response, thus assuming background activity was sustained during the stimulus period. For mechanical responses, the quantitative analysis of unit discharges was carried out by counting total action potentials in a response and by averaging responses in 0.3-s bins. For chemical responses, total action potentials were averaged during baseline and during chemical application. A unit was considered activated (responsive) when it discharged greater than 0.1 imp/s during chemical stimulation. If background activity was present, the unit was regarded as responsive if the activity was increased at least two standard deviations greater than the background activity during the chemical stimulation period.

Statistical Analyses

Among behavioral data, paw withdrawal frequencies to von Frey filament were analyzed using two-way ANOVA with repeated measures on one factor; significant main effects of genotype or interactions were followed by a separate unpaired t-test at each force level. Differences between strains for single variables were compared using the unpaired t-test. Proportions between strains were compared using the χ² test. The stimulus–response relationship for the mechanical responses was compared using two-way ANOVA with repeated measures on one factor followed by post-hoc unpaired t-test. Analysis of the mechanical stimulus–response relationship before and after the chemical stimulus was performed using two-way repeated measures.
ANOVA followed by post-hoc paired t-tests. Data are presented as mean ± SEM or proportions for each group. Statistical analysis was performed using SPSS 13.0 for Windows (SPSS Inc., Chicago, IL). *p < 0.05 was considered statistically significant.

Results

Isolated DRG Neurons from ASIC 1a/2/3 Null Mice Lack Transient Acid-evoked Currents

We first studied acid-evoked responses of isolated DRG neurons from ASIC TKO mice and compared them to WT. Figure 1A demonstrates typical ASIC-like currents recorded from a WT neuron: a rapid drop in pH evoked large rapidly activating and desensitizing (transient) currents that were followed by smaller sustained currents in a majority of cells. Transient currents were recorded in 68% (20 of 29) of WT neurons, and mean amplitude of pH 5-evoked currents was 2310 ± 382 pA. In comparison, acid-evoked transient currents were absent in all ASIC TKO neurons studied (n = 32, Fig. 1B), and the sustained currents were significantly smaller than those from WT (Fig. 1C). Despite lacking ASIC-like currents, DRG neurons from ASIC TKO mice displayed normal appearing voltage-activated currents evoked by voltage ramp (Fig. 1D).

Simultaneous Disruption of ASIC1a, 2 and 3 Increased the Behavioral Sensitivity to Mechanical Stimuli

ASIC TKO mice showed significantly enhanced mechanosensitivity compared to WT mice, measured as the frequencies of withdrawal responses to the application of von Frey filaments (Fig. 2B). When separated by sex, both female (n = 8) and male (n = 7) ASIC TKO mice showed increased sensitivity to mechanical stimuli compared to WT mice (12 females and 12 males) (Data not shown). In contrast, the mean withdrawal latency to a slow radiant heat ramp was not statistically different between the two genotypes (Fig. 2B).

Electrophysiological Studies

General properties of afferents. Extracellular recordings were made from a total of 277 cutaneous primary afferents from 32 mice; 141 fibers were studied from 16 WT control mice (8 female and 8 male) and 136 fibers were studied from 16 ASIC TKO (8 female and 8 male) (Table 2 and Fig. 3A). Fibers were classified according to their conduction velocity (CV), mechanical threshold and adaptation responses. The proportion of fiber classes was not statistically different between the two genotypes (Fig. 3B, C–E and Table 2). The percentage of Aδ-mechanonociceptors (AMs) that conducted in the Aβ range in this study was about 40% in both genotypes, in agreement with data previously reported in rats [22] and guinea pigs [23]. A CV distribution histogram is shown in Figure 3B, and the mean CVs of the different fiber classes are shown in Table 2. The mean CV of Aδ-AMs from ASIC TKO mice was significantly slower compared to that of Aδ-AMs from WT control (Table 2). There was no difference in the CV of other classes between the two genotypes.

Simultaneous disruption of ASIC1a, -2 and -3 increased the mechanosensitivity of AMs. An ascending series of sustained force stimuli was applied to evaluate the suprathreshold mechanosensitivity. With the exception of the AMs, the stimulus-response functions of all other fiber types were not significantly different between the two genotypes (Fig. 4). The example traces in Figure 4A show the responses of AMs to the series of computer-controlled mechanical stimuli. A summary of mechanical responses of AMs is shown in Figure 4B–D. When compared to WT control, AMs from ASIC TKO showed significantly greater mechanosensitivity (Fig. 4B). When AM fibers were subclassified based on their CV in order to further clarify the TKO, Aδ-AMs showed a significant increase in the stimulus-response function in ASIC TKO mice compared to WT control (Fig. 4C, D). The Aβ-AM fibers also had a significant interaction between force and genotype, and post-hoc tests were not significant at each force. Although the response of Aβ-AM fibers at each force was not statistically different between the genotypes, this may be because these fibers are rare,
approximately 10% of all fibers recorded. Another explanation is that disruptions of the ASIC genes might have resulted in a greater alteration in the electrophysiological phenotype of Aδ-AMs than Aβ-AMs.

When separated by sex, stimulus-response function showed enhanced mechanosensitivity of AMs from ASIC TKOs compared to those from WTs in both female and male mice. The stimulus-response functions of C-, DH, RA and SA fibers in the ASIC TKOs did not differ significantly from their corresponding WT controls in either female or male mice (data not shown).

The mean CV of Aδ-AM fibers from ASIC TKOs was reduced compared to WTs (Table 2). A conduction velocity histogram revealed an increase in the number of Aδ-AM fibers that conducted relatively slowly (1.2 ≤ CV ≤ 2.0 m/s) in ASIC TKOs (Fig. 3B). While only 1 of 21 Aδ-AMs from WTs conducted at velocities of 1.2 to 2.0 m/s, 8 of 25 from ASIC TKOs conducted at this velocity range (Fig. 3A). To test whether these slower Aδ-AM fibers in ASIC TKOs contributed to an enhanced mechanosensitivity, we subdivided Aδ-AMs into two CV groups (< 2.0 m/s, > 2.0 m/s), and compared mechanical stimulus-response functions (Fig. 5B, C). As shown in Figure 5C, faster fibers (CV > 2 m/s), rather than slower fibers (CV ≤ 2 m/s), contributed to the enhanced mechanosensitivity of Aδ-AMs from ASIC TKOs. Therefore, it is unlikely that decreased CV of Aδ-AMs in ASIC TKOs is directly related to the enhanced mechanosensitivity.

Adaptation properties of each fiber class were assessed by comparing the average action potentials in 0.3-s bins during a sustained, mechanical force stimulus. Binned data from the 80 mN stimulus were used for mechanonocceptors (C-fibers and AMs), and 20 mN force was used for low-threshold mechanoreceptors (DH, RA and SA fibers). AMs from ASIC TKO mice showed significantly increased firing initially and during the entire duration of the 80 mN stimulus, compared to WT control (Fig. 6A). When the action potentials in each bin were normalized to total action potentials generated during the entire duration of stimulus, there was no significant difference between the two genotypes (Fig. 6B). When AM fibers were subclassified based on their CV, Aδ-AMs but not Aβ-AMs from ASIC TKO mice showed significantly increased firing initially and throughout the duration of the stimulus, compared to WT controls (Fig. 6C, D). C-, DH, RA and SA fibers did not show any statistically significant differences in the adaptation properties between the two genotypes (Fig. 6E–H).

**The effects of acid on mechanosensitive responses of cutaneous primary afferents.** The sample recording in Figure 7A shows the experimental protocol used to test chemosensitivity and the effect of...
pH 5.0 lactic acid/ATP on mechanical responses of afferents. A C-fiber from a WT mouse in this example was a chemosensitive unit that was activated during application of pH 5.0 lactic acid/ATP. Four of 33 C-fibers (12.1%) from WT control mice and none of 20 C-fibers (0%) from ASIC TKO mice were responsive to the chemical stimulus. No AM, DH, RA or SA fiber in either genotype was chemosensitive. C-fiber responses to mechanical stimuli were not sensitized or desensitized by pH 5.0 lactic acid/ATP or the control K-H solution in either genotype (Fig. 7B,C). Mechanical stimulus response function curves of AM (Fig. 8A, B), DH (Fig. 8C, D), RA (Fig. 8E, F) and SA (Fig. 8G, H) fibers before and during exposure to pH 5.0 lactic acid/ATP or the control K-H solution are shown in Figure 8. In some fiber groups from both genotypes, a slightly decreased mechanosensitivity was noted during exposure to the test or control solutions, suggesting some degree of fatigue (Fig. 8A, B, F, G). It is unlikely that this change is related to alteration in skin compliance because C-fibers maintained their mechanical stimulus-response properties during the second stimulus.

Discussion

ASIC Gene Disruption and Mechanosensitivity

Combined knockout of ASIC1a, -2 and -3 increased the behavioral sensitivity to mechanical stimuli and the mechanosensitivity of AMs compared to WTs. The responses of all other fiber types were not different between the two genotypes.

Earlier work localized ASIC subunits in specialized cutaneous mechanosensory structures [8–10], and sequence comparisons showed similarity between ASIC subunits and the mechanosensory Mec-4 and Mec-10 subunits of C. elegans [4,5]. Therefore, we hypothesized that disrupting all three ASIC genes would markedly impair mechanosensation. We found just the opposite; enhanced mechanosensitivity in ASIC TKO mice. One possible explanation for this intriguing discovery could be that ASIC channels are mechanosensors, but that they somehow reduce or inhibit mechanosensitivity. An inhibitory mechanism is consistent with a previous study demonstrating enhanced mechanosensitivity in visceral afferents from ASIC1 null mice using single-fiber nerve recordings [11]. Yet other studies have shown that mechanical displacement of ASIC-expressing neurons generated greater depolarization compared to ASIC-nonexpressing neurons [24]. ASIC activation depolarizes the cell membrane [25,26], consistent with their Na+ and, to a lesser extent, Ca2+ permeability [27,28]. That, plus their peripheral localization would argue that if they were mechanoreceptors, they would activate rather than inhibit mechanosensory neurons.

We speculate that in the sensory extensions of DRG neurons, ASICs interact with and dampen the function of either mechanosensory channels or molecules required to generate maximal neuronal activity following a mechanical stimulus. Thus, disruption of ASIC genes might enhance mechanosensitivity. Previous studies have shown that ASIC subunits associate with a variety of
scaffolding proteins, including PICK1 [29], PSD-95 [30,31], Lin-7b [30], α-actinin [32], CIPP [33], NHERF-1 [34], MAGI-1b, PIST [30], and stomatin [35]. In addition, ASICs physically and/or functionally interact with other membrane ion channels that have been associated with mechanosensation, including BK channels [36], P2X channels [37], and TRP channels [17,38,39].

Although our conclusion that ASICs contribute to mechanosensation is consistent with some previous data, the phenotypes of

---

**Figure 4. Mechanosensitivity of cutaneous primary afferents in ASIC triple-knockout (TKO) and wild-type (WT) mice.**

**A.** Sample recording traces showing responses of A-mechanonociceptors (AM) from WT and ASIC TKO mice to mechanical stimuli. The upper, middle and lower panels show the digitized oscilloscope tracing, the spike density histograms (bin width = 1 s), and the force stimuli applied, respectively. CV = conduction velocity.

**B.** Stimulus-response function of AM from ASIC TKO (n = 40, ○) vs. WT (n = 35, ●), showing enhanced mechanosensitivity in ASIC TKO mice (p = 0.006 between genotypes by two-way ANOVA with repeated measures on one factor. **p < 0.01; ***p < 0.001 vs. WT by unpaired t-test).

**C, D.** When AM fibers were subclassified based on CV, not Aβ-AM (C) but Aδ-AM (D) showed a significant increase in the stimulus-response function in ASIC TKO mice (p = 0.028 between genotypes by two-way ANOVA with repeated measures on one factor. *p < 0.05; **p < 0.01 vs. WT by unpaired t-test).

**E-H.** Stimulus-response function of C-fibers (E), D-hair receptors (DHs) (F), rapidly adapting (RAs) (G), and slowly adapting low-threshold mechanoreceptors (SAs) (H). Data are presented as mean ± SEM.

doi:10.1371/journal.pone.0035225.g004
the ASIC TKO mice could not have been predicted from studies using ASIC single-knockout mice. For example, ASIC TKOs showed increased paw withdrawal frequencies to a range of von Frey filaments, suggesting enhanced behavioral mechanosensitivity. In contrast, ASIC single-knockout mice had no alteration in paw withdrawal responses to these stimuli [10,11,14]. Our data

Figure 5. Mechanosensitivity of cutaneous Aδ-mechanonociceptors (Aδ-AM) in ASIC triple-knockout (TKO) and wild-type (WT) mice. Aδ-AM fibers were further subdivided into two groups based on conduction velocity (CV) range (CV ≤ 2.0 m/s vs. CV > 2.0). A. Conduction velocity distribution histogram of AM fibers. B. Mechanical stimulus-response function of Aδ-AM from WT mice. Only one fiber conducted slower than 2 m/s ( ). C. Mechanical stimulus-response function of Aδ-AM from ASIC TKO mice. Fibers that conducted faster than 2 m/s (n = 17, r) showed a tendency toward enhanced mechanosensitivity, compared to those that conducted slower than 2 m/s (n = 8, □) (p = 0.090 between CV groups by two-way ANOVA with repeated measures on one factor). Data are presented as mean ± SEM.

doi:10.1371/journal.pone.0035225.g005

Figure 6. Adaptation properties of cutaneous primary afferents in ASIC triple-knockout (TKO) and wild-type (WT) mice. A. AM from ASIC TKO mice showed increased firing initially and throughout the duration of sustained, 80-mN stimulus (P = 0.001 between genotypes by two-way ANOVA with repeated measures on one factor. *P < 0.05; **P < 0.01; ***P < 0.001 vs. WT by unpaired t-test). B. When the action potentials in each 0.3-s bin were normalized to total spikes generated during the entire duration of stimulus, there was no significant difference between the two genotypes. C. When AM fibers were subclassified based on conduction velocity, not Aβ-AM (C) but Aδ-AM (D) from ASIC TKO mice showed a significantly increased firing initially and throughout the duration of the stimulus (p = 0.005 between genotypes by two-way ANOVA with repeated measures on one factor. *P < 0.05; **P < 0.01 vs. WT by unpaired t-test). E-H. Average action potentials in 0.3-s bins during a sustained force stimulus in C-fibers (E), D-hair receptors (DHs) (F), rapidly adapting (RAs) (G), and slowly adapting low-threshold mechanoreceptors (SAs) (H). Data are presented as mean ± SEM.

doi:10.1371/journal.pone.0035225.g006
are consistent with a previous behavioral study of transgenic mice expressing a dominant-negative ASIC3 subunit that was predicted to inactivate all ASIC-like transient currents [40]; transgenic mice exhibited a decreased paw withdrawal threshold to von Frey filaments. On the other hand, paw withdrawal frequencies to von Frey filaments were not different between these transgenic mice and WTs under non-pathological conditions. Our data demonstrated greater paw withdrawal frequencies in ASIC TKOs compared to WTs, suggesting further enhanced mechanosensitivity of ASIC TKOs. In addition, the enhanced mechanosensitivity of ASIC TKO AM fibers could not have been predicted from studies using ASIC single-knockout mice. As stated in the Introduction section, previous studies have demonstrated reduced mechanosensitivity of RAs and SAs in ASIC2 nulls [9], enhanced mechanosensitivity of RAs and reduced mechanosensitivity of Aδ-AMs in ASIC3 nulls [10], and no difference in the mechanosensitivity of cutaneous afferents in ASIC1 nulls [11]. Thus, our results suggest some functional redundancy of ASIC subunits in conferring a normal mechanosensory phenotype. ASIC channels are composed of three ASIC subunits, and in DRG neurons, previous data suggest that ASIC channels are predominantly heteromultimers [7]. Our data together with earlier studies suggest that any two of the subunits may be sufficient to confer normal mechanosensation.

Other potential mechanisms that could be considered for the enhanced mechanosensitivity in ASIC TKOs are as follows. First, ASIC1a and ASIC2 subunits are expressed in the central nervous system (CNS) [18,41,42]. It is possible that ASIC channels in brain might play an inhibitory role in the response to mechanosensory stimuli, and hence their loss might enhance the behavioral response to peripheral stimulation. While that could be the case, our finding that loss of ASIC function increased mechanical responsiveness to a specific fiber population in a preparation lacking connections to the CNS, and even to cell bodies in the DRG, argues that loss of ASIC subunits had a more direct effect on the mechanosensory process. If ASICs in the CNS were inhibiting behavioral responsiveness, heat latencies should have been affected. Second, it remains possible that there could be functional redundancies between ASICs and other candidate mechanosensory channels including transient receptor potential (TRP) channels, two-pore-domain K+ (K2P) channels [43] and piezo proteins [44]. Perhaps the loss of ASIC leads to compensatory changes in other mechanosensory channels. However, if that were the case, it would require “overcompensation” by other channels to produce the
enhanced mechanosensitivity in ASIC TKO mice. Third, the increased mechanosensitivity in AM fibers is not likely caused by differences in skin compliance or thickness because the mechanosensitivity of other fibers was not affected.

Behavioral Responses and Afferent Fiber Activity

Our data suggest that increased AM fiber mechanosensitivity is responsible for the increased paw withdrawal frequencies to punctate mechanical stimuli. Although it is not clear whether the paw withdrawal response to von Frey filaments reflects the response of nociceptive primary afferents [10,46]. In a human study, selective conduction blockade of A-fibers by non-ischemic nerve compression significantly reduced the magnitude of pain elicited by punctate mechanical probes in normal skin [47]. Similarly, in neonatal rats treated with 50 mg/kg of capsaicin, 95% of C-fibers were destroyed [48,49], and the paw withdrawal responses to punctate mechanical stimuli of these rats were not different from normal rats, implying a minimal role of C-fibers in this assay [50]. Thus, the behavioral and electrophysiological responses in ASIC TKOs may represent an increased response to a noxious mechanical stimulus.

Consistent with the increased mechanosensitivity of the AM fibers, ASIC2 and ASIC3 immunoreactivities were detected in the intraepidermal free nerve endings [8,10]. On the other hand, while previous studies have shown ASIC2 and ASIC3 immunoreactivities in specialized cutaneous mechanosensory structures [8–10], mechanosensitivity of low-threshold mechanoreceptors in ASIC TKOs did not differ significantly from their corresponding WT controls.

Considering that different ASIC subunits are expressed in various subsets of DRG neurons [6], it is unclear why the altered mechanosensitivity was only observed in AMs but not other fibers in ASIC TKOs. First, it could be that disrupting ASIC1a, -2 and -3 genes might affect some other channel/process that is unique to AM neurons. Second, we cannot absolutely exclude some contribution of ASIC1b. However, although ASIC1b mRNA was detected in mice with a disruption of ASIC1a, no evidence of either ASIC1a or ASIC1b protein was found in these animals [11]. In addition, no transient acid-evoked currents were found in ASIC TKO DRG neurons, suggesting that presence of ASIC1b is very unlikely. Third, while the feedback-controlled constant-force mechanical stimulus used in the present study is optimal for evaluating nociceptors [21,31], it might have limited sensitivity for detecting differences in RA or SA responses, mainly velocity- or displacement-detectors [32]. Because fiber classifications were finalized after the complete array of mechanical stimuli, we could only apply one modality and we selected force. This should also be considered when comparing mechanosensitivity data among studies, since displacement-controlled stimuli were used in the previous ASIC single-null mice studies [9–11].

ASICs and Chemosensitivity

Chemosensitivity of primary afferents was assessed using pH 5.0 lactic acid/ATP. Lactate and ATP were added in an attempt to optimize the ASIC-mediated responses of primary afferents to low pH [17,37,53,54]. We found very little activation of sensory fibers by lactic acid/ATP and no potentiation of mechanical sensitization in acidic conditions. Previously, in a study using the rat skin-nerve preparation, Steen et al. reported low-pH-induced sensitization of nociceptors to mechanical stimulation [55]. These seemingly opposite findings are likely attributed to the differences in the experimental protocols, including the frequency and duration of low-pH exposure. Also, the differences in species and the composition of the acid solution might have contributed to the discrepancy [55].

Conclusion and Future Studies

Combined knockout of ASIC1a, -2 and -3 increased the behavioral sensitivity to mechanical stimuli and the mechanosensitivity of AMs. Our data indicate that ASICs are involved in cutaneous mechanosensation. Future studies seeking to understand why ASIC TKOs have enhanced mechanosensitivity, such as identifying transcripts that are up- or down-regulated in AMs from TKOs, may help identify other components of the mechanosensory complex and evaluate possible compensatory effects. It would be also interesting for future studies to investigate mechanically activated current in dissociate DRG neurons to characterize other mechanosensory phenotypes of ASIC TKOs. We have shown slower mean conduction velocity (CV) of Aδ-AMs from ASIC TKOs. Previous studies have demonstrated the expression of ASIC2 or other channels from DEG/ENaC superfamily by human or rat Schwann cells [56,57]. Therefore, while the exact underlying mechanism remains unclear, warranting further investigation, this might suggest a possible role of ASICs in Schwann cell function and axonal conduction in subpopulations of afferents.

Author Contributions

Conceived and designed the experiments: SK JHJ MPP CJB TJW. Performed the experiments: SK JHJ MPP MG HG. Analyzed the data: SK CJB TJW. Wrote the paper: SK CJB TJW.

References

1. Waldmann R, Lazdunski M (1998) H(+)-gated cation channels: neuronal acid sensors in the Na/C/DEG family of ion channels. Curr Opin Neurobiol 8: 418–424.
2. Alvarez de la Rosa D, Canessa CM, Fyfe GK, Zhang P (2000) Structure and regulation of amiloride-sensitive sodium channels. Annu Rev Physiol 62: 573–594.
3. Geffeney SL, Cueva JG, Glauzer DA, Doll JC, Lee TH, et al. (2011) DEG/ENaC but Not TRP Channels Are the Major Mechanoelectrical Transduction Channels in a C. elegans Nociceptor. Neuron 71: 845–857.
4. Huang M, Chalfie M (1994) Gene interactions affecting mechanosensory transduction in Caenorhabditis elegans. Nature 367: 467–470.
5. O’Hagan R, Chalfie M, Goodman MB (2005) The MEC-4 DEG/ENaC channel of Caenorhabditis elegans touch receptor neurons transduces mechanical signals. Nat Neurosci 8: 43–50.
6. Alvarez de la Rosa D, Zhang P, Shao D, White F, Canessa CM (2002) Functional implications of the localization and activity of acid-sensitive channels in rat peripheral nervous system. Proc Nat Acad Sci U S A 99: 2326–2331.
7. Benson CJ, Nie J, Wennie JA, Price MP, Heuss JM, et al. (2002) Heteromultimers of DEG/ENaC subunits form H+–gated channels in mouse sensory neurons. Proc Natl Acad Sci U S A 99: 2338–2343.
8. Garcia-Anoveros J, Samad TA, Zuelda-Jelaska L, Woolf CJ, Corey DP (2001) Transport and localization of the DEG/ENaC ion channel BNaC1alpha to
peripheral mechanosensory terminals of dorsal root ganglion neurons. J Neurosci 21: 2678-2686.
9. Price MP, Lewin GR, McIlwrath SL, Cheng C, Xie J, et al. (2000) The mammalian sodium channel BNC1 is required for normal touch sensation. Nature 407: 1007-1011.
10. Price MP, McIlwrath SL, Xie J, Cheng C, Qiao J, et al. (2001) The DRASIC cation channel contributes to the detection of cutaneous touch and acid stimuli in mice. Neuron 32: 1071–1083.
11. Page AJ, Brierley SM, Martin CM, Martín-Salgado C, Wemmie JA, et al. (2004) The ion channel ASIC1 contributes to visceral but not cutaneous mechanoreceptor function. Gastroenterology 127: 1739-1747.
12. Roza C, Puel JL, Kress M, Baron A, Dieckot S, et al. (2004) Knockout of the ASIC2 channel in mice does not impair cutaneous mechanosensation, visceral nociception and hearing. J Physiol 556: 659-669.
13. Driscoll M, Chalfie M (1991) The mec-4 gene is a member of a family of Ca+2 permeable ion channel genes in C. elegans. Nature 354: 580-585.
14. Staniland AA, McMahon SB (2009) Mice lacking acid-sensing ion channels ASIC1 or 2, but not ASIC3, show increased pain behaviour in the formalin test. Eur J Pain 13: 554–563.
15. Drew LJ, Rohrer DK, Price MP, Blaver KE, Cockayne DA, et al. (2004) Acid-sensing ion channels ASIC2 and ASIC3 do not contribute to mechanically activated currents in mammalian sensory neurons. J Physiol 556: 691–710.
16. Welsh MJ, Price MP, Xie J (2002) Biochemical basis of touch perception: mechanosensory function of degenerin/epithelial Na+ channels. J Biol Chem 277: 2369-2372.
17. Light AR, Hughes RW, Zhang J, Rainier J, Liu Z, et al. (2008) Dorsal root ganglion neurons innervating skeletal muscle respond to physiological combinations of protons, ATP, and lactate mediated by ASIC, P2X, and TRPV1. J Neurophysiol 100: 1184–1201.
18. Wemmie JA, Chen J, Aksvig CC, Hruska-Hagerman AM, Price MP, et al. (2002) The acid-activated ion channel ASIC contributes to synaptic plasticity, learning, and memory. Neuron 34: 463-477.
19. Zimmermann M (1983) Ethical guidelines for investigations of experimental pain in conscious animals. Pain 16: 119-160.
20. Kolzenburg M, Stucky CL, Lewin GR (1997) Receptive properties of mouse sensory neurons innervating hairy skin. J Neurophysiol 78: 2033-2041.
21. Lu Y, Ma X, Sahbarwal R, Srinavase V, Morgan D, et al. (2009) The ion channel ASIC2 is required for baroreceptor and autonomic control of the circulation. Neuron 64: 835-849.
22. Baron A, Waldmann R, Lanzlunds M (2002) ASIC-like, proton-activated currents in rat hippocampal neurons. J Physiol 543: 483–494.
23. Lilley S, LeTissier P, Robbins J (2004) The discovery and characterization of a C-terminal interaction. J Biol Chem 284: 2697–2705.
24. Lu Y, Ma X, Sahbarwal R, Srinavase V, Morgan D, et al. (2009) The ion channel ASIC2 is required for baroreceptor and autonomic control of the circulation. Neuron 64: 835-849.
25. Arias H, Sauerwald M, Schonberger A, Hoffmann G, et al. (2006) Acid-sensing ion channels 1 and 2 in human sensory neuron-specific acid-sensing ion channel 3 by the adaptor protein Na+/H+ exchanger regulatory factor-1. J Biol Chem 281: 1796–1807.
26. Price MP, Thompson RJ, Edcol JO, Wemmie JA, Benson CJ (2004) Stomatin modulates gating of acid-sensing ion channels. J Biol Chem 279: 53806–53891.
27. Petroff EY, Price MP, Srinavase V, Gong H, Kosowksina V, et al. (2000) Acid-sensing ion channels interact with and inhibit BK K+ channels. Proc Natl Acad Sci U S A 105: 3140-3144.
28. Birdsong WT, Fierro L, Williams FG, Spekta V, Naves LA, et al. (2010) Sensing muscle ischemia: coincident detection of acid and ATP via interplay of two ion channels. Neuron 68: 739–749.
29. Gao Z, Henig O, Kehoe V, Sinoway LI, Li J (2006) Vanilloid type 1 receptor and the acid-sensing ion channel mediate acid phosphate activation of muscle afferent nerves in rats. J Appl Physiol 100: 421–426.
30. Gao Z, Li JD, Sinoway LI, Li J (2007) Effect of muscle interstitial pH on P2X and TRPV1 receptor-mediated pressor response. J Appl Physiol 102: 2209–2293.
31. Mogil JS, Breese NM, Witty MF, Ritchie J, Rainville ML, et al. (2005) Transgenic expression of a dominant-negative ASIC3 subunit leads to increased sensitivity to mechanical and inflammatory stimuli. J Neurosci 25: 9893–9901.
32. Alvarado de la Rosa D, Krueger SR, Kolar A, Shao D, Fitzsimmonds RM, et al. (2003) Distribution, subcellular localization and ontology of ASIC1 in the mammalian central nervous system. J Physiol 546: 77–87.
33. Garcia-Averinos J, Dörfler B, Neville-Golden J, Hyman BT, Corey DP (1997) BNA1 and BNA2 constitute a new family of human neuronal sodium channels related to degenerin and epithelial sodium channels. Proc Natl Acad Sci U S A 94: 1459–1464.
34. Chaffie M (2009) Neurosensory mechanotransduction. Nat Rev Mol Cell Biol 10: 44–52.
35. Coste B, Mathur J, Schmidt M, Earley TJ, Ranade S, et al. Piezo1 and Piezo2 are essential components of distinct mechanically activated cation channels. Science 330: 53–60.
36. Bennett GJ (1993) An animal model of neuropathic pain: a review. Muscle Nerve 16: 1040–1048.
37. Mogil JS, Wilson SG, Bon K, Lee SF, Chung K, et al. (1999) Heteritiatity of nociception I: responses of 11 inbred mouse strains on 12 measures of nociception. Pain 80: 67–76.
38. Gage W, Fuchs PN, Meyer RA, Treede RD (2001) Roles of capsaicin-insensitive nociceptors in cutaneous pain and secondary hyperalgesia. Brain 124: 1754–1764.
39. Nage JI, Hunt SP, Iversen LL, Emson PC (1981) Biochemical and anatomical observations on the degeneration of peptide-containing primary afferent neurons after neonatal capsaicin. Neuroscience 6: 1925–1934.
40. Nage JI, Iversen LL, Goedert M, Chapman D, Hunt SP (1983) Dose-dependent effects of capsaicin on primary sensory neurons in the neonatal rat. J Neurosci 3: 399–406.
41. Shir Y, Selzer Z (1990) A-fibers mediate mechanical hyperesthesia and allodynia and C-fibers mediate thermal hyperalgesia in a new model of caudalform pain disorders in rats. Neurosci Lett 115: 63–67.
42. Khalas PS, Zhang C, Qin YX (2000) Encoding of location and intensity ofnoxious indentation into rat skin by spatial populations of cutaneous mechano-nociceptors. J Neurophysiol 83: 3049–3061.
43. Ge W, Khalas PS (2002) Encoding of compressive stress during indentation by slowly adapting type I mecanoreceptors in rat hairy skin. J Neurophysiol 87: 1616–1635.
44. Inmuke DC, McIclesley EW (2001) Lactate enhances the acid-sensing Na+ channel on ischemia-sensing neurons. Nat Neurosci 4: 899–907.
45. Naves LA, McIclesley EW (2005) An acid-sensing ion channel that detects ischemic pain. Braz J Med Biol Res 38: 1561–1569.
46. Steen KH, Reeh PW, Anton F, Handwerker HO (1992) Protons selectively induce lasting excitation and sensitization to mechanical stimulation of nociceptors in rat skin, in vitro. J Neurosci 12: 86–95.
47. Calavia MG, Montano JA, Garcia-Sazuez O, Frito J, Guervas MA, et al. (2006) Differential localization of Acid-sensing ion channels 1 and 2 in human cutaneous pacinian corpocales. Cell Mol Neurobiol 30: 841–848.
48. Hitomi Y, Suzuki A, Kawano Y, Nozawa-Inoue K, Inoue M, et al. (2009) Acid-sensing ion channels: a C-terminal interaction. J Biol Chem 284: 2697–2705.