Published in final edited form as:

Pain. 2011 October ; 152(10): 2348–2356. doi:10.1016/j.pain.2011.06.027.

Selective targeting of ASIC3 using artificial miRNAs inhibits primary and secondary hyperalgesia after muscle inflammation

Roxanne Y. Walder^{a,b}, Mamta Gautam^{a,b}, Steven P. Wilson^c, Christopher J. Benson^d, and Kathleen A. Sluka^{a,b,*}

^aPhysical Therapy and Rehabilitation Sciences Graduate Program, Roy J. and Lucille A. Carver College of Medicine, The University of Iowa, Iowa City, IA, USA

^bNeuroscience Graduate Program, Pain Research Program, The University of Iowa, Iowa City, IA, USA

^cDepartment of Pharmacology, Physiology and Neuroscience, University of South Carolina School of Medicine, Columbia, SC, USA

^dDepartment of Internal Medicine, Pain Research Program, Roy J. and Lucille A. Carver College of Medicine, The University of Iowa, Iowa City, IA, USA

Abstract

Acid-sensing ion channels (ASICs) are activated by acidic pH and may play a significant role in the development of hyperalgesia. Earlier studies show ASIC3 is important for induction of hyperalgesia after muscle insult using ASIC3^{-/-} mice. ASIC3^{-/-} mice lack ASIC3 throughout the body, and the distribution and composition of ASICs could be different from wild-type mice. We therefore tested whether knockdown of ASIC3 in primary afferents innervating muscle of adult wild-type mice prevented development of hyper-algesia to muscle inflammation. We cloned and characterized artificial miRNAs (miR-ASIC3) directed against mouse ASIC3 (mASIC3) to downregulate ASIC3 expression in vitro and in vivo. In CHO-K1 cells transfected with mASIC3 cDNA in culture, the miR-ASIC3 constructs inhibited protein expression of mASIC3 and acidic pH-evoked currents and had no effect on protein expression or acidic pH-evoked currents of ASIC1a. When miR-ASIC3 was used in vivo, delivered into the muscle of mice using a herpes simplex viral vector, both muscle and paw mechanical hyperalgesia were reduced after carrageenan-induced muscle inflammation. ASIC3 mRNA in DRG and protein levels in muscle were decreased in vivo by miRASIC3. In CHO-K1 cells co-transfected with ASIC1a and ASIC3, miR-ASIC3 reduced the amplitude of acidic pH-evoked currents, suggesting an overall inhibition in the surface expression of heteromeric ASIC3-containing channels. Our results show, for the first time, that reducing ASIC3 in vivo in primary afferent fibers innervating muscle prevents the development of inflammatory hyperalgesia in wild-type mice, and thus, may have applications in the treatment of musculoskeletal pain in humans.

Keywords

ASIC; HSV; Hyperalgesia; Inflammation; Muscle; Pain

© 2011 Published by Elsevier B.V. on behalf of International Association for the Study of Pain.

*Corresponding author. Address: Department of Physical Therapy and Rehabilitation Science, 1-252 Medical Education Building, The University of Iowa, Iowa City, IA 52242, USA. Tel.: +1 319 335 9791; fax: +1 319 335 9707. kathleen-slukauiowa.edu (K.A. Sluka).

Conflict of interest statement

The authors have no conflicts of interest with regard to the studies described in this manuscript.

1. Introduction

Acid-sensing ion channels (ASICs) are activated by protons and belong to the epithelial sodium channel/degenerin (ENaC/DEG) family of amiloride-sensitive proteins [8,27,50,62]. Four genes within mammalian genomes encode several splice variants including ASIC1a, ASIC1b, ASIC1b2, ASIC2a, ASIC2b, ASIC3, and ASIC4, which are thought to combine as heteromers to form functional ion channels [5,14,23]. Each of the ASICs display unique biophysical properties, such as different pH sensitivities, current kinetics, ion selectivities, and pharmacological sensitivities [1,4,16,17,28].

ASICs are expressed on neurons including nociceptors, and are ideally located to be activated by the acidic pH that occurs after inflammation or ischemia [20,21,32,37,40,59,60]. Previous studies of ASICs and inflammatory hyperalgesia have yielded conflicting results, often depending on the location or the inflammatory animal model used. In models of paw inflammation, ASIC3 null or knockdown mice show no change or enhanced hyperalgesia when compared with wild-type controls [6,31,40,51]. Models of cutaneous inflammation measure hyperalgesia at the site of injury, ie, primary hyperalgesia, but not at sites distant from the injury, ie, secondary hyperalgesia. In contrast, models of musculoskeletal pain have examined the roles of ASICs in both primary and secondary hyperalgesia. Prior work shows that ASIC1 and ASIC3 play differential roles in the development of both primary and secondary hyperalgesia after deep-tissue injury. Specifically, after muscle inflammation, ASIC3^{-/-} mice do not develop secondary hyperalgesia, but still develop primary hyperalgesia, whereas ASIC1^{-/-} mice do not develop primary hyperalgesia, but still develop secondary hyperalgesia [20,49,58,65].

Although the ASIC knockout mice are invaluable tools for examining the role of ASICs and pain, they lack specific ASIC subunits throughout the body, and the distribution and composition of their ASIC channels would be different from wild-type mice. We therefore tested whether knockdown of ASIC3 in primary afferents innervating muscle of adult wild-type mice would prevent the development of hyperalgesia to muscle inflammation. To test this hypothesis, we developed and characterized artificial miRNAs to mouse ASIC3 *in vitro* and tested a mouse miRNA against ASIC3 *in vivo*.

2. Materials and methods

2.1. Cloning of miRNAs

Synthetic oligonucleotides to generate pre-miRNA sequences against mouse ASIC3, 844 and 847, within the endogenous murine miR-144 flanking sequences were selected by the proprietary BLOCK-iT software program (Invitrogen, Carlsbad, CA). The oligonucleotides were purchased and cloned into the plasmid, pcDNA6.2-GW EmGFP-miR (Invitrogen) (Table 1). The DNA sequence of each construct was verified by bidirectional DNA sequencing. The target sequences on ASIC3 mRNA and their respective locations are also listed in Table 1. As a negative control, an miRNA sequence that is not predicted to target any known mammalian gene, also inserted into pcDNA6.2-GW EmGFP-miR, was obtained from Invitrogen (Carlsbad, CA).

2.2. Cell culture and transient transfections

Chinese hamster ovary (CHO-K1) cells were maintained and transfected with mouse hemagglutinin (HA) tagged ASIC1a and HA tagged ASIC3 cDNAs as previously described [26]. miR844 or miR847 in pcDNA6.2-GW EmGFP-miR (0 to 25 μ g) were added at the same time as cDNA to either ASIC3 (4 μ g) or ASIC1a (4 μ g). Cell lysates were analyzed by Western blot after 48 h in culture.

For electrophysiology experiments, cells were plated in 35-mm tissue culture dishes (40,000 cells/plate) as previously described [11]. After 24 h, CHO-K1 cells were transfected (Transfast, Promega, Madison, WI) with one of the miRNA-carrying plasmids (control, miR847, miR844) and ASIC1a, ASIC3, or ASIC1a and ASIC3 cDNA at varying concentrations (Fig. 1). For ASIC3 or ASIC1a, we tested a ratio of 1:1 ASIC:miRNA ratio (1 μ g each), and a ratio of 1:5 ASIC:miRNA ratio (0.2 and 0.9 μ g). A reporter plasmid expressing dsRed (Express-C1, Clontech, Mountain View, CA) was also transfected at the same time. ASIC currents were recorded at 48 h after transfection.

2.3. Western blot

Equal amounts of protein (20 μ g) from transfected CHO-K1 cell lysates were run on 7.5% SDS-PAGE mini-gels (Bio-Rad, Richmond, CA) and transferred to nitrocellulose as previously described [26]. The membranes were blocked with 5% bovine serum albumin in 10 mM Tris-HCl and 100 mM NaCl pH 7.5 containing 0.1% (wt/vol) Tween 20 (TBST), for 1 h at room temperature and probed with anti-hemagglutinin-horseradish peroxidase (HRP) conjugate monoclonal antibody (1:750, Roche, Indianapolis, IN) and developed with the ECL Plus western detection system (GE Healthcare, Piscataway, NJ). Subsequently, the blots were re-probed with mouse monoclonal β -actin antibody (1:15,000, Sigma, St. Louis, MO) and HRP-conjugated goat antimouse antibody (1:2000, Millipore (Upstate), Billerica, MA) and developed with the ECL Plus reagent to verify equal loading of protein. Gels were scanned and analyzed in Image J (National Institutes of Health, Bethesda, MD) for densitometric quantification of the immunoreactive bands.

2.4. Electrophysiology

Recordings of ASIC currents were measured in transfected CHO-K1 cells as previously described [11]. Whole-cell ASIC currents (-70 mV) were recorded at room temperature from cells identified with red (dsRed) and green (eGFP) fluorescence. Test solutions with different pH (pH 7.2, pH 6.5, pH 6.0, pH 5.5, and pH 5.0) contained (in mM) 120 NaCl, 5 KCl, 2 CaCl_2 , 1 MgCl_2 , 10 HEPES, 10 MES, 5 glucose. Test solutions were applied directly to the cells using a BPS 8 perfusion system (ALA Scientific, Westbury, NY) that was controlled by the AXON Digidata 1200 and pCLAMP 8 software (Molecular Devices, Union City, CA). Patch pipettes were filled with (in mM) 100 KCl, 10 EGTA, 40 HEPES, 5 MgCl_2 . Patch clamp data were analyzed using Clampfit (Axon instruments) and Origin 7 software programs (Northampton, MA). Current densities were calculated by dividing the peak current amplitude (pico amperes) by the cell capacitance (pico farads). Kinetics of desensitization were fit to a single or double exponential equations and time constants (τ) reported. Data are mean $\pm$ SEM.

2.5. Virus construction

Recombinant herpes viruses were constructed as previously described [66]. The pre-miRNA sequences against mouse ASIC3, miR844, and the control miRNA were excised from their respective plasmids (pcDNA6.2-GW EmGFP-miR) along with the EmGFP sequence, and inserted downstream of the human cytomegalovirus (CMV) immediate-early enhancer-promoter in a shuttle plasmid containing portions of the HSV-1 genes UL36 and UL37 flanking the expression cassette. We chose to use the human CMV promoter to transcribe the artificial miRNA because it allows for more robust transcription than the class III promoter typically used to transcribe shRNA [34,53]. The expression cassette also contained the woodchuck hepatitis virus element to enhance RNA stability. The resultant HSV are more selective for infecting the peripheral nervous system [66]. The cloned pre-miRNA sequences were verified by DNA sequencing. High titers of the recombinant HSV viruses were produced at the University of South Carolina. HSV stocks were used as 10^7 plaque-forming units (PFU)/ μ L. Viruses were stored at -70°C until used.

2.6. Animal care and use

All experiments with laboratory animals were approved by the Animal Care and Use Committee at the University of Iowa and were conducted in accordance with National Institutes of Health guidelines. Male C57Bl/6J mice (n = 40, Jackson Laboratories, Bar Harbor, ME) were bred at the University of Iowa Animal Care Facility and used in these studies at 6 to 10 weeks of age.

2.7. Virus administration

Mice were anesthetized with 2% to 4% isoflurane, and the skin overlying the left gastrocnemius muscle was excised to expose the muscle as previously described [49]. Recombinant viruses (20 μ L) were injected at a flow rate of 5 μ L/min into the left gastrocnemius muscle with a Hamilton syringe connected to PE-10 tubing and a 30-gauge needle. After the virus was injected, saline-moistened gauze was placed over the wound for 10 min to allow the virus to be absorbed into the muscle and prevent leakage into the skin. The wound was closed and mice recovered for 4 weeks prior to behavioral testing or examination for ASIC3 mRNA or protein expression. The 4-week time point was chosen to allow the animals to recover from surgery, minimize interference between the immune responses of the HSV infection and behavior measurement, and allow for expression of the miRNAs. We previously determined that HSV-1 has no effect on pain behaviors at 4 weeks after injection, and there is adequate and continued expression of the transgene after 4 weeks [49].

2.8. Induction of inflammation

Mice were anesthetized with 2% to 3% isoflurane and injected with 3% carrageenan in normal saline into the left gastrocnemius muscle as previously described [58].

2.9. Behavioral assessments

Behavioral measurements were made before and 4 weeks after HSV-1 injection, and 24 h, 72 h, and 1 week after carrageenan injection into the muscle. Mice injected with HSV-miR844 (n = 12) were tested against mice injected with HSV-control miRNA (n = 12) for responses to mechanical sensitivity of the paw and mechanical muscle sensitivity. The tester was blinded to group in all behavioral measurements.

2.9.1. Mechanical sensitivity of the muscle—Mechanical sensitivity of the muscle was tested by squeezing the gastrocnemius muscle of the mice with a calibrated pair of tweezers until the mouse withdrew from the stimulus as previously described [45,58]. The force at which the mouse withdrew was measured in millinewtons and called the muscle withdrawal threshold. Each hindlimb was tested 3 times, and the 3 trials were averaged. A decrease in threshold was interpreted as primary muscle hyperalgesia.

2.9.2. Mechanical sensitivity of the paw—Mechanical sensitivity of the paw was measured with a von Frey filament (0.4 mN) as previously described [58]. The filament was applied 5 times, and 10 trials were averaged. An increase in the number of withdrawals was interpreted as secondary cutaneous hyperalgesia.

2.10. Immunohistochemistry

Immunohistochemistry was done on muscle tissue 4 weeks after HSV-1 injection. Mice were injected with either HSV-control miRNA (n = 4) or HSV-miR844 (n = 4) in the gastrocnemius muscle as previously described. The mice were deeply anesthetized and transcardially perfused with 4% paraformaldehyde. The gastrocnemius muscle was immediately dissected and postfixed with 4% paraformaldehyde in phosphate-buffered

saline (PBS) (137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 2 mM KH₂PO₄, pH 7.4) for 24 h at 4 °C. The muscle was placed in 10% sucrose in PBS at 4 °C for 2 h, 20% sucrose in PBS at 4 °C for 2 h, and 30% sucrose in PBS at 4 °C for 24 h. The tissues were then rapidly frozen in OCT and stored at –80 °C until needed. Ten-micrometer sections were cut on a cryostat and mounted on glass slides. The sections were fixed in acetone for 10 min at 4 °C, and then blocked with 5% normal goat serum (NGS) for 30 min. Sections were incubated with guinea pig anti-ASIC3 (Millipore, 1:100) in 1% NGS containing 0.1% Triton X-100 for 18 h at room temperature. Sections were washed with PBS and then incubated with biotinylated goat anti-guinea pig immunoglobulin G (Vector Laboratories, Burlingame, CA; 1:400) for 1 h. Sections were subsequently incubated for 1 h with streptavidin-conjugated Alexa 568 (Invitrogen, 1:400). AlexaFluor 568 was chosen to detect ASIC3 expression to avoid background immunofluorescence and fluorescence resulting from the coexpression of eGFP from the recombinant viruses. Sections were coverslipped with Vectashield containing DAPI (Vector Laboratories). Importantly, all sections were immunoreacted at the same time using the same solutions to minimize variability in staining among animals.

Muscle sections were viewed with an LSM 710 confocal microscope (Zeiss, Jena, Germany) in the Central Microscopy Facility at the University of Iowa. For quantification of staining, all sections were taken at the same time, on the same day, and under the same lighting conditions. Images for quantification were taken at 63× magnification. We used Image J software (NIH, Bethesda, Md., version 1.45g, 2011, <http://imagej.nih.gov/ij>) to quantify the amount of fluorescent staining in each section. The confocal images were separated so that we only viewed the 568-nm (red) channel. All images were normalized to the same background by adjusting the brightness (60) to the same value. Images were then analyzed for the number of pixels at 568 nm fluorescence (red pixels) using the histogram function, which gave a mean value for the entire image. Five sections per animal were quantified and averaged to obtain 1 number per animal. We compared muscles from HSV-control miRNA (n = 4) or HSV-miR844 (n = 4), as well as from ASIC3^{–/–} mice (n = 2).

2.11. Quantitative real-time polymerase chain reaction (PCR)

The ipsilateral and contralateral L4–L6 DRGs were isolated from mice 24 h after carrageenan-induced muscle inflammation and that had received either HSV-1 control miRNA (n = 8) or HSV-miR844 (n = 8) 4 weeks previously. Total cellular RNA was isolated from DRGs using the Trizol method, and the quantitative PCR (qPCR) method was performed as previously described [58]. First-strand cDNA synthesis was performed with 0.2 to 1 µg of RNA and VILO polymerase (Invitrogen, Carlsbad, CA). Reverse transcriptase was omitted in negative controls. The qPCRs were run for 40 cycles on an ABI Prism 7900HT sequence detector instrument (Applied Biosystems, Foster City, CA) at the University of Iowa DNA Facility. Validated, predesigned TaqMan assays for ASIC1 (ASIC1a and ASIC1b) (Mm01305997_m1), ASIC2 (ASIC2a and ASIC2b) (Mm00475691_m1), ASIC3 (Mm00805460_m1), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were used (Applied Biosystems). Each cDNA sample was run in triplicate, and the expression of ASIC1, ASIC2, and ASIC3 mRNAs was normalized to the expression of GAPDH mRNA, based on cycle thresholds (C_T). The results of the triplicate samples are expressed as means and SEM of the relative abundances ($2^{-\Delta C_T}$) for both the ipsilateral and the contralateral DRGs. As a comparison, the relative abundance of ASIC3 mRNA in DRG from ASIC3^{–/–} mice, which should be undetectable, was also calculated to show the relative amount of knockdown of ASIC3 mRNA by HSV-miR844.

2.12. Data analysis

Statistical analysis for both the qPCR and the Western blot data was performed with a 1-way analysis of variance followed by Tukey post hoc tests to compare significance between

groups. Behavioral data and patch-clamp data were analyzed with repeated-measures analysis of variance for differences across time and between groups. Post hoc testing with a Tukey test analyzed differences between groups. Differences between the fluorescence intensities of ASIC3 staining in muscle tissue of HSV-control miRNA or HSV-miR844 injected animals were analyzed by Student *t* test. All data are represented as the mean $\pm$ SEM. Differences were considered significant if $P < .05$.

3. Results

3.1. miRNA for targeting ASIC3 inhibits mASIC3

To examine whether miR-ASIC3 inhibited ASIC3, we tested the ability of each pre-miRNA containing plasmid to reduce ASIC3 protein expressed in CHO-K1 cells. The sequences of 844 and 847 directed against mouse ASIC3 are shown in Table 1. Fig. 1A illustrates a dose-dependent inhibition of expression of mASIC3. Fig. 1B shows this inhibition resulted in a nearly complete reduction in ASIC3 with the highest dose of miR844. The percentage knockdown with the highest dose was $> 90\%$ when compared with controls (Fig. 1B). The control miRNA did not inhibit the expression of mouse ASIC3, even when tested at a concentration at which the other miR-ASIC3s were able to produce a $>90\%$ knockdown of mouse ASIC3 expression (data not shown).

To test whether miR-ASIC3 could affect the expression of ASIC1a, we tested the ability of miR844 and miR847 to reduce ASIC1a expression in CHO-K1 cells. Neither miR844 nor miR847 inhibited mouse ASIC1a (Fig. 1C). Thus, the 2 miR-ASIC3 are selective for ASIC3.

To test whether miR844 or miR847 reduced functional ASIC3 activity, we tested pH currents in CHO-K1 cells expressing ASIC3 or ASIC1a. In cells expressing ASIC3, pH-evoked currents were significantly reduced with both miR844 and miR847 when given at 2 different concentrations (Fig. 2A–C). However, in cells expressing ASIC1a, neither miR844 nor miR847 reduced pH-activated currents (Fig. 2D–F).

3.2. miR844 reduces ASIC3 protein expression in muscle tissue

ASIC3 protein levels in the injected muscle were reduced by HSV-miR844 administration in vivo. Immunohistochemical analysis of gastrocnemius muscle tissue removed 4 weeks after injection of either HSV-control miRNA or HSV-miR844 revealed that ASIC3 expression was decreased in tissues from HSV-miR844 injected animals, relative to HSV-control miRNA injected animals (Fig. 3). Quantitation of the fluorescence at 568 nm (ASIC3) in the images from the tissues of the 2 groups shows that the HSV-control miRNA injected tissues was 25.32 ± 4.55 , whereas the HSV-miR844 tissues was 5.51 ± 1.06 ($P = .001$) (Fig. 3G). The decrease in staining of muscle injected with HSV-miR844 was similar to that from ASIC3 $^{-/-}$ mice, which showed intensities of 3.38 ± 1.25 .

3.3. miR844 reduces ASIC3 expression in DRG neurons

To confirm that ASIC3 mRNA expression was reduced in vivo, we performed qPCR of the lumbar DRGs 4 weeks after injection of HSV-miR844 or HSV-control miRNA into the gastrocnemius muscle of mice. In DRGs from mice that had been injected with HSV-miR844, ASIC3 mRNA was significantly decreased (8-fold) relative to the controls injected with HSV-control miRNA in the ipsilateral DRG (Fig. 3H) or the contralateral DRG ($F_{3,12} = 45.2$, $P = .0001$). In comparison, there was an approximate 64-fold decrease in ASIC3 mRNA in ASIC3 $^{-/-}$ DRG, which is a relative number because it is well established that the ASIC3 $^{-/-}$ DRG contain no ASIC3. HSV-miR844, however, had no effect on ASIC1 or ASIC2 mRNA expression in the ipsilateral or contralateral DRGs when compared with

those injected with HSV-control miRNA. These data support the significant knockdown of ASIC3 = expression after treatment with HSV-miR844.

3.4. miR844 reduces inflammatory hyperalgesia in vivo

We tested whether ASIC3 knockdown in muscle afferents would prevent the development of hyperalgesia induced by muscle inflammation. In control mice injected with HSV-control miRNA, muscle inflammation resulted in a decrease in withdrawal threshold of the ipsilateral muscle (Fig. 4A and B) and an increased number of withdrawals to repeated stimulation of the paw bilaterally (Fig. 4C and D). The withdrawal thresholds of the muscle were significantly greater in the group injected with HSV-miR844 when compared with those injected with HSV-control miRNA (ipsilateral; $F_{1,22} = 4.9$, $P = .037$) (Fig. 4A). However, mice injected with HSV-miR844 showed no change in the number of withdrawals to repeated stimulation of the paw ipsilaterally ($F_{1,22} = 50.2$, $P = .0001$) or contralaterally ($F_{1,22} = 28.5$, $P = .0001$) after muscle inflammation when compared with mice injected with HSV-control miRNA (Fig. 4C and D). The data after injection of HSV-miR844 (Fig. 4, B2) were not significantly different than that before injection (Fig. 4, B1). Baseline mechanical withdrawal thresholds of the muscle and number of withdrawals to repeated von Frey stimulation were similar between the group treated with HSV-miR844 4 weeks after infection when compared with the group treated with HSV-1 control miRNA (see times B1 and B2 in Fig. 4). Thus, HSV-miR844 prevented the increased mechanical sensitivity of the paw and muscle induced by muscle inflammation.

3.5. miR844 inhibits ASIC1a/ASIC3 heteromeric channels

Our previous data defined a role for both ASIC1a and ASIC3 isoforms in the development of hyperalgesia after muscle insult [58]. In addition, we have previously shown that most ASIC channels in mouse DRG neurons consist of heteromultimers of multiple different ASIC isoforms [4,16]. Therefore, we tested whether miR844 would affect ASIC1a/3 heteromeric channels. Acidic pH generated large, rapidly desensitizing currents in CHO-K1 that coexpressed both ASIC1a and ASIC3 (Fig. 5A). The rapid desensitization kinetics (see control miRNA data in Fig. 5D) indicate that ASIC1a and ASIC3 formed heteromeric channels because the heteromeric combination has been shown to generate channels with faster kinetics than either of the homomeric channels [4,17]. Most importantly, miR844 significantly reduced the current density when compared with cells transfected with the control miRNA ($P < .05$) (Fig. 5B). To test whether knockdown of ASIC3 also changed the composition of the channels in these cells, we measured whether miR844 altered the pH sensitivity of activation and the desensitization kinetics of the currents. miR844 had no effect on the pH sensitivity of activation (Fig. 5C). However, miR844 tended to slow the desensitization kinetics of the currents, although these were not significantly different ($P > .05$) (Fig. 5D). Closer inspection of the currents from cells transfected with miR844 revealed that the currents generally had 2 components to desensitization (see Fig. 5A). Thus, we fit the desensitization phase of these same currents to double exponential equations and the 2 time constants (fast and slow) for cells treated with miR844 (Fig. 5E). Interestingly, the faster desensitization constant is consistent with the kinetics for ASIC1a/ASIC3 heteromers and was statistically similar between groups ($\tau = 122.8 \pm 8.1$ ms miR844 vs $\tau = 122.8 \pm 4.9$ ms control miRNA; $P > .05$). On the other hand, the slower desensitization constant in cells treated with miR844 was statistically different than that for those treated with control miRNA ($\tau = 1312 \pm 149$ ms miR844 vs $\tau = 865 \pm 147$ ms, $P = .03$). This slower desensitization constant in those cells treated with miR844 matches that of ASIC1a homomeric channels [4,17]. In conclusion, these data suggest that selective knockdown of ASIC3 reduces the current of ASIC1a/ASIC3 heteromeric channels, and could alter the subunit composition of native ASIC channels.

4. Discussion

In this study, we show for the first time that use of artificial miRNAs to inhibit expression of ASIC3 *in vivo* prevents development of chronic inflammatory pain. Inhibition of both muscle and cutaneous hyperalgesia by HSV-miR844 is a different pattern than that observed for muscle inflammation in ASIC3^{-/-} mice, in which only the secondary cutaneous hyperalgesia is reduced [49]. Our prior studies show that ASIC1^{-/-} mice do not develop muscle hyperalgesia, but still develop cutaneous hyperalgesia. Because the behavioral response to HSV-miR844 shows a pattern of both ASIC3^{-/-} and ASIC1^{-/-} mice, we tested whether ASIC3 downregulation affected expression of heteromeric ASICs. We show that downregulation of ASIC3 reduces currents of heteromeric ASIC1/ASIC3, likely a result of decreased surface receptor expression. This suggests that manipulation of ASIC3 expression in adult mice may affect the function of heteromeric ASICs to reduce normal pH sensitivity, resulting in a reduction in hyperalgesia after muscle inflammation, as observed in the current study.

Prior studies suggest that the HSV system is particularly suited for delivery of RNAi to sensory neurons [2,3,18] and show efficacy of HSV-mediated gene transfer into neurons [15,66]. It is highly likely that the combination of HSV vector and the human CMV polymerase II promoter, a promoter known to direct robust transcription [34,53], allowed for the high expression of miR844 resulting in significant knockdown of both ASIC3 mRNA and protein *in vivo*. Further, muscle afferent fibers express substantially more ASIC3 than skin afferent fibers, 50% vs 10% to 20% [32]. In addition, the gastrocnemius muscle in mice is large, with a strong innervation from L4 to L6 when compared with other structures innervated by these afferents in mice. Thus, knockdown of ASIC3 in a large structure such as the gastrocnemius muscle, which has the greatest proportion of ASIC3-containing afferent fibers, is easily detected as a significant decrease similar to that observed in the present study.

Artificial miRNAs, when introduced *in vivo*, generate short hairpin RNAs, which participate in RNA interference, a highly evolutionary conserved mechanism. RNA interference was first described in plants [33,57], but is also found in mammalian cells [10]. An endogenous miRNA is incorporated into a RNA-induced silencing complex and catalytically degrades copies of the targeted message. Artificial miRNAs mimic endogenous short hairpin RNAs and use endogenous intracellular RNA interference pathways to target specific mRNAs for cleavage. The targeted mRNAs are cleaved and thus mRNA and protein levels are decreased, as we show for ASIC3.

4.1. ASICs in inflammatory pain

Previous animal studies of ASICs and pain primarily examine behavior in ASIC knockout mice or test efficacy of ASIC antagonists to decrease hyperalgesia. Cutaneous inflammatory hyperalgesia, induced by paw inflammation, has yielded conflicting results. In general, paw inflammation models measure hyperalgesia at the site of inflammation, and show no differences or increases in pain behaviors in ASIC3^{-/-} mice [6,31,40,51]. Despite conflicting results in ASIC3^{-/-} mice, systemic delivery of nonspecific ASIC antagonists reduces cutaneous hyperalgesia after paw inflammation in mice [9]. In rats, however, the role of ASIC3 in detection of cutaneous pain is clearer [7]. Thermal hyperalgesia after paw inflammation is prevented by a selective ASIC3 antagonist (APETx2) injected at the site of inflammation or by intrathecal administration of a short hairpin RNA (shRNA) against ASIC3 [7]. Together, these data in adult mice and rats suggest that ASICs, in particular ASIC3, plays a critical role in cutaneous inflammatory pain behaviors.

In models of muscle and joint pain, ASIC3^{-/-} mice do not develop secondary mechanical hyperalgesia after muscle and joint insult [20,40,48,49,58,65]. However, these mice still develop secondary thermal hyperalgesia and primary mechanical hyperalgesia [20,49]. In contrast, ASIC1a^{-/-} mice do not develop primary mechanical hyperalgesia after muscle insult [48,58]. Together, these prior data suggest that different ASICs = play different roles in the development of primary and secondary hyperalgesia. However, the current data surprisingly show that knockdown of ASIC3 in primary afferent fibers innervating muscle in mice prevents the development of both primary and secondary mechanical hyperalgesia induced by muscle inflammation. This would suggest a different role for ASIC3 in wild-type mice when compared with ASIC3^{-/-} mice. The development and survival of ASIC knockout mice may result in compensatory mechanisms resulting in distinct differences in ASIC function in wild-type animals. However, Price et al. show no changes in ASIC1 or ASIC2 expression in ASIC3^{-/-} mice [40]. As an alternative, a channel other than an ASIC1 or ASIC2 could compensate for the pH sensitivity.

4.2. ASIC3 in primary afferents innervating muscle is critical for development and maintenance of hyperalgesia

The location of ASIC3 at the site of muscle inflammation is critical for both development and maintenance of both the cutaneous and the muscle hyperalgesia. These data suggest that ASICs serve as a pH sensor at the site of injury to set up the nervous system for the development of both primary muscle and secondary cutaneous hyperalgesia. Activation of ASIC3 would send increased input to the central nervous system to result in central sensitization that is manifested as bilateral hypersensitivity of the paw. We and others show that insult to muscle results in central sensitization of dorsal horn neurons with expansion of receptive fields to include the contralateral hindlimb and increased sensitivity to mechanical stimulation [44,61]. This central sensitization of dorsal horn neurons does not occur in ASIC3^{-/-} mice [48]. Repeated intramuscular injection of acidic saline produces a bilateral mechanical hyperalgesia; prior treatment with a selective ASIC3 inhibitor (APETx2) prevents the development of the hyperalgesia in rats [25]. The current study shows that unilateral infection with HSV-miR844 prevents development of bilateral hyperalgesia and is similar to our prior study showing a unilateral injection of nonspecific ASIC antagonists at the site of muscle insult prevents hyperalgesia bilaterally [48,58]. Further re-expression of ASIC3 into ASIC3^{-/-} mice unilaterally restores hyperalgesia bilaterally [49]. Together, these data suggest that ASIC3 expression in muscle afferents is important for initiating, as well as maintaining, central sensitization and the accompanying bilateral long-lasting hyperalgesia.

4.3. ASICs are primarily heteromeric channels in DRG neurons

In DRG neurons, ASICs are heteromeric and ASIC3 is an essential constituent of ASIC currents [4,16,54,64]. ASIC3 and ASIC1a are responsible for most of the currents in rat DRG neurons activated by moderate acidification [7]. In mice, pH-sensitive currents in cardiac DRG neurons are composed of ASIC2a and ASIC3 heteromers [16]. The current study also shows this preferential formation of heteromers in cells co-transfected with ASIC1a and ASIC3, despite a 90% or greater knockdown of ASIC3. We further show the current density is decreased in miR844-treated CHO cells that were co-transfected with ASIC1a and ASIC3, suggesting an overall decrease in surface expression of heteromeric ASICs. One would predict a longer desensitization constant when more ASIC1 subunits are expressed, ie, in the group transfected with miR844 [17]. Alternatively, ASIC3 could facilitate cell surface expression of ASICs by participating in intracellular trafficking of ASICs to the cell surface.

4.4. Muscle pain and cutaneous pain are differentially processed

Nociceptive inputs from muscle and skin are processed differently both peripherally and centrally. Prior studies show enhanced or no change in nociceptive responses after cutaneous inflammation in ASIC3^{-/-} mice [6,31,40], whereas consistent loss of hyperalgesia has been found after muscle inflammation in ASIC3^{-/-} mice [48,49,58,65] and in the current study using miRNA knockdown of ASIC3. Peripherally, primary afferent fibers innervating muscle express high levels of ASIC3, calcitonin gene-related peptide and substance P, and lower levels of isolectin B4 and somatostatin when compared with afferent fibers innervating skin [32,35,39].

Centrally, electrical stimulation of C-fibers innervating muscle produces longer-lasting enhancement of the flexion reflex, a measure of central excitability, when compared with electrical stimulation of C-fibers innervating skin [61]. In parallel, intramuscular injection of capsaicin produces a longer-lasting secondary hyperalgesia (1 to 4 weeks) when compared with peripheral activation of cutaneous afferents with an intradermal injection of capsaicin (3 to 4 h) [46]. As in the current study, deep tissue stimulation often produces not only an effect on the side of the insult, but also contralaterally [46,47,61,63], which may reflect differences in central processing.

Spinally, deep tissue afferent fibers project to and activate deeper laminae in the dorsal horn and a larger number of spinal cord segments [29,30,42]. Supraspinally, noxious muscle stimuli result in activation of more brain areas than noxious cutaneous stimuli [36]. Thus, differential processing of information in the peripheral and central nervous system after cutaneous or muscle injury could explain the differences between the role of ASIC3 in the development of cutaneous and muscle hyperalgesia.

4.5. ASICs and pain in humans

Many painful conditions, such as rheumatoid arthritis, cardiac ischemia, and skeletal muscle soreness after exhaustive exercise are associated with local tissue acidosis, suggesting a relationship between pain and acidosis [13,19,38,41,43,55]. Studies in human subjects show infusion of acidic pH solutions into skin or muscle produces pain and hyperalgesia [12,22,24,41,52,56]. In fact, infusion of acidic buffer into muscle produces pain and referred pain, as well as primary and secondary hyperalgesia [12]. The pain in response to cutaneous infusion of acidic solutions is inhibited by local application of amiloride, a nonspecific ASIC inhibitor, suggesting ASICs mediate pain to decreases in pH in human subjects [56].

Acknowledgments

This work was funded by the National Institutes of Health, National Institute of Arthritis and Musculoskeletal and Skin Diseases (R01AR053509 and R01AR053509S1).

References

1. Alvarez de la Rosa D, Krueger SR, Kolar A, Shao D, Fitzsimonds RM, Canessa CM. Distribution, subcellular localization and ontogeny of ASIC1 in the mammalian central nervous system. *J Physiol.* 2003; 546:77–87. [PubMed: 12509480]
2. Anesti AM. Delivery of RNA interference to peripheral neurons in vivo using herpes simplex virus. *Methods Mol Biol.* 2010; 617:347–361. [PubMed: 20336434]
3. Anesti AM, Peeters PJ, Royaux I, Coffin RS. Efficient delivery of RNA interference to peripheral neurons in vivo using herpes simplex virus. *Nucleic Acids Res.* 2008; 36:e86. [PubMed: 18583367]
4. Benson CJ, Xie J, Wemmie JA, Price MP, Henss JM, Welsh MJ, Snyder PM. Heteromultimers of DEG/ENaC subunits form H⁺-gated channels in mouse sensory neurons. *Proc Natl Acad Sci USA.* 2002; 99:2338–2343. [PubMed: 11854527]

5. Carnally SM, Dev HS, Stewart AP, Barrera NP, Van Bemmelen MX, Schild L, Henderson RM, Edwardson JM. Direct visualization of the trimeric structure of the ASIC1a channel, using AFM imaging. *Biochem Biophys Res Commun.* 2008; 372:752–755. [PubMed: 18514062]
6. 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; 99:8992–8997. [PubMed: 12060708]
7. Deval E, Noel J, Lay N, Alloui A, Diochot S, Friend V, Jodar M, Lazdunski M, Lingueglia E. ASIC3, a sensor of acidic and primary inflammatory pain. *EMBO J.* 2008; 27:3047–3055. [PubMed: 18923424]
8. Dube GR, Elagoz A, Mangat H. Acid sensing ion channels and acid nociception. *Curr Pharm Des.* 2009; 15:1750–1766. [PubMed: 19442188]
9. Dube GR, Lehto SG, Breese NM, Baker SJ, Wang X, Matulenko MA, Honore P, Stewart AO, Moreland RB, Brioni JD. Electrophysiological and in vivo characterization of A-317567, a novel blocker of acid sensing ion channels. *Pain.* 2005; 117:88–96. [PubMed: 16061325]
10. Elbashir SM, Harborth J, Lendeckel W, Yalcin A, Weber K, Tuschl T. Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. *Nature.* 2001; 411:494–498. [PubMed: 11373684]
11. Eshcol JO, Harding AM, Hattori T, Costa V, Welsh MJ, Benson CJ. Acid-sensing ion channel 3 (ASIC3) cell surface expression is modulated by PSD-95 within lipid rafts. *Am J Physiol Cell Physiol.* 2008; 295:C732–C739. [PubMed: 18579798]
12. Frey Law LA, Sluka KA, McMullen T, Lee J, Arendt-Nielsen L, Graven-Nielsen T. Acidic buffer induced muscle pain evokes referred pain and mechanical hyperalgesia in humans. *Pain.* 2008; 140:254–264. [PubMed: 18835099]
13. Geborek P, Saxne T, Pettersson H, Wollheim FA. Synovial fluid acidosis correlates with radiological joint destruction in rheumatoid arthritis knee joints. *J Rheumatol.* 1989; 16:468–472. [PubMed: 2746586]
14. Gonzales EB, Kawate T, Gouaux E. Pore architecture and ion sites in acid-sensing ion channels and P2X receptors. *Nature.* 2009; 460:599–604. [PubMed: 19641589]
15. Goss JR, Gold MS, Glorioso JC. HSV vector-mediated modification of primary nociceptor afferents: an approach to inhibit chronic pain. *Gene Ther.* 2009; 16:493–501. [PubMed: 19357694]
16. Hattori T, Chen J, Harding AM, Price MP, Lu Y, Abboud FM, Benson CJ. ASIC2a and ASIC3 heteromultimerize to form pH-sensitive channels in mouse cardiac dorsal root ganglia neurons. *Circ Res.* 2009; 105:279–286. [PubMed: 19590043]
17. Hesselager M, Timmermann DB, Ahring PK. pH Dependency and desensitization kinetics of heterologously expressed combinations of acid-sensing ion channel subunits. *J Biol Chem.* 2004; 279:11006–11015. [PubMed: 14701823]
18. Hong CS, Goins WF, Goss JR, Burton EA, Glorioso JC. Herpes simplex virus RNAi and neprilysin gene transfer vectors reduce accumulation of Alzheimer's disease-related amyloid-beta peptide in vivo. *Gene Ther.* 2006; 13:1068–1079. [PubMed: 16541122]
19. Hood VL, Schubert C, Keller U, Muller S. Effect of systemic pH on pHi and lactic acid generation in exhaustive forearm exercise. *Am J Physiol.* 1988; 255:F479–F485. [PubMed: 3414804]
20. 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.* 2008; 137:662–669. [PubMed: 18343037]
21. Ikeuchi M, Kolker SJ, Sluka KA. Acid-sensing ion channel 3 expression in mouse knee joint afferents and effects of carrageenan-induced arthritis. *J Pain.* 2009; 10:336–342. [PubMed: 19185546]
22. Issberner U, Reeh PW, Steen KH. Pain due to tissue acidosis: a mechanism for inflammatory and ischemic myalgia? *Neurosci Lett.* 1996; 208:191–194. [PubMed: 8733302]
23. Jasti J, Furukawa H, Gonzales EB, Gouaux E. Structure of acid-sensing ion channel 1 at 1.9 Å resolution and low pH. *Nature.* 2007; 449:316–323. [PubMed: 17882215]
24. Jones NG, Slater R, Cadiou H, McNaughton P, McMahon SB. Acid-induced pain and its modulation in humans. *J Neurosci.* 2004; 24:10974–10979. [PubMed: 15574747]

25. Karczewski J, Spencer RH, Garsky VM, Liang A, Leidl MD, Cato MJ, Cook SP, Kane S, Urban MO. Reversal of acid-induced and inflammatory pain by the selective ASIC3 inhibitor, APETx2. *Br J Pharmacol.* 2010; 161:950–960. [PubMed: 20860671]
26. Kolker SJ, Walder RY, Usachev Y, Hillman J, Boyle DL, Firestein GS, Sluka KA. ASIC3 expressed in type B synoviocytes and chondrocytes modulates hyaluronan expression and release. *Ann Rheum Dis.* 2010; 69:903–909. [PubMed: 19933746]
27. Lingueglia E. Acid-sensing ion channels in sensory perception. *J Biol Chem.* 2007; 282:17325–17329. [PubMed: 17430882]
28. Lingueglia E, de Weille JR, Bassilana F, Heurteaux C, Sakai H, Waldmann R, Lazdunski M. A modulatory subunit of acid sensing ion channels in brain and dorsal root ganglion cells. *J Biol Chem.* 1997; 272:29778–29783. [PubMed: 9368048]
29. Mense S. Nociception from skeletal muscle in relation to clinical muscle pain. *Pain.* 1993; 54:241–289. [PubMed: 8233542]
30. Mense S, Craig AD Jr. Spinal and supraspinal terminations of primary afferent fibers from the gastrocnemius-soleus muscle in the cat. *Neuroscience.* 1988; 26:1023–1035. [PubMed: 3200424]
31. Mogil JS, Breese NM, Witty MF, Ritchie J, Rainville ML, Ase A, Abbadi N, Stucky CL, Seguela P. Transgenic expression of a dominant-negative ASIC3 subunit leads to increased sensitivity to mechanical and inflammatory stimuli. *J Neurosci.* 2005; 25:9893–9901. [PubMed: 16251436]
32. 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.* 2005; 1:35. [PubMed: 16305749]
33. Napoli C, Lemieux C, Jorgensen R. Introduction of a chimeric chalcone synthase gene into petunia results in reversible co-suppression of homologous genes in trans. *Plant Cell.* 1990; 2:279–289. [PubMed: 12354959]
34. Nielsen TT, Marion I, Hasholt L, Lundberg C. Neuron-specific RNA interference using lentiviral vectors. *J Gene Med.* 2009; 11:559–569. [PubMed: 19434604]
35. 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.* 1989; 32:493–502. [PubMed: 2555742]
36. Ohtori S, Takahashi K, Chiba T, Takahashi Y, Yamagata M, Sameda H, Moriya H. Fos expression in the rat brain and spinal cord evoked by noxious stimulation to low back muscle and skin. *Spine.* 2000; 25:2425–2430. [PubMed: 11013492]
37. Olson TH, Riedl MS, Vulchanova L, Ortiz-Gonzalez XR, Elde R. An acid sensing ion channel (ASIC) localizes to small primary afferent neurons in rats. *Neuroreport.* 1998; 9:1109–1113. [PubMed: 9601677]
38. Pan JW, Hamm JR, Rothman DL, Shulman RG. Intracellular pH in human skeletal muscle by ^1H NMR. *Proc Natl Acad Sci USA.* 1988; 85:7836–7839. [PubMed: 3186694]
39. Plenderleith MB, Snow PJ. The plant lectin *Bandeiraea simplicifolia* I-B4 identifies a subpopulation of small diameter primary sensory neurones which innervate the skin in the rat. *Neurosci Lett.* 1993; 159:17–20. [PubMed: 8264961]
40. 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; 32:1071–1083. [PubMed: 11754838]
41. Reeh PW, Steen KH. Tissue acidosis in nociception and pain. *Prog Brain Res.* 1996; 113:143–151. [PubMed: 9009732]
42. Schaible HG, Grubb BD. Afferent and spinal mechanisms of joint pain. *Pain.* 1993; 55:5–54. [PubMed: 8278210]
43. Shah JP, Phillips TM, Danoff JV, Gerber LH. An in vivo microanalytical technique for measuring the local biochemical milieu of human skeletal muscle. *J Appl Physiol.* 2005; 99:1977–1984. [PubMed: 16037403]
44. Skyba DA, King EW, Sluka KA. Effects of NMDA and non-NMDA ionotropic glutamate receptor antagonists on the development and maintenance of hyperalgesia induced by repeated intramuscular injection of acidic saline. *Pain.* 2002; 98:69–78. [PubMed: 12098618]

45. Skyba DA, Lisi TL, Sluka KA. Excitatory amino acid concentrations increase in the spinal cord dorsal horn after repeated intramuscular injection of acidic saline. *Pain*. 2005; 119:142–149. [PubMed: 16297556]
46. 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*. 2002; 22:5687–5693. [PubMed: 12097520]
47. Sluka KA, Kalra A, Moore SA. Unilateral intramuscular injections of acidic saline produce a bilateral, long-lasting hyperalgesia. *Muscle Nerve*. 2001; 24:37–46. [PubMed: 11150964]
48. 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; 106:229–239. [PubMed: 14659506]
49. 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*. 2007; 129:102–112. [PubMed: 17134831]
50. Sluka KA, Winter OC, Wemmie JA. Acid-sensing ion channels: a new target for pain and CNS diseases. *Curr Opin Drug Discov Dev*. 2009; 12:693–704.
51. 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; 13:554–563. [PubMed: 18801682]
52. Steen KH, Reeh PW. Sustained graded pain and hyperalgesia from harmless experimental tissue acidosis in human skin. *Neurosci Lett*. 1993; 154:113–116. [PubMed: 8361622]
53. Stegmeier F, Hu G, Rickles RJ, Hannon GJ, Elledge SJ. A lentiviral microRNA-based system for single-copy polymerase II-regulated RNA interference in mammalian cells. *Proc Natl Acad Sci USA*. 2005; 102:13212–13217. [PubMed: 16141338]
54. Sutherland SP, Benson CJ, Adelman JP, McCleskey EW. Acid-sensing ion channel 3 matches the acid-gated current in cardiac ischemia-sensing neurons. *Proc Natl Acad Sci USA*. 2001; 98:711–716. [PubMed: 11120882]
55. Sutherland SP, Cook SP, McCleskey EW. Chemical mediators of pain due to tissue damage and ischemia. *Prog Brain Res*. 2000; 129:21–38. [PubMed: 11098679]
56. 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*. 2002; 110:1185–1190. [PubMed: 12393854]
57. van der Krol AR, Mur LA, Beld M, Mol JN, Stuitje AR. Flavonoid genes in petunia: addition of a limited number of gene copies may lead to a suppression of gene expression. *Plant Cell*. 1990; 2:291–299. [PubMed: 2152117]
58. Walder RY, Rasmussen LA, Rainier JD, Light AR, Wemmie JA, Sluka KA. ASIC1 and ASIC3 play different roles in the development of hyperalgesia after inflammatory muscle injury. *J Pain*. 2010; 11:210–218. [PubMed: 20015700]
59. Waldmann R, Bassilana F, de Weille J, Champigny G, Heurteaux C, Lazdunski M. Molecular cloning of a non-inactivating proton-gated Na⁺ channel specific for sensory neurons. *J Biol Chem*. 1997; 272:20975–20978. [PubMed: 9261094]
60. Waldmann R, Champigny G, Bassilana F, Heurteaux C, Lazdunski M. A proton-gated cation channel involved in acid-sensing. *Nature*. 1997; 386:173–177. [PubMed: 9062189]
61. Wall PD, Woolf CJ. Muscle but not cutaneous C-afferent input produces prolonged increases in the excitability of the flexion reflex in the rat. *J Physiol*. 1984; 356:443–458. [PubMed: 6520794]
62. Wemmie JA, Price MP, Welsh MJ. Acid-sensing ion channels: advances, questions and therapeutic opportunities. *Trends Neurosci*. 2006; 29:578–586. [PubMed: 16891000]
63. Woolf CJ, Wall PD. Relative effectiveness of C primary afferent fibers of different origins in evoking a prolonged facilitation of the flexor reflex in the rat. *J Neurosci*. 1986; 6:1433–1442. [PubMed: 3711988]
64. Xie J, Price MP, Berger AL, Welsh MJ. DRASIC contributes to pH-gated currents in large dorsal root ganglion sensory neurons by forming heteromultimeric channels. *J Neurophysiol*. 2002; 87:2835–2843. [PubMed: 12037186]

65. Yen YT, Tu PH, Chen CJ, Lin YW, Hsieh ST, Chen CC. Role of acid-sensing ion channel 3 in sub-acute-phase inflammation. *Mol Pain*. 2009; 5:1. [PubMed: 19126241]
66. Zhang G, Mohammad H, Peper BD, Raja S, Wilson SP, Sweitzer SM. Enhanced peripheral analgesia using virally mediated gene transfer of the mu-opioid receptor in mice. *Anesthesiology*. 2008; 108:305–313. [PubMed: 18212576]

Fig. 1. miRNA targeting acid-sensing ion channel 3 (ASIC3) selectively decreases heterologously expressed ASIC3. (A) Western blot showing a dose-dependent inhibition of ASIC3 expression by miR844. CHO-K1 cells were transfected with hemagglutinin (HA)-tagged ASIC3 (4 μg) and increasing concentrations of miR844-containing plasmid: 0, 8, 12, 16, 20, and 24 μg, lanes 1 to 6, respectively. The blot was first probed with anti-HA-horseradish peroxidase for HA-tagged proteins, and subsequently with anti-beta actin, and goat anti-mouse-horseradish peroxidase as a loading control. (B) Densitometric quantitation of ASIC3 protein levels. Immunoreactive bands in Western blots were measured in Image J. (* $P < .0001$; $n = 4$ per miRNA concentration, significantly different from 0 μg miRNA group, ^+P

< .05; significantly different from the 8 μ g miRNA group). (C) Western blot showing no inhibition of ASIC1a by miR844 or miR847. HA-tagged ASIC1a (4 μ g) plus 0, 10, 20 μ g miR847 (lanes 1 to 3); HA-tagged ASIC1a (4 μ g) plus 0, 10, 20 μ g miR844 (lanes 4 to 6).

Fig. 2. miRNA targeting acid-sensing ion channel 3 (ASIC3) selectively decreases ASIC3 current. (A) Representative pH-evoked currents from CHO cells expressing ASIC3 along with control miRNA, miR847, or miR844. (B) Mean pH 6-evoked current density from cells expressing ASIC3 and control miRNA, miR847, or miR844 transfected at 1:1 ASIC3:miRNA ratio (1 μ g each; *significantly different from control miRNA), or (C) at 1:5 ASIC3:miRNA ratio (0.2 and 0.9 μ g, respectively; *significantly different from control miRNA). Control currents in C are less than those in B because there was less ASIC3 transfected. (D) Representative pH-evoked currents from CHO cells expressing ASIC1a along with control miRNA, miR847, or miR844. (E) Mean pH 5-evoked current density

from cells expressing ASIC1a and control miRNA, miR847, or miR844 transfected at 1:1 ASIC1a:miRNA ratio (1 μ g each), or (C) at 1:5 ASIC1a:miRNA ratio (0.2 and 0.9 μ g, respectively).

Fig. 3. HSV-miR844 injected into mouse muscle decreases acid-sensing ion channel 3 (ASIC3) expression. (A–F) HSV-miR844 inhibits ASIC3 protein expression in muscle tissue. Representative tissue sections showing ASIC3 staining (red) in animals injected with HSV-control miRNA (A and D) and HSV-miR844 (B, E), in comparison to staining in muscle tissue from ASIC3^{-/-} mice (C, F). The nuclei are stained with DAPI (blue). Lower magnification of ASIC3 staining in muscle is shown in A–C (scale bar, 100 μ m), and higher magnification of these images (boxed area) is shown in D–F (scale bar, 20 μ m). Positive ASIC3 staining is marked with white arrows (D). (G) The intensity of ASIC3 immunoreactivity was measured in the muscle tissue from 4 animals in each group, and the

mean $\pm$ SEM values are presented ($**P = .001$). (H) HSV-miR844 selectively inhibits ASIC3 mRNA expression in the ipsilateral DRG (8-fold decrease). Graph shows quantitative polymerase chain reaction of ASIC1, ASIC2, and ASIC3 expression in DRGs ($n = 8$ per group) in groups treated with HSV-control miRNA and HSV-miR844. For comparison, ASIC3 expression in DRGs from ASIC3 $^{-/-}$ mice were calculated and added to the graph (approximately 64-fold decrease). Data are mean $\pm$ SEM for the relative abundance normalized to GAPDH ($2^{-\Delta C_T}$) ($*P < .05$).

Fig. 4.

HSV-miR844 prevents the development of primary and secondary hyperalgesia in vivo. (A and B) Primary hyperalgesia (muscle withdrawal thresholds) measured in HSV-control miRNA or HSV-miR844 injected animals tested before (B1) and at 4 weeks after HSV injection (B2), and 24 h, 72 h, and 1 week after induction of muscle inflammation. HSV-miR844 injected animals do not develop hyperalgesia at 24 h, 72 h, and 1 week after muscle inflammation ($*P < .05$ on the ipsilateral side). (C and D) Secondary hyperalgesia (responses to repeated von Frey stimulation of the paw) show that HSV-miR844 injected animals do not develop paw hyperalgesia at 24 h, 72 h, and 1 week after muscle inflammation ($*P < .05$ on the ipsilateral and contralateral sides).

Fig. 5. miR844 inhibits acid-sensing ion channel (ASIC)1a/ASIC3 heteromeric channels in CHO-K1 cells. (A) Representative pH-evoked currents from CHO cells coexpressing both ASIC1a and ASIC3 (at 1:1 cDNA ratio) along with control miRNA (black traces) or miR844 (at ASIC:miRNA ratio of 1:5, gray traces). Normalized currents show the difference in desensitization kinetics with different pH stimuli. Amplitudes differed at each pH between control miRNA (pH 5.0 = 8.3 nA; pH 6.0 = 6.9 nA; pH 6.5 = 3.3 nA) and miR844 (pH 5.0 = 3.5 nA; pH 6.0 = 2.4 nA; pH 6.5 = 2.1 nA). (B) Mean current density from above cells evoked by the indicated pH solutions showing that miR844 significantly inhibits the current density at all tested pHs ($*P < .05$). (C) pH dose response data of transient currents from the

above cells. (D) Mean desensitization time constants (τ) measured from fits of single exponential equations of currents evoked by pH 5.0, 6.0, and 6.5 were not significantly different ($P > .05$). Currents were normalized to currents evoked at pH 5. (E) Mean fast and slow desensitization time constants (τ) measured from fits of double exponential equations of currents evoked by pH 5.0, 6.0, and 6.5 from the cells transfected with miR844.

Table 1

Targeted sequences for the miRNA against ASIC3 in mouse.

miRNA	Pre-miRNA sequences	Target sequence on ASIC3 RNA	Location of target on ASIC3 ^a
844	Top: GCTGTGAAGTTCACAGGTCCACAGGGTTTTGGCCACTGACTGACCTGTGGATGAGAACTTCA	CCCUGUGGACCUGAGAACUUCA	511–532
	Bottom: CCTGTGAAGTTCTCATCCACAGGGTCAAGTCAGTGGCCAAAACCCCTGTGGACCTGAGAACTTCAC		
847	Top: TGCTGTACACAAAAGTGACAGCTGGGAGTTTTGGCCACTGACTGCCAGCTCACTTTGTGTA	UCCCAGCUGUCACUUUGUGUA	260–280
	Bottom: CCTGTACACAAAGTGAGCTGGGAGTCAGTCAGTGGCCAAAACCTCCAGCTGTCACTTTGTGTAC		

ASIC3 = acid-sensing ion channel 3.

^aBased on the distance from the CDS start; NM_183000 (mouse ASIC3).