Transient receptor potential vanilloid 1 mediates pain in mice with severe sickle cell disease

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Pain is the leading cause of emergency department visits, hospitalizations, and daily suffering in individuals with sickle cell disease (SCD). The pathologic mechanisms leading to the perception of pain during acute RBC sickling episodes and development of chronic pain remain poorly understood and ineffectively treated. We provide the first study that explores nociceptor sensitization mechanisms that contribute to pain behavior in mice with severe SCD. Sickle mice exhibit robust behavioral hypersensitivity to mechanical, cold, and heat stimuli. Mechanical hypersensitivity is further exacerbated when hypoxia is used to induce acute sickling. Behavioral mechanical hypersensitivity is mediated in part by enhanced excitability to mechanical stimuli at both primary afferent peripheral terminal and sensory membrane levels. In the present study, inhibition of the capsaicin receptor transient receptor potential vanilloid 1 (TRPV1) with the selective antagonist A-425619 reversed the mechanical sensitization at both primary afferent terminals and isolated somata, and markedly attenuated mechanical behavioral hypersensitivity. In contrast, inhibition of TRPA1 with HC-030031 had no effect on mechanical sensitivity. These results suggest that the TRPV1 receptor contributes to primary afferent mechanical sensitization and a substantial portion of behavioral mechanical hypersensitivity in SCD mice. Therefore, TRPV1-targeted compounds that lack thermoregulatory side effects may provide relief from pain in patients with SCD.

Introduction

Sickle cell disease (SCD) is a major health care and socioeconomic problem that affects millions of people worldwide. In the United States alone, SCD affects >80,000 people, the majority of whom are African American. Pain is the hallmark symptom of SCD and the leading cause of emergency department visits, hospitalizations, and daily suffering.1 Patients suffer unpredictable, incapacitating acute pain episodes that are believed to result from red blood cell (RBC) sickling and “vaso-occlusion.” However, many features of this pain are not explained by hemoglobin polymerization and vascular obstruction. Furthermore, individuals with SCD often develop chronic pain syndromes that are poorly understood and ineffectively treated.1,2 The frequency and severity of pain is associated with increased mortality and profoundly erodes patients’ quality of life.3 Because they are often from minority and lower socioeconomic groups, SCD patients are commonly underserved and suboptimally treated.

The pathologic mechanisms leading to the perception of pain during RBC sickling episodes and the transition from acute to chronic pain remain poorly understood.1,2 Patient descriptors of SCD pain include neuropathic pain attributes such as “aching,” “shooting,” and “stabbing,” as well as noxious pain qualities such as “sharp,” “throbbing,” and “pounding.” These descriptors depict spontaneous pain or nonstimulus-evoked pain.2,4 Furthermore, increased hospital admissions and reports of acute pain have been associated with high environmental wind speed on the day of admission.5,6 These data suggest that SCD patients may have hypersensitivity to touch stimuli. The wide array of pain descriptors and precipitating factors suggest that both peripheral and central neural mechanisms likely contribute to pain in SCD.

We sought to quantify SCD-associated touch sensitivity and pain behavior using the well-characterized Berkeley mouse model of severe SCD.3 SCD mice exhibited persistent hypersensitivity to mechanical touch that was further exacerbated when hypoxia was used to induce acute RBC sickling. Functional recordings of mechanistically sensitive neurons in ex vivo skin-nerve preparations and isolated dorsal root ganglion (DRG) somata demonstrated that the behavioral mechanical hypersensitivity is driven by increased primary afferent input to the CNS. Pharmacologic inhibition of the transient receptor potential vanilloid 1 (TRPV1) channel completely reversed the mechanical sensitization at both the primary afferent terminals and isolated DRG somata and partially reversed the behavioral mechanical hypersensitivity. These data suggest that future TRPV1 antagonists that lack thermoregulatory side effects may provide novel strategies for the treatment of the pain suffered by patients with severe SCD.

Methods

Mouse model of severe SCD

All procedures were approved by the Medical College of Wisconsin (MCW) Institutional Animal Care and Use Committee. We used the well-characterized Berkeley mouse model of severe sickle cell disease (SCD) developed by Paszty et al.7 Mice for this study were generated by...
breeding at the MCW animal facility. Adult Berkeley SCD mice (HbSS) exclusively express the human sickle hemoglobin in erythroid cells. Control (HbAA) mice express only normal human hemoglobin (HbAA) on the same mixed Berkeley genetic background. HbSS mice exhibit pathology and phenotypes that closely mimic many features of severe SCD in humans.\(^7\)

Male and female mice were used for all behavioral experiments, and male mice were used for skin-nerve and patch-clamp experiments. All mice used were adults to determine the effects of SCD on a developmentally mature population; ages ranged from 10-25 weeks (average age at testing, 16 weeks for HbAA mice and 14 weeks for HbSS mice). For all behavioral and electrophysiological tests, the experimenter was blinded to the genotype and chemical treatment of the mice.

**Behavioral assays**

Mechanical hind paw withdrawal thresholds were measured using a series of calibrated von Frey filaments (0.38-37 mN) applied to the plantar hind paw to determine the 50% paw withdrawal threshold.\(^8\) Heat sensitivity was determined by calculating the latency to paw withdrawal from focal radiant heat.\(^9\) Cold sensitivity was measured by determining the latency to a response from a cold plate held at 20°C. A response to the cold plate was defined as a paw lift, lick, or flinch. The number of cold-evoked responses was quantified over the initial 2 minutes. Mechanical, heat, and cold responses were quantified from both hind paws and averaged per mouse. To test the effects of TRPV1 inhibition, mice were dosed (intraperitoneal) with either the TRPV1 antagonist A-425619 (100 μmol/kg; Abbott Laboratories)\(^10\) or vehicle (10% DMSO + 34% 2-hydroxypropyl β-cyclodextrin in dH2O; Sigma), which was followed by measuring behavioral mechanical thresholds at 30-minute intervals. The 100 μmol/kg dose of A-425619 was selected because it had the greatest antinociceptive effect in the complete Freund's adjuvant model of peripheral inflammation in rats and did not induce any effect on motor activity, general CNS function, or spontaneous exploratory activity.\(^11\) We did not measure changes in body temperature after administration of A-425619; however, transient hyperthermia followed by hypothermia has been observed in rats administered this compound.\(^12\) Recent evidence indicates that the hyperthermia-inducing effects of TRPV1 antagonists including A-425619 is due to their ability to block the proton-binding site on TRPV1.\(^13\)

**Induction of acute sickling via hypoxia**

Mice were placed in a closed chamber that mixes nitrogen inflow with room air to achieve hypoxia at 10% F O2 (normoxia = 21% F O2); F O2 was continuously monitored with an O2 sensor (Biospherix E702). HbSS or HbAA control mice were exposed to 10% F O2 for 2 hours, followed by return to ambient air for reoxygenation (21% F O2). Mechanical von Frey thresholds were tested before hypoxia treatment (baseline) and then after 1, 2, and 3 hours of reoxygenation.

**Teased fiber recordings in skin-nerve preparation**

Single fibers were teased and recorded from the ex vivo hairy skin-saphenous nerve preparation, as described previously.\(^16\) Receptive fields were located using electrical search stimuli.\(^14\) A constant pH of 7.45 ± 0.05 was maintained throughout the recordings. Fibers were classified by their conduction velocity, von Frey thresholds, and response properties to mechanical stimuli. Recordings were focused on nociceptor groups including myelinated Aδ fibers (conduction velocity range, 1.2-10 m/s) and unmyelinated C fibers (conduction velocity < 1.2 m/s). For treatment with compounds, the receptive field was isolated by sealing a metal ring to the skin and then the TRPV1 antagonist A-425619 (10μM; Abbott Laboratories), the TRPA1 antagonist HC-030031 (100μM; Hydra Biosciences), or vehicle (0.1% N-methyl-2-pyrrolidone for A-425619; 0.1% DMSO for HC-030031) was applied to the ring for 10 minutes. The concentrations of antagonists for TRPV1 and TRPA1 were selected based on effective and selective concentrations used in prior studies.\(^10,11,15,16\) Quantitative mechanical stimuli were then applied in the presence of the compound using a computer-driven, feedback-controlled probe placed on the receptive field and sustained mechanical stimuli were applied with increasing intensity (5-245 mN; 10-second duration; 1-minute interstimulus interval).

**Patch-clamp electrophysiology**

Lumbar 1-6 DRGs were isolated bilaterally from adult male HbSS or HbAA control mice, dissociated, and plated on laminin-coated coverslips with no added growth factors, as described previously.\(^17\) Whole-cell voltage-clamp recordings were performed 12-36 hours after plating. A constant pH of 7.4 was maintained throughout the recordings. Small-diameter neurons < 27 μm were targeted because the majority have unmyelinated C-fiber axons in vivo and many C fibers are nociceptors. Focal mechanical indentation was applied to the soma membrane using a fire-polished closed glass pipette (tip diameter, 2-3 μm) positioned above the neuron at a 45° angle and driven by a piezoelectric micromanipulator (MM3A; Kleindiek Nanotechnik) controlled via NanoControl software (version 4.0) as described previously.\(^17\) The stimulator pipette was advanced toward the cell (velocity, 3.5 μm/ms; duration, 200 ms) and then moved back to its original position. The displacement was increased by 2 μm for each consecutive stimulation (indentation range, 2.0-10 μm; 1-minute intervals) until the patch seal became unstable. An inward current ≥ 30 pA was considered a response. The current profile was characterized as "slowly adapting" if the half-time of inactivation was > 10 ms, and "transient" if < 10 ms. For each neuron, the current exhibiting the largest amplitude was analyzed and characterized for profile and peak amplitude. For treatment with compounds, the neuron was superfused with the TRPV1 antagonist A-425619 (1μM) or vehicle (0.01% ETOH) for 2 minutes and mechanical stimuli were applied in the presence of the compound. Neurons were incubated with IB4-FITC (10 μg/mL) for 10 minutes and visualized as described previously.\(^17\)

**Data analyses and statistics**

Because of the discrete nature of mechanical threshold data, paw withdrawal thresholds to mechanical stimuli were compared using the Mann Whitney U test for 2 groups or the Friedman repeated-measures test with post hoc Dunn analyses when testing the same group of mice over time. All other data passed tests for normalcy except where specifically noted. Latency for response to heat or cold, or number of responses was compared using independent samples Student t test. Mechanically evoked action potentials were compared between groups using 2-way repeated-measures ANOVA. Percentage responses were compared using a χ2 test or Fisher exact test. Data are presented as means ± SEM or the percentage responding of the total tested unless otherwise noted.

**Results**

**SCD mice display persistent hypersensitivity to mechanical and thermal stimuli**

We found that male HbSS mice display marked hypersensitivity to mechanical stimuli with mechanical thresholds that were 4-fold lower than control HbAA mice (Figure 1A). Thermal modalities were also affected in HbSS mice because paw withdrawal latencies from both heat and cold stimuli were markedly reduced (Figure 1B-C). Similarly, female HbSS mice also showed marked hypersensitivity to mechanical, heat, and cold stimuli (supplemental Figure 1A-C, available on the Blood Web site; see the Supplemental Materials link at the top of the online article). Therefore, male and female mice with severe SCD have persistent hypersensitivity to multiple physical modalities.

**RBC sickling exacerbates mechanical hypersensitivity**

We next investigated whether induction of RBC sickling would exacerbate the mechanical hypersensitivity in an experimental
model of acute vasoocclusion. RBC sickling was induced by exposing mice to hypoxia (10% FiO2) for 2 hours, followed by reoxygenation (21% FiO2) for 1-3 hours. Behavioral tests showed that mechanical thresholds were progressively reduced in a time-dependent manner 1-3 hours after reoxygenation in male HbSS mice compared with no change in HbAA controls exposed to similar hypoxia (Figure 1D). We found a similar effect in female HbSS mice (supplemental Figure 1D). In contrast, neither heat nor cold hypersensitivity was affected by exposure to hypoxia in male or female HbSS mice (data not shown). Given the selective exacerbation of mechanical hypersensitivity in response to hypoxia-induced acute sickling, as well as reports of hypersensitivity to light touch stimuli from individuals with SCD,5,6 we focused our functional recording studies on mechanical stimulation.

Enhanced nociceptor excitability underlies behavioral hypersensitivity to mechanical stimuli

To determine whether enhanced primary afferent input drives the behavioral mechanical sensitization in HbSS mice, we recorded mechanically evoked action potentials from identified cutaneous sensory neurons in an ex vivo skin-nerve preparation. We focused on myelinated (Aβ) and unmyelinated (C-fiber) nociceptors because these afferents typically provide nociceptive input for behavioral sensitization to evoked external stimuli. C fibers from HbSS mice exhibited marked sensitization to mechanical force across the entire force range, and at 200 mN, fired nearly 2-fold more action potentials than C fibers from controls (Figure 2A-B; *P < .0001). There was no difference in the proportion of C fibers...
located by electrical stimuli that were mechanically sensitive in HbSS mice (13 of 15) versus HbAA controls (13 of 13). Myelinated A-mechanoreceptors (AMs) showed no overall changes in firing triggered by mechanical stimuli (Figure 2C). However, when AM fibers were stratified into high-threshold (≥ 4 mN) and low-threshold (< 4 mN), high-threshold AM fibers from HbSS mice fired more action potentials in response to sustained mechanical force than those from HbAA controls (Figure 2D). The response of low-threshold AM fibers was similar in both groups (Figure 2E). There were no differences between HbSS or HbAA mice in von Frey thresholds, electrical thresholds, or conduction velocity in any fiber type (supplemental Table 1). Therefore, both myelinated and unmyelinated nociceptors provide increased input, in the form of enhanced action potential firing to suprathreshold cutaneous mechanical stimuli, to the spinal cord pain pathways in HbSS mice.

TRPV1 mediates sickle cell–mediated mechanical sensitization of nociceptor terminals

To address mechanisms underlying mechanical hypersensitivity and afferent sensitization, we investigated whether ion channels involved in sensory transduction might contribute. First, we investigated whether the Transient Receptor Potential Ankyrin 1 (TRPA1) channel mediates the mechanical sensitization in SCD mice because TRPA1 is important for normal mechanical firing in C fibers14,15 and mediates mechanical hypersensitivity in models of peripheral inflammation and nerve injury.16 We determined whether the TRPA1 antagonist HC-030031 (100μM for 10 minutes; Hydra Biosciences) applied to skin receptive fields altered mechanically evoked firing. HC-030031 had no effect on mechanical firing in C fibers from HbSS mice compared with naive HbSS mice (Figure 3A-E), indicating that the TRPA1 channel does not contribute to sickle cell–sensitized mechanical firing in nociceptors.

A relative of TRPA1, the TRPV1 channel is more extensively expressed on many C-fiber nociceptors and is the chief mediator of inflammatory heat pain.19,20 Evidence indicates that TRPV1 contributes to mechanical sensitization and behavioral hypersensitivity in animal models of nerve injury and inflammation.21,22 Because SCD exhibits characteristics of chronic inflammation,23 we investigated the effects of pharmacologic inhibition of TRPV1 on mechanical firing using the TRPV1 antagonist A-425619 (10μM for 10 minutes; Abbott Laboratories). A-425619 was selected because it blocks all modalities of TRPV1 channel activation, including capsaicin, protons, heat, and endogenous ligands such as NADA and anandamide.24 A-425619 substantially reduced the sensitized mechanical firing in C fibers from HbSS mice to normal levels (Figure 3A-B). Vehicle for A-425619 (0.1% NMP) had no effect in HbSS mice (Figure 3C). A-425619 had no effect in control HbAA mice (Figure 3D), indicating that TRPV1 specifically mediates the sickle cell-sensitized mechanical firing, but not the normal mechanical response properties in these C fibers. These data suggest that TRPV1 mediates the mechanical sensitization of C fiber nociceptors in mice with severe SCD and may underline the behavioral mechanical hypersensitivity in these mice.

TRPV1 mediates sickle cell–induced mechanical sensitization at the sensory membrane

To investigate the cellular site of sickle cell–mediated mechanical sensitization, we recorded mechanically evoked currents at the level of the sensory neuron membrane. Mechanical currents were induced by focal mechanical force to the plasma membrane of small-diameter neurons isolated from lumbar ganglia, which contain the somata of sensory neurons that project to the hind limb areas stimulated in behavioral and skin-nerve recording assays. Small-diameter, putative C-fiber neurons responded to a sustained probe application with either a slowly adapting or a transient inward current, as described previously (Figure 4A).17 Whereas 41% of small neurons from HbAA control mice responded to mechanical force, significantly more (76%) small neurons from HbSS mice responded to the focal stimulus (Figure 4B). There was no difference in the proportion of slowly adapting versus transient currents, as 20% of all mechanically gated currents were slowly adapting and 80% were transient in both cohorts.

We next classified small neurons as isolectin B4 (IB4) positive or IB4 negative, because these 2 neuronal classes depend on distinct growth factors, respond differently to noxious stimuli, terminate in discrete regions of the spinal dorsal horn, and contribute to diverse pain pathways to the cortex.24 In control mice,
IB4-negative neurons were more likely to respond to focal mechanical force than IB4-positive neurons, as described previously (Figure 4C). Sickle cell IB4-positive neurons were 3-fold more likely to respond to mechanical stimulation than IB4-positive control neurons (Figure 4C). There were no differences in resting membrane potential, threshold for generation of an action potential, or cell diameter between the 2 genotypes (supplemental Table 2).

To determine whether TRPV1 underlies the enhanced mechanical response at the soma membrane level, we treated neurons with A-425619 (1 μM) during mechanical stimulation. A-425619 completely reversed the increased mechanical responsiveness in IB4-positive neurons from HbSS mice compared with vehicle-treated controls, but had no significant effect on responsiveness in the IB4-negative group (Figure 4B-C). These data suggest that the TRPV1 receptor mediates sickle cell–induced sensitization of mechanically gated ion currents at the level of the sensory neuron membrane.

TRPV1 mediates sickle cell–induced mechanical behavioral hypersensitivity

To determine whether inhibition of TRPV1 could reduce the persistent mechanical hypersensitivity behavior, HbSS mice were treated with the TRPV1 antagonist A-425619 (100 μmol/kg IP) and paw withdrawal thresholds were tested over time. A-425619 significantly reversed the mechanical hypersensitivity in HbSS mice 30-90 minutes after administration (Figure 5). By 2 hours after injection, the effect of TRPV1 inhibition on mechanical hypersensitivity returned to baseline. These data show that a single systemic injection with a selective TRPV1 antagonist reversibly

**Figure 4. Isolated DRG neurons from HbSS mice exhibit greater mechanical responsiveness.** (A) Examples of slowly adapting and transient inward currents induced by mechanical stimulation in HbAA control neurons. (B) The percentage of isolated DRG neurons that respond to focal mechanical stimulation applied to their soma is significantly greater in HbSS mice compared with HbAA controls. Inhibition of TRPV1 (1 μM A-425619) reduced the mechanical responsiveness of DRG neurons from HbSS mice to control levels (**P < .01, Fisher exact test). (C) The increased mechanical responsiveness occurred selectively in the IB4-positive population of small-diameter neurons (*P < .05, Fisher exact test). Inhibition of TRPV1 reduced the mechanical responsiveness of IB4-positive neurons from HbSS mice to control levels (*P < .05, Fisher exact test).

**Figure 5. TRPV1 antagonist reversibly improves mechanical hypersensitivity in HbSS mice.** The TRPV1 antagonist A-425619 (100 μmol/kg IP) reversibly improved mechanical hypersensitivity in HbSS mice compared with vehicle control injection (10% DMSO, 34% 2-hydroxypropyl β-cyclodextrin; †P < .001, Friedman repeated measures test; ††P < .01 and †P < .05, posthoc Dunn test comparing HbSS baseline with HbSS treated with A-425619 at 30 and 60 minutes, respectively; posthoc Dunn test also showed P < .001 for 30 minutes vs 120 minutes and P < .01 for 60 vs 120 minutes within the HbSS group treated with A-425619; HbSS n = 14). There was no significant difference in mechanical hypersensitivity in HbSS mice treated with vehicle (Friedman test, n = 12 HbSS-Vehicle). HbSS mice treated with A-425619 mice exhibited significantly improved mechanical hypersensitivity compared with HbSS mice treated with vehicle mice at 30, 60, and 90 minutes (P < .001, P < .001, P < .01, respectively; Mann Whitney U test), but not at 120 minutes (P > .05). All data are shown as means ± SEM.
reduces the persistent mechanical hypersensitivity in mice with severe SCD.

Discussion

Acute pain during RBC sickling is the leading cause of emergency room visits and hospitalizations among patients with SCD, and many patients cope with underreported, incapacitating chronic pain on a daily basis.1,2 Despite detailed understanding of the genetics, molecular biology, and biochemistry of sickle hemoglobin and its effects on the erythrocyte, the pathogenesis of the profound pain syndromes in SCD remains inadequately understood and likely involves complex peripheral and central mechanisms. The diversity of spontaneous and stimulus-evoked pain sensations cannot be fully explained by simple vascular obstruction of selected tissue beds and suggests that multiple pathologic mechanisms underlie the sensory phenomena in SCD. Furthermore, the specific array of pain descriptors and precipitating factors for acute painful events in SCD implies that both peripheral and central mechanisms may contribute to the pain.

In the present study, we used a mouse model of severe SCD to quantify the magnitude and modalities of pain behavior and to uncover underlying cellular and molecular mechanisms that drive the pain behavior in primary sensory neurons. We show that SCD mice exhibit persistent hypersensitivity to mechanical, heat, and cold stimuli, and that mechanical hypersensitivity is specifically exacerbated in the setting of experimental vasocclusion induced by hypoxia. The mechanical hypersensitivity appears to be driven by enhanced responsiveness in nociceptive-type sensory neurons. First, both C fibers and Aδ fiber nociceptors exhibited increased firing to suprathreshold mechanical stimuli. Second, small-diameter C-fiber-type neurons exhibited an increased number of neurons that responded to mechanical stimuli applied to the soma plasma membrane.

Several apparent inconsistencies in the data should be noted. First, in the skin-nerve recordings, we found no increase in the proportion of C fibers that were mechanically sensitive in HbSS mice, a finding that did not parallel the increased number of isolated small-diameter sensory neurons from HbSS mice that were mechanically responsive. It is difficult to make a direct correlation between these 2 techniques because they differ substantially with respect to the site that is stimulated (peripheral terminal vs soma), the type of mechanical stimulation used (sustained force to the terminal vs focal force to the plasma membrane), the environment of the neuron (terminals in skin vs isolated soma in culture), and the function measured (action potential firing vs inward current).

Furthermore, neither C fibers nor Aδ fibers in skin-nerve recordings from SCD mice exhibited sensitized mechanical thresholds, a finding that did not coincide with the decreased behavioral mechanical thresholds. We have rarely observed a decrease in the threshold of individual nociceptive afferents in skin nerve recordings. This is likely because the mechanical thresholds are low due to stimulation of the dermal side “inside” of the skin against a flat bottom surface of the recording chamber, whereas behavioral thresholds are higher because mechanical stimuli are applied to the epidermal side and pliable deep muscle, tendon, and bone tissue support the skin in vivo. Moreover, behavioral mechanical thresholds may be mediated by many properties of primary afferent fibers including their thresholds, number of neurons responding that summate onto second-order spinal neurons, and their firing rate to suprathreshold stimuli. Behavioral responses also involve spinal reflexes that include influences from primary afferent input, spinal interneurons, motor neurons, and descending inhibitory or facilitatory neurons from supraspinal sites that modulate spinal neurons. Sensitization at any of these sites could contribute to a sensitized behavioral response as measured by decreased withdrawal threshold. Acute pharmacologic inhibition of the TRPV1 channel completely reverses the mechanical sensitization of nociceptors at the terminal level in skin and at the membrane level in isolated somata, and it partially reverses the mechanical hypersensitivity behavior. These data provide compelling evidence for a novel pathway mediating pain in SCD.

Our results suggest that the TRPV1 receptor contributes to the mechanical hypersensitivity in SCD at the cellular, primary afferent terminal, and behavioral levels. The TRPV1 receptor is classically known as a receptor for inflammatory heat.20 Growing recent evidence in several injury and disease models indicates that TRPV1 also mediates mechanical hypersensitivity in inflamed bladder or colon, bone cancer pain, and after nerve injury or cutaneous inflammation.21,22 Interestingly, patients with SCD report “burning” pain ~10% of the time and less frequently compared with other pain descriptors such as “aching,” “stabbing,” or “sharp.” This suggests that the TRPV1 receptor in SCD may also mediate sensitization of the primary afferent neurons in a manner distinct from the classic TRPV1 heat receptor, possibly by forming a complex or heteromer with other channels involved in mechanotransduction or mechanical sensitization.

Mechanically evoked currents in DRG somata from SCD mice were selectively increased in IB4-positive, C-fiber-type nociceptors. Furthermore, TRPV1 on these IB4-positive nociceptors mediates part of the mechanical hypersensitivity in SCD mice. IB4-positive neurons overlap extensively with nociceptors that express the G-protein-coupled receptor Mrgprd, and Mrgprd-positive nociceptors have been shown to mediate mechanical pain in inflammatory and neuropathic pain models. The mechanical pain is selectively inhibited by δ opioid receptor agonists.23 Therefore, δ opioid agonists may selectively inhibit the mechanical pain in SCD. Another study found decreased expression of μ opioid receptors in the skin and spinal cord of Berkeley SCD mice,26 a finding consistent with evidence that patients with SCD require unusually high doses of morphine to treat their pain.27 These data suggest that δ family opioid agonists may be efficacious for treating chronic, touch-evoked pain in patients with SCD.

Berkeley SCD mice have a thinner epidermis and dermis and increased expression of CGRP and substance P in the skin compared to control mice.26 One explanation for the increased mechanical firing in C and Aδ nociceptors might be that thinner skin results in increased force to the terminal. However, the fact that mechanical currents were increased in the isolated soma indicates that the mechanical sensitization is an inherent property of the sensory nerve membrane. Further, our finding that the mechanical sensitization was acutely blocked by TRPV1 inhibition supports that the increased mechanical firing was not because of thinner skin.

Several key mechanisms may drive and maintain the persistent increased TRPV1 function in SCD. First and foremost, the repeated ischemic reperusions that occur with SCD cause hypoxia and likely local tissue acidosis. The TRPV1 channel has both a site for activation and sensitization by protons,28,29 and short-term anoxia sensitizes Ca2+ responses in isolated DRG neurons in a TRPV1-dependent manner.30

Second, the repeated vaso-occlusive events that result in ischemia-reperfusion cycles induce a persistent chronic inflammatory state in SCD patients.23 Therefore, SCD is associated with
elevated levels of pro-inflammatory cytokines including TNF-α, IL-1β, 12-HPETE, Endothelin-1, LTβ-4, and PGE2.31 TNF-α appears to initiate the inflammatory cytokine cascade that sensitizes TRPV1,32 and 12-HPETE and LTβ-4 are documented endogenous direct activators of TRPV1.33 ET-1 is also known to sensitize TRPV1.34 Furthermore, during inflammation, endogenous oxidized linoleic acid lipid metabolites such as 9-hydroxyoctadecadienoic acid potently activate TRPV1 and result in mechanical allodynia.35

Third, the NO pathway is disturbed in SCD.36 Whereas there may be decreased NO bioavailability in circulating blood because of intravascular hemolysis with the release of cell-free hemoglobin that scavenges plasma NO, there is also evidence for increased NO production in extravascular tissues in SCD.36 This includes increased nitric oxide synthase (NOS) activity and associated second messenger cGMP levels in the kidneys,36,37 and lungs of SCD mice,38 as well as human sickle RBCs.39 The source of increased NOS activity may be because of either tissue-specific NOS or emigrated inflammatory cell iNOS that have been induced in extravascular tissue by inflammatory mediators or oxidative injury associated with SCD. Elevated tissue NO can activate TRPV1 and mediate NO-dependent peripheral nociception.40,41 Therefore, tissue-specific increases in NO production may contribute to the TRPV1-mediated pain behavior observed in SCD mice.

Episodes of acute pain during RBC sickling begin in early childhood and increase in frequency throughout life, often transitioning into a chronic pain syndrome that affects patients daily.1 The mechanisms that underlie the transition from acute episodes to chronic pain in any disease are just beginning to be investigated and likely involve persistent sensitization of both peripheral and central neurons and their associated cells. One possibility is that nociceptors in SCD become chronically sensitized in a phenomenon called “hyperalgesic priming.” In this condition, a transient insult triggers long-lasting changes in nociceptors that prime them to become persistently hyperresponsive to future non-nociceptive stimuli.42 Indeed, the finding that somata exhibited mechanical sensitization even 36 hours after isolation suggests that nociceptors from SCD mice have an inherent, long-lasting sensitization that does not require the continued presence of in vivo factors. A strong candidate for hyperalgesic priming is PKCε, which is highly expressed in nociceptors and mediates cytokine-induced nociceptor hyperexcitability.43 Remarkably, this PKCe-mediated priming is restricted to IB4-positive nociceptors, the population enhanced in cellular mechanical responsiveness in SCD (Figure 4).44 Therefore, IB4/Mrgprd–positive nociceptors may be a key population to selectively target for relief of chronic SCD pain. Given the acute to chronic development of the SCD pain phenomena in patients, our results suggest that Berkeley SCD mice may be a good model for studying the pathologic cellular and molecular mechanisms that drive long-lasting alterations in the excitability of nociceptors during the transition from acute to chronic pain.

In summary, the findings from this study should help us to better understand the complex pain syndromes that are only recently becoming recognized in human SCD and that are not all explained by an “acute vascular occlusion” model. Current treatment of acute and chronic pain in SCD relies heavily on opioid-based drug regimens that work centrally and have numerous deleterious physiologic and social effects. Therefore, there is tremendous need for novel therapies based on well-delineated mechanisms. The data in this study suggest that the TRPV1 pathway is selectively enhanced in SCD and specifically contributes to the pain phenotype. Several TRPV1 antagonists are in phase 1/2 clinical trials for treatment of osteoarthritic, neuropathic, and nociceptive pain.45,46 Although initial TRPV1 compounds had limited success because of thermoregulatory side effects,47,48 novel TRPV1 antagonists are being developed that may provide pain relief with fewer side effects.49 In addition, TRPV1 may play protective roles in the healthy vasculature, including increased eNOS activity and improved endothelial-dependent vasodilation.50 Therefore, it will be important to assess the vascular effects as well as the analgesic effects of TRPV1 antagonists in preclinical SCD mouse models before human trials are considered. Our data suggest that these future novel and less-toxic TRPV1-targeted compounds should be considered for studies in human trials to determine whether they may provide safe and efficacious pain relief for patients suffering from SCD.

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Authorship

Contribution: C.A.H. and C.L.S. designed, planned, and supervised the study, analyzed and interpreted the data, and wrote the manuscript; P.C.K. performed the skin-nerve experiments and contributed to writing the manuscript; D.V. performed the patch-clamp experiments and contributed to writing and editing the manuscript; M.E.B. assisted with the behavioral experiments and contributed to editing the manuscript; D.R. performed the mouse behavior assays and assisted with the statistical analysis; A.M.B. offered advice regarding clinical aspects of SCD pain and contributed to writing and editing the manuscript; and N.J.W. provided expertise regarding the generation of large number of SCD mice and mouse models and contributed to writing and editing the manuscript.

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Transient receptor potential vanilloid 1 mediates pain in mice with severe sickle cell disease

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