Published in final edited form as:

Pain. 2016 December ; 157(12): 2798–2806. doi:10.1097/j.pain.0000000000000704.

CaMKII α underlies spontaneous and evoked pain behaviors in Berkeley sickle cell transgenic mice

Ying He^{1,3}, Yan Chen¹, Xuebi Tian¹, Cheng Yang¹, Jian Lu¹, Chun Xiao¹, Joseph DeSimone^{2,3}, Diana J. Wilkie⁴, Robert E. Molokie^{1,2,3,5}, and Zaijie Jim Wang^{1,3,*}

¹Department of Biopharmaceutical sciences, University of Florida, Gainesville, FL

²Division of Hematology/Oncology, Department of Medicine, University of Florida, Gainesville, FL

³University of Illinois Sickle Cell Center, University of Florida, Gainesville, FL

⁴Department of Biobehavioral Nursing Science, University of Florida, Gainesville, FL

⁵Jesse Brown Veteran's Administration Medical Center, Chicago, IL 60612

Abstract

Pain is one of the most challenging and stressful conditions to patients with sickle cell disease (SCD) and their clinicians. Patients with SCD start experiencing pain as early as three months old and continue having it throughout their lives. Although many aspects of the disease are well understood, little progress has been made in understanding and treating pain in SCD. This study aimed to investigate the functional involvement of Ca²⁺/calmodulin-dependent protein kinase II (CaMKII α) in the persistent and refractory pain associated with SCD. We found non-evoked ongoing pain as well as evoked hypersensitivity to mechanical and thermal stimuli were present in Berkeley sickle cell transgenic mice (BERK mice), but not non-sickle control littermates.

Prominent activation of CaMKII α was observed in the dorsal root ganglia and spinal cord dorsal horn region of BERK mice. Intrathecal administration of KN93, a selective inhibitor of CaMKII, significantly attenuated mechanical allodynia and heat hyperalgesia in BERK mice. Meanwhile, spinal inhibition of CaMKII elicited conditioned place preference in the BERK mice, indicating the contribution of CaMKII in the ongoing spontaneous pain of SCD. We further targeted CaMKII α by siRNA knockdown. Both evoked pain and ongoing spontaneous pain were effectively attenuated in BERK mice. These findings elucidated, for the first time, an essential role of CaMKII α as a cellular mechanism in the development and maintenance of spontaneous and evoked pain in SCD, which can potentially offer new targets for pharmacological intervention of pain in SCD.

Keywords

Sickle cell disease; pain; spontaneous pain; phosphorylation; Ca²⁺/calmodulin-dependent protein kinase II

*Corresponding author: Zaijie Jim Wang, Ph.D., University of Illinois, Chicago, IL, 60607 (MC865), (312)996-0888 (voice); (312)996-0098 (fax); zjwang@uic.edu.

Conflict of Interest Statement

The authors declare no conflict of interest.

1. Introduction

Sickle cell disease (SCD) is a group of autosomal recessive genetic disorders that are caused by mutations in the hemoglobin genes and result in polymerization of deoxyhemoglobin and red cell sickling. Pain is a life-long companion of people living with SCD and is a predictor of disease severity and mortality.³⁹ A nationwide epidemiological study reported that over 60% of SCD patients have at least one pain crisis episode annually.³⁹ In addition to severe acute pain that is associated with vaso-occlusive crises, chronic pain is also prevalent in SCD. A longitudinal diary survey found over half of patients with SCD reported the presence of chronic pain on more than 50% of the days.⁴² In our self-reported, computerized McGill Pain Questionnaire study conducted in the clinic for non-urgent visits (i.e., not on crisis days), over 60% of subjects reported continuous or constant pain.⁴⁸ Strikingly, 90% of these subjects also chose pain quality descriptors that are consistent with the presence of neuropathic pain. On average, patients reported using 4.9 analgesics, yet at least one third of patients were not satisfied with their pain control.⁴⁸ As better treatment and care are extending the life expectancy of patients with SCD, pain is increasingly becoming a big problem that negatively impacts the health and quality of life of these patients.

Limited progress, however, has been made in understanding the basic neurobiological mechanisms underlying chronic pain in SCD. In this study, we employed Berkeley sickle cell transgenic mice (BERK mice),³⁷ a model of severe SCD, to identify and characterize neurobiological mechanisms of chronic pain in SCD. BERK mice express exclusively human sickle hemoglobin and have a phenotype that closely mimics many features of severe SCD in humans, including severe hemolytic anemia, irreversibly sickled red cells, increased rigidity of erythrocytes, extensive multiple organ damage, vascular ectasia, intravascular hemolysis, exuberant hematopoiesis, cardiomegaly, glomerulosclerosis, visceral congestion, hemorrhages, multiorgan infarcts, pyknotic neurons, progressive siderosis, gallstones, and priapism.^{25,33,37}

Ongoing spontaneous pain is frequently reported by patients with SCD; however, it is rarely studied in preclinical research.²⁸ In our ongoing work with other, but not all, chronic pain conditions, we found the Ca^{2+} /calmodulin-dependent protein kinase II α (CaMKII α) to be a critical molecular mechanism in experimental models of chronic inflammatory and nerve injury neuropathic pain.^{6,7,32} In this study, we examined the role of CaMKII α in chronic pain behaviors including ongoing spontaneous pain in BERK mice.

2. Materials and methods

2.1. Materials

2-[N-(2-hydroxyethyl)]-N-(4-methoxybenzenesulfonyl)amino-N-(4-chlorocinnamyl)-N-methylbenzylamine (KN93) and 2-[N-(4-Methoxybenzenesulfonyl)]amino-N-(4-chlorocinnamyl)-N-methylbenzylamine (KN92) were purchased from Tocris Bioscience (Ellisville, MO). Lidocaine HCl (2%) was from Hospira (Lake Forest, IL). Other chemicals were purchased from Sigma-Aldrich (St. Louis, MO). CaMKII α siRNA (sense, 5'-CACCACCAUUGAGGACGAAdTdT-3', antisense, 5'-

UUCGUCCUCAAUGGUGdTdT-3') and scrambled RNA duplex control (sense, 5'-AUACGCGUAUUAUACGCGAUUACGAC-3'; antisense, 5'-CGUUAUUCGCGUAUAAUACGCGUAT-3') were synthesized by Integrated DNA Technologies (Coralville, IW). Lidocaine, KN93, KN92 and RNA duplexes were administered intrathecally (*i.t.*) in a volume of 5 μ L by percutaneous puncture through the L5–L6 intervertebral space.^{26,32} The RNAs were mixed with a transfection reagent i-Fect (Neuromics, Minneapolis, MN) at a ratio of 1:5 (w/v).⁷

2.2. Animals

BERK mouse³⁸ breeding colony was established from breeders that were obtained from the Jackson Laboratories (Bar Harbor, MA). These mice have been crossed once with C57Bl/6J mice in the Jackson Laboratories, so they have > 50% C57Bl/6J background. We used the following breeding schedule that has been found to be most efficient: female breeders: heterozygous for *Hbb* (non-sickle); male breeders: homozygous for *Hbb* (sickle). Genotyping was performed as previously published³⁸ to obtain the BERK mice (sickle) and non-sickle wild-type control littermate mice. The latter serves as control for BERK mice. Age- and sex- matched adult mice (3–5 months old; 20–30g) were used in the study.

Prior to actual experimental procedures, mice were provided with food and water *ad libitum*. Experiments were carried out in accordance with the International Association for the Study of Pain (IASP) recommendations and the NIH Guide for the Care and Use of Laboratory Animals after securing approval from the University of Illinois Institutional Animal Care and Use Committee. The researchers who performed tests were blinded to genotype information before and during the experiments.

2.3. Assessment of ongoing spontaneous pain

The conditioned place preference (CPP) method was employed to unmask the ongoing spontaneous pain as we have previously described.²¹ The CPP apparatus (San Diego Instruments, San Diego, CA) consists of 3 Plexiglas chambers separated by manual doors. A center chamber (6 1/4" W $\times$ 8 1/8" D $\times$ 13 1/8" H) connects the two end-chambers that are identical in size (10 3/8" W $\times$ 8 1/8" D $\times$ 13 1/8" H), but can be distinguished by the texture of the floor (rough *vs.* smooth) and wall pattern (vertical *vs.* horizontal stripes). Movement of mice and time spent in each chamber were monitored by 4 $\times$ 16 photobeam arrays and automatically recorded in SDI CPP software.

Preconditioning was performed across 3 days for 30 min each day when mice were exposed to the environment with full access to all chambers. On day 3, a pre-conditioning bias test was performed to determine whether a preexisting chamber bias exists. In this test, mice were placed into the middle chamber and allowed to explore open field with access to all chambers for 15 min. Data were collected and analyzed for duration spent in each chamber. Animals spending more than 80% or less than 20% of the total time in an end-chamber were eliminated (~ 10% of total animals) from further testing.

A single trial conditioning protocol was used in the experiments. On conditioning day (day 4), mice first received vehicle control (saline, *i.t.*) paired with a randomly chosen chamber in the morning and, 4 h later, either KN93 (45 nmol, *i.t.*) or lidocaine (0.04 %, *i.t.*) was paired

with the other chamber in the afternoon for 15 min (lidocaine) or 30 min (KN93). Whereas the pairing time with lidocaine was based on previous experience,²¹ pairing condition with KN93 was determined in pilot experiments. On the test day, 20 h after the afternoon pairing, mice were placed in the middle chamber of the CPP box with all doors open so the animals had free access to all chambers. The movement and duration that each mouse spent in each chamber were recorded for 15 min for analysis of chamber preference. Difference scores were calculated as (test time-preconditioning time) spent in the drug chamber.

2.4. Assessment of cold sensitivity

Sensitivity to cold stimulus was examined as previously described.⁸ Mice were placed in individual Plexiglas containers to adapt to the environment for 30 min. A cold stimulus was applied by a brief application (1 s) of tetrafluoroethane to the ventral surface of left hindpaw. Mice were observed for 5 min and the number of licks and the duration of lifting of the sprayed paw were recorded.

2.5. Assessment of heat sensitivity

Sensitivity to heat stimulus was determined by the following methods: 1) paw withdrawal latency to radiant heat using a plantar tester (UGO BASILE Model 7372, Stoelting, Wood Dale, IL).^{7,20} Mice were allowed to acclimate within Plexiglass enclosures on a clear glass plate maintained at 30°C. Radiant heat stimulation was applied to the center of the planter surface of the hindpaw and the latency to paw withdrawal was recorded. A cut-off time of 20s was applied to avoid tissue damage, which was appropriate as the mean control latency is about 12 sec, although the control baseline can be adjusted by varying the intensity of the stimulus. The presence and degree of hyperalgesia were determined by comparing the withdrawal latencies of the test animals and those of controls; 2) The hotplate test was conducted by placing the mice in a glass cylinder on a cold/hot plate analgesia meter (Stoelting) with floor temperature controlled to 50, 52 or 55°C and determining the latency to hindpaw licking or escaping. A 60, 45, and 30 s cutoff time was set for 50°C, 52°C, and 55°C plate temperature, respectively, to prevent injury; 3) The tail immersion test was performed by dipping the distal half of the tail into a water bath maintained at 48, 52, or 55 °C and recording the latency to a rapid tail flick response. A 15, 12, or 10 s cutoff was applied to 48, 52, or 55 °C test, respectively.

2.6. Assessment of mechanical sensitivity

Non-noxious mechanical sensitivity was assessed by probing with von Frey filaments.⁷ Mice were placed in individual Plexiglas containers on a wire mesh platform and tested after 30 min acclimation to the environment. Calibrated von Frey filaments (Stoelting, Wood Dale, IL) were used to press upward to the midplantar surface of the hindpaw for 5 s or until a withdrawal response happened. Using the “up-down” algorithm,¹¹ 50% probability of paw withdrawal threshold was determined.

2.7. Formalin-induced inflammatory pain model

Tonic inflammatory pain was induced in BERK mice and non-sickle littermate controls (~ 3 months) by a subcutaneous injection of formalin (2% in saline, 20 µL/mouse) into the dorsal

surface of the left hindpaw, as described previously.^{44,46} The hindpaw was observed for 60 min for the number and duration of paw flinching and the results tabulated for successive 5 min intervals. This test produces a distinct biphasic response. The total number of flinches during the early phase (0–10 min) and late phase (10.01–60 min) were summed, respectively.

2.8. Immunohistochemistry

Immunostaining of spinal activated CaMKII α (pCaMKII α) was performed as described previously.⁷ After deep anesthesia with ketamine (100 mg/kg) and xylazine (5 mg/kg, *i.p.*), mice were initially perfused with phosphate buffer (0.1 M, pH 7.4) for 5 min, followed by 4% paraformaldehyde in phosphate buffer for 20 min (~10 mL/min). Spinal cord lumbar regions were dissected out and sectioned at 20 μ m thickness with a cryostat. The floating sections were incubated with the primary antibody for pCaMKII α Thr286 (1:1000, Santa Cruz Biotechnology, Santa Cruz, CA) overnight at room temperature, followed by another incubation with biotinylated goat anti-rabbit IgG secondary antibody (1:200, Vector Laboratories, Burlingame, CA) at room temperature for 2 h. The sections were developed using Elite Vectastain ABC kit (Vector Laboratories). Diaminobenzidine (DAB) stained sections were imaged using Olympus IX71 inverted fluorescence microscope (Olympus Corp., Lake Success, NY) and quantified by the MetaMorph Imaging Software (Molecular Devices, Sunnyvale, CA). For each animal, 5–10 consecutive sections (depending on the size of the region) were imaged. For each group, 5 sections and 6 areas from each section were analyzed and averaged.

2.9. Immunoblotting

The lumbar spinal cord and dorsal root ganglia (DRG) tissues were harvested for western blot analysis as previously described.²³ Briefly, tissue samples were homogenized in a modified RIPA buffer (1% NP-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 5 mM EDTA, 1.0 mM NaF, 10mM sodium pyrophosphate, and 2 mM sodium vanadate in PBS, pH 7.4) in the presence of protease inhibitors and centrifuged (11,000g, 60 min). Supernatants were separated by 12% SDS-PAGE and electro-transferred onto PVDF membrane. The membrane was probed with a rabbit antibody against pCaMKII α (1:1000, Santa Cruz Biotechnology) at room temperature overnight, followed by incubation with HRP-conjugated donkey anti-rabbit secondary antibody (1:1000, GE Healthcare, Marlborough, MA) at room temperature for 1 h. An enhanced chemiluminescence detection system (ECL, GE Healthcare) was applied for development. The membrane was then stripped and reprobed with a mouse anti-CaMKII α antibody (1:1000, Santa Cruz Biotechnology) followed by a HRP-conjugated anti-mouse secondary antibody (1:1000, GE Healthcare) and developed as above. Finally, the membrane was stripped again and probed with a mouse anti- β -actin antibody (1:10,000, Sigma) followed by a HRP-conjugated anti-mouse secondary antibody (1:10,000, GE Healthcare). ECL signals were detected using a ChemiDoc system and analyzed using the Quantity One program (Bio-Rad). CaMKII immunoreactivity was expressed as the ratio of the optical densities of pCaMKII α or CaMKII α to those of β -actin.

2.10. Statistical Analysis

All data are presented as Mean $\pm$ SEM. For evoked pain behavior data, differences between groups were analyzed using a one-way ANOVA (treatment) followed by Tukey *post hoc* test (multiple groups) or Student's *t* test (two groups). To analyze the CPP data, two-way ANOVA (pairing vs. treatment) was applied followed by Bonferroni *post hoc* test. Difference scores were analyzed using Student's paired *t* test by computing the differences between test time and preconditioning time for each mouse. Statistical significance was established at the 95% confidence limit.

3. Results

3.1. Presence of ongoing spontaneous pain in BERK mice

Patients with SCD experience ongoing pain that is different from the sickle cell crisis pain. We established a conditioned place preference (CPP) paradigm that has been validated in mice to detect non-evoked pain in SCD mice.²¹ The chronic pain in mice with SCD may present as an aversive state, and the suppression of which may produce CPP, as we have observed in mice with tissue or nerve injury.^{10,22} First, we performed a preconditioning test to ensure that there was no existing chamber bias for any of the mice with different genotypes. After a single trial conditioning with saline and lidocaine, BERK mice spent significantly more time in the chambers that were paired with lidocaine (420 ± 32 s) than in saline-paired chamber (256 ± 37 s, $P < 0.01$), indicating that lidocaine induced CPP in the sickle mice (Fig. 1A). On the contrary, non-sickle littermate mice spent equal amount of time in the saline (330 ± 25 s) or lidocaine (294 ± 34 s) paired chambers, suggesting the absence of lidocaine-CPP in the non-sickle mice (Fig. 1A). This observation was in agreement with what we reported for naïve ICR and C57Bl/6 mice in the absence of spontaneous pain.^{10,21,22}

We also analyzed the different score for each mouse in each chamber. In comparison to the time spent in the chambers during pre-conditioning, the sickle mice displayed significant difference scores in lidocaine-paired chamber ($P < 0.05$). None of the other mice groups, including the sickle mice paired with saline treatment, exhibited significant difference scores after conditioning (Fig. 1B). These scores further confirmed the presence of ongoing spontaneous pain in BERK sickle cell mice.

3.2. Hypersensitivity to noxious cold stimulus in BERK mice

Patients with SCD have enhanced pain sensitivity to cold environments.^{2,35,41,47} To assess the noxious cold hyperalgesia in BERK mice, the plantar surface of the left hindpaw was exposed to tetrafluoroethane for 1 s.⁸ Under this condition, it produced reflex responses as measured by the paw licking and lifting behaviors. We counted the number of licks occurred in 5 min triggered by the cold stimulus. As compared with the non-sickle littermates, BERK mice displayed significantly enhanced licking behavior, with an increased number of licks by more than 2.6 fold ($P < 0.01$, Fig. 2A). The total time of hindpaw lifting and licking in BERK mice (139.9 ± 5.0 s) was increased by around 3.5 fold of that in the non-sickle mice (40.5 ± 10.0 s) ($P < 0.001$, Fig. 2B). Consistent with the findings from other cold pain

measurements from other investigators,^{24,34} these observations demonstrated the existence of cold-induced hypersensitivity in BERK mice.

3.3. Evoked mechanical and heat pain behaviors in BERK mice

We also determined the baseline nociceptive response of BERK mice towards mechanical and heat stimuli. As compared with the age/sex-matched non-sickle control mice (1.52 ± 0.14 g), BERK mice displayed significantly reduced pain threshold (0.10 ± 0.05 g) to normally innocuous mechanical stimulus by von Frey filament probing ($P < 0.001$, Fig. 2C), indicative of the presence of tactile allodynia in BERK mice. In response to the radiant heat challenge, BERK mice exhibited decreased latencies to noxious heat stimuli applied to the hindpaw (6.32 ± 0.78 s in BERK mice vs. 11.97 ± 0.78 s in non-sickle mice, $P < 0.001$, Fig. 2D), indicating the presence of heat hyperalgesia. The enhanced thermal sensitivity was further confirmed in the tail-flick assay, as BERK mice consistently displayed shortened tail-withdrawal latencies to the water bath maintained at 48°C (6.28 ± 0.56 s in BERK mice vs. 8.35 ± 0.63 s in non-sickle mice, $P < 0.05$), 52°C (1.84 ± 0.11 s in BERK mice vs. 2.31 ± 0.15 s in non-sickle mice, $P < 0.05$), and 55°C (1.02 ± 0.07 s in BERK mice vs. 1.33 ± 0.09 s in non-sickle mice, $P < 0.05$) (Fig. 2E). In addition, BERK mice showed a significant decrease in the latency to noxious input in the hot plate test at 52°C (6.61 ± 0.90 s in BERK mice vs. 8.90 ± 0.55 s in non-sickle mice, $P < 0.05$) (Fig. 2F). Therefore, these data indicated that BERK mice have pronounced evoked hypersensitivity to mechanical and heat stimuli.

3.4. Inflammatory pain sensitivity in BERK mice

As seen in SCD patients, BERK mice have been reported to have increased inflammation and inflammatory mediators.^{1,17,33} A logical question to ask is whether BERK mice responded differently to commonly used inflammatory pain stimuli such as formalin. Intraplantar formalin injection induced typical biphasic spontaneous pain in both BERK mice and non-sickle littermate controls (Fig. 3A). There was no difference between the BERK and non-sickle mice in Phase I (1–10 min) response ($P > 0.05$, Fig. 3B). For Phase II (10.01–60 min), there was a significantly increased response in BERK mice ($P < 0.05$, Fig. 3A).

3.5. Activation of CaMKII α in BERK mice

We hypothesized that CaMKII α , a key cellular protein kinase and regulator of neuronal activity, may play an important role in promoting chronic pain in SCD. As a first step to address this question, we determined CaMKII α activity in the spinal cord and dorsal root ganglia (DRG) of BERK mice and non-sickle control mice. Western blot analysis showed the expression of activated or phosphorylated CaMKII α (pCaMKII α) was significantly elevated in the spinal cord in BERK mice (Fig. 4A). Compared with the non-sickle mice, the level of spinal pCaMKII α in BERK mice increased by 1.7-fold ($P < 0.01$). On the other hand, the expression of total CaMKII α in BERK and control mice were not different ($P > 0.05$). Similar observations were found in DRG region (Fig. 4B). There was a prominent activation of CaMKII α in DRG of the sickle mice, with a 2.3-fold increase of pCaMKII α as compared with that in the non-sickle mice.

We further conducted immunohistochemical analysis in BERK mice. The pCaMKII α immunoreactivity was found mostly in the superficial dorsal horn of the spinal cord (Fig. 4C). Quantitative analysis of pCaMKII α immunoreactivity was performed by counting the number of positively stained cells using the MetaMorph Imaging Software. The percentage of pCaMKII α -positive cells was drastically enhanced in the sickle mice (48%, 105/220, Fig. 4C, right panel) compared with that of non-sickle mice (29%, 45/155, Fig. 4C left panel). The latter was similar to the percentage of pCaMKII α -positive spinal neurons in the Sprague-Dawley rat.⁴ These data clearly demonstrated that spinal CaMKII α activity is enhanced in BERK sickle cell mice.

3.6. Pharmacologic intervention of CaMKII α in BERK mice

To assess a possible functional role of CaMKII α in sickle cell pain, we examined the effect of KN93, a potent and cell permeable inhibitor of CaMKII α in BERK and control mice. CPP test was performed first using KN93 as the pairing drug. If CaMKII α participates in the ongoing spontaneous pain in BERK mice, its inhibition is expected to suppress ongoing pain and produce CPP. In these experiments, BERK mice demonstrated a strong preference for KN93-paired chamber (415 ± 14 s) over the saline chamber (311 ± 17 s, $P < 0.05$, Fig. 5A), after pairing with KN93 (45 nmol, *i.t.*) for 30 min. In contrast, the non-sickle mice spent similar amount of time in the saline chamber (336 ± 20 s) and the KN93-paired chamber (361 ± 22 s) ($P > 0.05$). As compared before and after conditioning, BERK mice preferred to stay in the KN93-paired chamber as illustrated by the significant difference score generated in the KN93-paired chamber ($P < 0.05$, Fig. 5B). In addition, KN92, a kinase-inactive derivative of KN93 failed to produce CPP in either BERK or control mice (Fig. 5C–D). Since KN93 selectively elicited CPP in BERK mice, these data suggested a functional role for CaMKII α in mediating ongoing spontaneous pain in SCD.

We next examined the evoked pain behaviors in BERK and control mice after receiving a bolus injection of KN93 (45 nmol, *i.t.*). The mechanical hypersensitivity in BERK mice (0.06 ± 0.01 g) was transiently, but effectively, reversed by KN93 (Fig. 5E). With an initial onset at around 30 min, the peak anti-allodynic effect of KN93 was observed at 2 h, when the paw withdrawal threshold in BERK mice (1.55 ± 0.26 g, $P < 0.001$) was restored to a level that was indistinguishable from that in the non-sickle mice (1.59 ± 0.27 g). In response to the radiant heat stimuli, spinal administration of KN93 was capable of reversing thermal hyperalgesia in BERK mice (Fig. 5F). Significant attenuation was achieved at 2 h (11.17 ± 1.15 s in BERK mice treated with KN93 vs. 6.33 ± 0.79 s in BERK mice treated with KN92, $P < 0.01$). Moreover, KN93 did not change mechanical and thermal sensitivity in the non-sickle mice (Fig. 5E–F). Neither did KN92 affect baseline nociception in BERK or the non-sickle mice. Therefore, inhibiting spinal CaMKII α is effective in transiently reversing evoked hypersensitivity in BERK sickle cell mice.

3.7. CaMKII α silencing in BERK mice

To further investigate the essential role of CaMKII α in sickle cell pain, siRNA targeting CaMKII α (*i.t.*) was applied to knock down the expression of CaMKII α . This approach is known to produce a highly selective repression of the target protein. BERK and non-sickle littermate mice received CaMKII α siRNA or scrambled RNA duplex (2 μ g/injection, twice

per day for 3 consecutive days, *i.t.*). Sensitivities to mechanical and thermal stimuli were measured daily. Treatment with CaMKII α siRNA gradually attenuated mechanical allodynia (Fig. 6A) and thermal hyperalgesia (Fig. 6B) in BERK mice. When tested 24 h after first siRNA treatment, thermal hyperalgesia was significantly reversed in BERK mice (6.03 ± 0.33 s in siRNA group vs. 2.90 ± 0.37 g in scrambled RNA group, $P < 0.01$, Fig. 6B). After 3 days' treatment, CaMKII α knockdown was confirmed in the spinal cord by western blot analysis (Fig. 6A insert). In BERK mice, mechanical hypersensitivity was completely reversed (0.96 ± 0.11 g in siRNA group vs. 0.10 ± 0.03 g in scrambled RNA group, $P < 0.001$, Fig. 6A). Meanwhile, thermal hyperalgesia in BERK mice was significantly suppressed by CaMKII α siRNA and lasted for 5 d (Fig. 6B). On the other hand, CaMKII α siRNA did not alter mechanical or thermal sensitivity in the non-sickle mice (Fig. 6A–B).

Furthermore, BERK and non-sickle mice were subjected to the CCP paradigm to investigate ongoing spontaneous pain on Day 4. BERK mice treated with scrambled RNA spent significantly more time in the lidocaine-paired chamber (412 ± 44 s) than in the saline chamber (231 ± 38 s) ($P < 0.05$, Fig. 6C), similar to BERK mice without any treatment (Fig. 1). BERK mice received 3d CaMKII α siRNA failed to show preference for saline- (344 ± 36 s) or lidocaine-paired (340 ± 41 s) chambers ($P > 0.05$), which was in stark contrast to BERK mice received scrambled RNA or BERK mice received no treatment (Fig. 1). Lidocaine didn't produce CPP in the non-sickle mice treated with CaMKII α siRNA or scrambled RNA. Analysis of difference scores confirmed that CaMKII α siRNA disrupted lidocaine-CPP in BERK mice (Fig. 6D). Since persistent ongoing spontaneous pain and evoked hypersensitivities were no longer present in BERK mice after knocking down spinal CaMKII α , these data strongly indicated that CaMKII α is required for chronic pain behaviors in SCD.

4. Discussion

Pain in SCD is characterized by the presence of chronic pain with episodes of acute pain crises. The neurobiology of chronic pain in SCD is poorly understood. Studies employing animal models of SCD become an indispensable approach *in vivo* to unravel the complicated molecular mechanisms in the central and peripheral nervous systems. Early murine models of SCD, such as HbSAD mice and HbS/HbS-Antilles mice expressed a “supersickling” variant of HbS and developed organ and tissue characteristics of SCD. However, these mice retain the production of mouse globins, which interfere significantly with the sickling of erythrocytes. To overcome this limitation, the recent creation of transgenic mice, such as BERK mice completely replace mouse hemoglobins with human hemoglobins, producing a phenotype that closely mimics many features of severe SCD in humans.^{25,33,37}

In the present study, we profiled two distinctive pain components (ongoing spontaneous pain and evoked pain) in BERK mouse. When asked to characterize their pain, patients described daily, chronic pain without any known stimulus.^{42,48} As a dominant clinical manifestation, it is imperative to investigate the presence of ongoing spontaneous pain in animal models of SCD. Our study is the first to demonstrate the presence of ongoing spontaneous pain in BERK mice using the CPP paradigm that we have validated in mice with chronic tissue inflammation or nerve injury.²¹ Indeed, a single trial conditioning with lidocaine was

sufficient to produce CPP selectively in BERK mice, but not non-sickle mice (Fig 1). This is not only in agreement with the clinic characteristics of pain in patients, but also the first study to demonstrate the feasibility of applying CPP paradigm to detect ongoing spontaneous pain in a transgenic mouse model.

BERK mice showed heightened responses to formalin in the second phase, but not the first phase, suggesting a central mechanism. Unlike the Phase I that is mostly due to direct activation of primary afferent nociceptors, the second phase is considered as an index for inflammatory response involving central mechanisms.⁴⁴

To identify heat hypersensitivity in BERK mice, we used three different types of heat stimuli with varying intensities. These results corroborated with each other to demonstrate that BERK mice displayed prominent heat hyperalgesia as well as mechanical allodynia, as have been reported previously by two other groups,^{24,30} reflecting the presence of fully developed evoked pain behaviors in mice with SCD. The enhanced responses and/or lowered thresholds to heat and mechanical stimuli were also found in patients with SCD by thermal and mechanical quantitative sensory testing.^{2,12}

Allodynia to noxious cold was also found in the study. In an operant assay using two plates with different temperature settings, BERK mice spent less time on the plate set at 23 °C than that at 30 °C.⁴⁹ Another group observed changes in mouse facial expression, body length and back curvature in response to noxious cold (4 °C).³⁴ Together with the findings of the current study, it is clear that BERK mice exhibited hypersensitivity to both mild and extremely cold stimuli, which was also reported by patients with SCD.^{2,12,35}

Our findings provided the first evidence that CaMKII α is a critical mediator for chronic pain in SCD. CaMKII α is a multifunctional, Ca²⁺/calmodulin (CaM) activated serine/threonine protein kinase that is a key component of intracellular Ca²⁺ signaling pathways.^{9,16,31} It is involved in a variety of Ca²⁺-mediated cellular processes including the biosynthesis of neurotransmitters, hormone secretion, neurotransmitter release, gene expression and neuronal plasticity.^{18,31} Autophosphorylation at threonine 286/287 of CaMKII α renders the kinase fully active even in the absence of Ca²⁺. Robust activation of CaMKII α was identified in the spinal cord of BERK mice (Fig. 4), suggesting a correlation between increased CaMKII α activity and persistent pain state in SCD. Moreover, spinal inhibition or knock-down of CaMKII α was able to abolish both ongoing spontaneous pain and evoked hypersensitivities in BERK mice, further illustrating a functional role of spinal CaMKII α in promoting chronic pain in SCD.

CaMKII α is specifically expressed in the superficial laminae in the spinal dorsal horn and in the small to medium diameter primary sensory neurons in the primary afferent, where nociceptive signals are transmitted and processed.^{3,4} CaMKII α activity was significantly increased in the spinal cord within minutes after an intradermal injection of capsaicin.¹³ Spinally administered KN93 inhibited the enhanced response in the spinal nociceptive neurons and changes in exploratory behavior evoked by capsaicin.¹³ KN93 selectively and directly binds to the CaM-binding site of CaMKII, preventing the activation of CaMKII.⁴³ We have previously reported that CaMKII α is required for the initiation and/or maintenance

of chronic inflammatory pain, L5/L6 nerve ligation-induced neuropathic pain, and opioid-induced hyperalgesia.^{6,7,32} Furthermore, CaMKII α has been reported to contribute to hyperalgesia priming for transition from acute to chronic pain.^{14,15}

Establishing CaMKII α mechanisms will open a new scientific arena to explore in future studies the upstream and downstream signaling components of CaMKII α -mediated pain transmission in SCD. Both the release of Ca²⁺ from intracellular storages and Ca²⁺ influx from extracellular sources as a result of receptor or ion channel activation may activate CaMKII α . Activation of the transient receptor potential vanilloid 1 (TRPV1) or the N-methyl-D-aspartate (NMDA) receptors can lead to Ca²⁺ influx and CaMKII activation.^{27,47} The latter, in turn, phosphorylates and activates the TRPV1 and the NMDA receptors.^{19,29} Therefore, there exist potential feed-forward loops to keep CaMKII α -mechanisms active after the original Ca²⁺ signaling has subsided or disappeared, which is crucial to maintain chronicity in pain conditions such as that in SCD. Hargreaves and colleagues found that capsaicin increased CaMKII α activity in TRPV1-positive neurons in rat trigeminal ganglion neurons.⁴⁰ In these trigeminal ganglion neurons, capsaicin- or n-arachidonoyl-dopamine (NADA)-evoked calcitonin gene-related peptide (CGRP) release was inhibited by KN93.⁴⁰ Indeed, TRPV1 has been identified as a contributor to the mechanical hypersensitivity in SCD. TRPV1 antagonist A-425619 attenuated mechanical hypersensitivity in BERK mice 30–60 minutes after administration (*i.p.*), implicating a unique TRPV1-mediated mechanism for mechanical hypersensitivity in SCD.²⁴

Several other mechanisms have also been proposed for SCD pain. BERK mice were found to have decreased expression of the mu opioid receptor and increased immunoreactivity for CGRP, substance P, and several activators of neuropathic and inflammatory pain, which may contribute to hyperalgesia.^{5,30} These investigations found that morphine, the cannabinoid receptor agonist CP 5594015, or inhibitors of mast cell activation attenuated chronic pain behaviors in BERK mice.^{30,45} As an intracellular protein kinase, CaMKII α may be activated by some of these inflammatory or pronociceptive mediators, although future studies are needed to investigate potential interactions.

In summary, we characterized pain behaviors in BERK mice using age- and sex-controlled non-sickle littermate mice as controls. Moreover, a CaMKII α -mediated cellular mechanism for chronic pain in SCD was identified by two complementary approaches employing pharmacological inhibition and genetic silencing. These findings may lead to translational research targeting CaMKII α for alleviating chronic pain in SCD. The latter is poorly managed in the clinic because it is frequently refractory to the currently available pain medications, underscoring an urgent need for novel medications for treating chronic pain in SCD. Although specific inhibitors of CaMKII α have not made to the clinical testing, we have previously identified a FDA-approved antipsychotic drug trifluoperazine as a potent inhibitor of CaMKII α .^{6,32} In a small mechanism-based translational study, trifluoperazine was not only found to be safe, but also showed some promise in alleviating chronic pain after a single dose in patients with SCD.³⁶ Additional studies with trifluoperazine and other CaMKII α inhibitors may ultimately lead to rational designing, identifying, and optimizing clinically useful treatment for the chronic pain in SCD.

Acknowledgments

This work was supported by a grant (R01HL098141) from the National Heart Lung and Blood Institute (NHLBI), National Institutes of Health (NIH). Y.H. is a Sickle Cell Scholar supported by U01HL117658 from NHLBI. Its contents are solely the responsibility of the authors and do not necessarily represent the official views of the NHLBI or NIH.

References

1. Belcher JD, Bryant CJ, Nguyen J, et al. Transgenic sickle mice have vascular inflammation. *Blood*. 2003; 101:3953–3959. [PubMed: 12543857]
2. Brandow AM, Stucky CL, Hillery CA, Hoffmann RG, Panepinto JA. Patients with sickle cell disease have increased sensitivity to cold and heat. *Am J Hematol*. 2013; 88:37–43. [PubMed: 23115062]
3. Bruggemann I, Schulz S, Wiborny D, Holtt V. Colocalization of the mu-opioid receptor and calcium/calmodulin-dependent kinase II in distinct pain-processing brain regions. *Brain Res Mol Brain Res*. 2000; 85:239–250. [PubMed: 11146127]
4. Carlton SM. Localization of CaMKIIalpha in rat primary sensory neurons: increase in inflammation. *Brain Res*. 2002; 947:252–259. [PubMed: 12176168]
5. Cataldo G, Rajput S, Gupta K, Simone DA. Sensitization of nociceptive spinal neurons contributes to pain in a transgenic model of sickle cell disease. *Pain*. 2015; 156:722–730. [PubMed: 25630029]
6. Chen Y, Luo F, Yang C, Kirkmire CM, Wang ZJ. Acute inhibition of Ca²⁺/calmodulin-dependent protein kinase II reverses experimental neuropathic pain in mice. *J Pharmacol Exp Ther*. 2009; 330:650–659. [PubMed: 19478130]
7. Chen Y, Yang C, Wang ZJ. Ca²⁺/calmodulin-dependent protein kinase II alpha is required for the initiation and maintenance of opioid-induced hyperalgesia. *J Neurosci*. 2010; 30:38–46. [PubMed: 20053885]
8. Chen Y, Yang C, Wang ZJ. Proteinase-activated receptor 2 sensitizes transient receptor potential vanilloid 1, transient receptor potential vanilloid 4, and transient receptor potential ankyrin 1 in paclitaxel-induced neuropathic pain. *Neuroscience*. 2011
9. Colbran RJ. Targeting of calcium/calmodulin-dependent protein kinase II. *Biochem J*. 2004; 378:1–16. [PubMed: 14653781]
10. Corder G, Doolen S, Donahue RR, et al. Constitutive mu-opioid receptor activity leads to long-term endogenous analgesia and dependence. *Science*. 2013; 341:1394–1399. [PubMed: 24052307]
11. Dixon WJ. Efficient analysis of experimental observations. *Annu Rev Pharmacol Toxicol*. 1980; 20:441–462. [PubMed: 7387124]
12. Ezenwa MO, Molokie RE, Wang ZJ, et al. Safety and Utility of Quantitative Sensory Testing among Adults with Sickle Cell Disease: Indicators of Neuropathic Pain? *Pain Pract*. 2016; 16:282–293. [PubMed: 25581383]
13. Fang L, Wu J, Lin Q, Willis WD. Calcium-calmodulin-dependent protein kinase II contributes to spinal cord central sensitization. *J Neurosci*. 2002; 22:4196–4204. [PubMed: 12019337]
14. Ferrari LF, Araldi D, Levine JD. Distinct terminal and cell body mechanisms in the nociceptor mediate hyperalgesic priming. *J Neurosci*. 2015; 35:6107–6116. [PubMed: 25878283]
15. Ferrari LF, Bogen O, Levine JD. Role of nociceptor alphaCaMKII in transition from acute to chronic pain (hyperalgesic priming) in male and female rats. *J Neurosci*. 2013; 33:11002–11011. [PubMed: 23825405]
16. Fink CC, Meyer T. Molecular mechanisms of CaMKII activation in neuronal plasticity. *Curr Opin Neurobiol*. 2002; 12:293–299. [PubMed: 12049936]
17. Frenette PS, Atweh GF. Sickle cell disease: old discoveries, new concepts, and future promise. *J Clin Invest*. 2007; 117:850–858. [PubMed: 17404610]
18. Fukunaga K, Miyamoto E. A working model of CaM kinase II activity in hippocampal long-term potentiation and memory. *Neurosci Res*. 2000; 38:3–17. [PubMed: 10997573]

19. Fukunaga K, Soderling TR, Miyamoto E. Activation of Ca²⁺/calmodulin-dependent protein kinase II and protein kinase C by glutamate in cultured rat hippocampal neurons. *J Biol Chem.* 1992; 267:22527–22533. [PubMed: 1358879]
20. Hargreaves K, Dubner R, Brown F, Flores C, Joris J. A new and sensitive method for measuring thermal nociception in cutaneous hyperalgesia. *Pain.* 1988; 32:77–88. [PubMed: 3340425]
21. He Y, Tian X, Hu X, Porreca F, Wang ZJ. Negative reinforcement reveals non-evoked ongoing pain in mice with tissue or nerve injury. *J Pain.* 2012; 13:598–607. [PubMed: 22609247]
22. He Y, Wang ZJ. Nociceptor Beta II, Delta, and Epsilon Isoforms of PKC Differentially Mediate Paclitaxel-Induced Spontaneous and Evoked Pain. *J Neurosci.* 2015; 35:4614–4625. [PubMed: 25788678]
23. He Y, Yang C, Kirkmire CM, Wang ZJ. Regulation of Opioid Tolerance by let-7 Family MicroRNA Targeting the {micro} Opioid Receptor. *J Neurosci.* 2010; 30:10251–10258. [PubMed: 20668208]
24. Hillery CA, Kerstein PC, Vilceanu D, et al. Transient receptor potential vanilloid 1 mediates pain in mice with severe sickle cell disease. *Blood.* 2011; 118:3376–3383. [PubMed: 21708890]
25. Hsu L, Diwan B, Ward JM, Noguchi CT. Pathology of “Berkeley” sickle-cell mice includes gallstones and priapism. *Blood.* 2006; 107:3414–3415. [PubMed: 16597602]
26. Hylden JL, Wilcox GL. Intrathecal morphine in mice: a new technique. *Eur J Pharmacol.* 1980; 67:313–316. [PubMed: 6893963]
27. Jones TL, Lustig AC, Sorkin LS. Secondary hyperalgesia in the postoperative pain model is dependent on spinal calcium/calmodulin-dependent protein kinase II alpha activation. *Anesth Analg.* 2007; 105:1650–1656. table of contents. [PubMed: 18042863]
28. King T, Vera-Portocarrero L, Gutierrez T, et al. Unmasking the tonic-aversive state in neuropathic pain. *Nat Neurosci.* 2009; 12:1364–1366. [PubMed: 19783992]
29. Kitamura Y, Miyazaki A, Yamanaka Y, Nomura Y. Stimulatory effects of protein kinase C and calmodulin kinase II on N- methyl-D-aspartate receptor/channels in the postsynaptic density of rat brain. *J Neurochem.* 1993; 61:100–109. [PubMed: 7685812]
30. Kohli DR, Li Y, Khasabov SG, et al. Pain-related behaviors and neurochemical alterations in mice expressing sickle hemoglobin: modulation by cannabinoids. *Blood.* 2010; 116:456–465. [PubMed: 20304807]
31. Lisman J, Schulman H, Cline H. The molecular basis of CaMKII function in synaptic and behavioural memory. *Nat Rev Neurosci.* 2002; 3:175–190. [PubMed: 11994750]
32. Luo F, Yang C, Chen Y, et al. Reversal of chronic inflammatory pain by acute inhibition of Ca²⁺/calmodulin-dependent protein kinase II. *J Pharmacol Exp Ther.* 2008; 325:267–275. [PubMed: 18178903]
33. Mancini EA, Hillery CA, Bodian CA, Zhang ZG, Luty GA, Collier BS. Pathology of Berkeley sickle cell mice: similarities and differences with human sickle cell disease. *Blood.* 2006; 107:1651–1658. [PubMed: 16166585]
34. Mittal A, Gupta M, Lamarre Y, Jahagirdar B, Gupta K. Quantification of pain in sickle mice using facial expressions and body measurements. *Blood Cells Mol Dis.* 2016; 57:58–66. [PubMed: 26852657]
35. Molokie RE, Wang ZJ, Wilkie DJ. Presence of neuropathic pain as an underlying mechanism for pain associated with cold weather in patients with sickle cell disease. *Med Hypotheses.* 2011; 77:491–493. [PubMed: 21763079]
36. Molokie RE, Wilkie DJ, Wittert H, et al. Mechanism-driven phase I translational study of trifluoperazine in adults with sickle cell disease. *Eur J Pharmacol.* 2014; 723:419–424. [PubMed: 24211787]
37. Paszty C. Transgenic and gene knock-out mouse models of sickle cell anemia and the thalassemias. *Curr Opin Hematol.* 1997; 4:88–93. [PubMed: 9107524]
38. Paszty C, Brion CM, Mancini E, et al. Transgenic knockout mice with exclusively human sickle hemoglobin and sickle cell disease. *Science.* 1997; 278:876–878. [PubMed: 9346488]
39. Platt OS, Thorington BD, Brambilla DJ, et al. Pain in sickle cell disease. Rates and risk factors. *N Engl J Med.* 1991; 325:11–16. [PubMed: 1710777]

40. Price TJ, Jeske NA, Flores CM, Hargreaves KM. Pharmacological interactions between calcium/calmodulin-dependent kinase II alpha and TRPV1 receptors in rat trigeminal sensory neurons. *Neurosci Lett*. 2005; 389:94–98. [PubMed: 16095822]
41. Smith WR, Bauserman RL, Ballas SK, et al. Climatic and geographic temporal patterns of pain in the Multicenter Study of Hydroxyurea. *Pain*. 2009; 146:91–98. [PubMed: 19683393]
42. Smith WR, Penberthy LT, Bovbjerg VE, et al. Daily assessment of pain in adults with sickle cell disease. *Ann Intern Med*. 2008; 148:94–101. [PubMed: 18195334]
43. Sumi M, Kiuchi K, Ishikawa T, et al. The newly synthesized selective Ca²⁺/calmodulin dependent protein kinase II inhibitor KN-93 reduces dopamine contents in PC12h cells. *Biochem Biophys Res Commun*. 1991; 181:968–975. [PubMed: 1662507]
44. Tang L, Chen Y, Chen Z, Blumberg PM, Kozikowski AP, Wang ZJ. Antinociceptive pharmacology of N-(4-chlorobenzyl)-N'-(4-hydroxy-3-iodo-5-methoxybenzyl) thiourea, a high-affinity competitive antagonist of the transient receptor potential vanilloid 1 receptor. *J Pharmacol Exp Ther*. 2007; 321:791–798. [PubMed: 17312187]
45. Vang D, Paul JA, Nguyen J, et al. Small-molecule nociceptin receptor agonist ameliorates mast cell activation and pain in sickle mice. *Haematologica*. 2015; 100:1517–1525. [PubMed: 26294734]
46. Wang Z, Gardell LR, Ossipov MH, et al. Pronociceptive actions of dynorphin maintain chronic neuropathic pain. *J Neurosci*. 2001; 21:1779–1786. [PubMed: 11222667]
47. Wang ZJ, Molokie RE, Wilkie DJ. Does cold hypersensitivity increase with age in sickle cell disease? *Pain*. 2014; 155:2439–2440. [PubMed: 25172823]
48. Wilkie DJ, Molokie R, Boyd-Seal D, et al. Patient-reported outcomes: descriptors of nociceptive and neuropathic pain and barriers to effective pain management in adult outpatients with sickle cell disease. *J Natl Med Assoc*. 2010; 102:18–27. [PubMed: 20158132]
49. Zappia KJ, Garrison SR, Hillery CA, Stucky CL. Cold hypersensitivity increases with age in mice with sickle cell disease. *Pain*. 2014; 155:2476–2485. [PubMed: 24953902]

Figure 1.

Lidocaine induced conditioned place preference (CPP) in BERK sickle cell mice. (A) BERK mice spent significantly more time in lidocaine-paired chamber, whereas the non-sickle control littermates showed no chamber preference to lidocaine. ** $P < 0.01$, saline paired vs. lidocaine paired group, two-way ANOVA followed by Bonferroni *post hoc* test; $n = 6$. (B) Difference score analysis confirmed that the sickle mice, but not non-sickle mice produced CPP to lidocaine. * $P < 0.05$, test time vs. preconditioning time in the lidocaine paired chamber, paired *t* test; $n = 6$.

Figure 2.

Evoked pain behaviors displayed in BERK sickle cell mice.

A cold stimulus was applied by a brief (1 s) spray of tetrafluoroethane to the ventral surface of hindpaw. (A) The number of licks and (B) the duration of lifting of the sprayed paw were recorded. *** $P < 0.001$, “sickle” group vs. “non-sickle”, $n = 9$. (C) When compared with the non-sickle control littermates, BERK mice exhibited significantly reduced threshold of response to probing by von Frey filaments, $P < 0.001$, $n = 12$. (D) BERK mice showed significantly reduced withdrawal latency to radiant heat, indicating the presence of thermal hyperalgesia, *** $P < 0.001$ vs. “non-sickle” mice group, $n = 12$. (E) In the tail immersion test performed at 48, 52, or 55 °C. BERK mice showed reduced latencies to a rapid tail flick response as compared with non-sickle control mice. * $P < 0.05$, $n = 9$. (F) BERK mice showed significantly reduced withdrawal latency to the hot plate maintained at 52 °C, * $P < 0.05$ vs. “non-sickle” group, $n = 9$.

Figure 3.

BERK sickle cell mice responded to the inflammatory pain stimulus induced by a subcutaneous injection of formalin (2% in saline, 20 μ L/mouse) into the dorsal surface of the left hindpaw. (A) Mice paw flinching was observed for 60 min, the number of flinches was recorded for successive 5 min intervals. (B) The total number of flinches during the early phase (0–10 min) and late phase (10.01–60 min) were summed, respectively.* $P < 0.05$ “sickle” vs. “non-sickle” group, $n = 10$.

Figure 4.

Activation of CaMKIIα was observed in BERK sickle cell mice. Western blot analysis showed significant increase of phosphorylated CaMKIIα (pCaMKIIα) in the spinal cord (A) and DRG (B) of BERK mice as compared with the non-sickle control mice, ** $P < 0.01$, $n = 3$. (C) Compared with the non-sickle mice (left panel), elevated immunohistochemical staining of pCaMKIIα was found in the superficial lamina region of the dorsal spinal cord in the sickle cell mice (right panel). Quantitative analysis of pCaMKIIα immunoreactivity was performed by counting the number of positively stained cells using the MetaMorph Imaging Software.

Figure 5.

KN93 suppressed chronic pain in BERK sickle cell mice. (A) KN93 (45nmol in 5 μ L saline, *i.t.*) produced CPP in the sickle mice. The sickle mice spent significantly more time in KN93-paired chamber, whereas the non-sickle control mice showed no chamber preference, spending similar amount of time in saline- and KN93-paired chambers. (B) Difference scores confirmed the presence of KN93-CPP in the sickle mice, but not the non-sickle control mice. * $P < 0.05$; $n = 6$. (C) KN92, an inactive analog of KN93 (45 nmol in 5 μ L saline, *i.t.*), did not produce CPP in the sickle mice. Neither the sickle mice nor the non-

sickle mice exhibited chamber preference. (D) Difference scores confirmed the absence of KN92-CPP in the sickle mice. $n = 6$. Mice paw withdrawal threshold to von Frey filament probing (E) and withdrawal latency to radiant heat (F) were measured before (0) and 0.5, 1, 2, 4 and 24 h after the injection of KN93 (45 nmol in 5 μ L of saline, *i.t.*). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, compared with the “non-sickle w/KN93” group; # $P < 0.05$, ## $P < 0.01$, ### $P < 0.001$, compared with the “sickle w/KN92” group; $n = 6$.

Figure 6.

CaMKII α knockdown by siRNA reversed mechanical allodynia (A), thermal hyperalgesia (B) and ongoing spontaneous pain (C–D) in BERK sickle cell mice. Mice were treated with CaMKII α siRNA or scrambled (scr) RNA duplex (2 μ g, *i.t.* twice per day for 3 days). Mechanical and thermal sensitivities were tested daily. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, compared with the “non-sickle w/siRNA” group; # $P < 0.05$, ## $P < 0.01$, ### $P < 0.001$, compared with the “sickle w/scr” group; scr: scrambled RNA duplex; $n = 6$. Arrows indicated siRNA/scr injections. The expression of CaMKII α was reduced by 3d-treatment

with CaMKII α siRNA, compared with the same treatment with scrambled siRNA (A-insert). (C) When tested on Day 4 by lidocaine-CPP paradigm, spontaneous ongoing pain was present in “sickle w/scr” mice, but not “sickle w/siRNA” mice. (D) Difference score confirmed the absence of chamber preference in BERK mice after CaMKII α siRNA treatment, * $P < 0.05$, n = 6.