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*J Clin Invest.* 2016;**126**(8):3053-3057. <https://doi.org/10.1172/JCI86165>.

**Brief Report**

**Hematology**

Pain is a life-long symptom in sickle cell disease (SCD) and a predictor of disease progression and mortality, but little is known about its molecular mechanisms. Here, we characterized pain in a targeted knockin mouse model of SCD (TOW mouse) that exclusively expresses human alleles encoding normal  $\alpha$ - and sickle  $\beta$ -globin. TOW mice exhibited ongoing spontaneous pain behavior and increased sensitivity to evoked pain compared with littermate control mice expressing normal human hemoglobins. PKC $\delta$  activation was elevated in the superficial laminae of the spinal cord dorsal horn in TOW mice, specifically in GABAergic inhibitory neurons. Functional inhibition and neuron-specific silencing of PKC $\delta$  attenuated spontaneous pain, mechanical allodynia, and heat hyperalgesia in TOW mice. Furthermore, we took a hematopoietic stem cell transplantation approach to generating a SCD model in PKC $\delta$ -deficient mice. Neither spontaneous pain nor evoked pain was detected in the mice lacking PKC $\delta$  despite full establishment of SCD phenotypes. These findings support a critical role of spinal PKC $\delta$  in the development of chronic pain in SCD, which may become a potential target for pharmacological interventions.

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# PKC $\delta$ -targeted intervention relieves chronic pain in a murine sickle cell disease model

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**Pain is a life-long symptom in sickle cell disease (SCD) and a predictor of disease progression and mortality, but little is known about its molecular mechanisms. Here, we characterized pain in a targeted knockin mouse model of SCD (TOW mouse) that exclusively expresses human alleles encoding normal  $\alpha$ - and sickle  $\beta$ -globin. TOW mice exhibited ongoing spontaneous pain behavior and increased sensitivity to evoked pain compared with littermate control mice expressing normal human hemoglobins. PKC $\delta$  activation was elevated in the superficial laminae of the spinal cord dorsal horn in TOW mice, specifically in GABAergic inhibitory neurons. Functional inhibition and neuron-specific silencing of PKC $\delta$  attenuated spontaneous pain, mechanical allodynia, and heat hyperalgesia in TOW mice. Furthermore, we took a hematopoietic stem cell transplantation approach to generating a SCD model in PKC $\delta$ -deficient mice. Neither spontaneous pain nor evoked pain was detected in the mice lacking PKC $\delta$  despite full establishment of SCD phenotypes. These findings support a critical role of spinal PKC $\delta$  in the development of chronic pain in SCD, which may become a potential target for pharmacological interventions.**

## Introduction

Sickle cell disease (SCD) is a deadly hereditary blood disorder, characterized by sickle-shaped red blood cells (rbc), anemia, and multiple organ failure (1). Pain is a hallmark and a predictor of disease progression and mortality in SCD. Besides acute painful vaso-occlusive crises, SCD is also accompanied by intractable chronic pain. This persistent, and often unrelieved, pain starts early in childhood and continues throughout life (2). Over half of patients reported experiencing chronic pain on more than 50% of the diary days and a third on 95% of days (3).

The neurobiological mechanisms of chronic pain in SCD remain unclear, which markedly limits effective pain management and the quality of life in patients with SCD. Transgenic mouse models have provided new insights into SCD research. Several studies have been started to characterize pain behaviors in Berkeley mice, a model of a severe form of SCD, which exhibit heat hyperalgesia, cold, and mechanical allodynia (4–6). However, Berkeley mice demonstrate unbalanced synthesis of  $\beta$ - and  $\alpha$ -globin chains, indicating these mice are  $\beta$ -thalassemic (7). Moreover, the copy number of the transgene is not controllable and may differ greatly. Recently, another transgenic SCD mouse strain (TOW) that was originally created by T.M. Townes' group became available to the research community (8). TOW mice were created by targeted knockin where the mouse  $\alpha$ - and  $\beta$ -globin genes were replaced by the human  $\alpha$ - and human  $\gamma$  and  $\beta^S$  (sickle) globin genes, respectively. Mimicking humans with SCD, TOW

mice complete the fetal hemoglobin switch after birth (9). Homozygous TOW mice for the human  $\beta^S$  allele ( $h\beta^S/h\beta^S$ ) remain viable for up to 18 months, but develop extensive rbc sickling, severe anemia, and multiple organ damage, similar to what is seen in the human disease (10).

In the present study, the TOW humanized mouse model of SCD is validated as a valuable rodent model for studying 2 distinctive pain components (nonevoked ongoing pain and evoked pain) in SCD and offers us the opportunity to gain insights into PKC $\delta$ -based mechanisms and intervention targets for chronic pain in SCD.

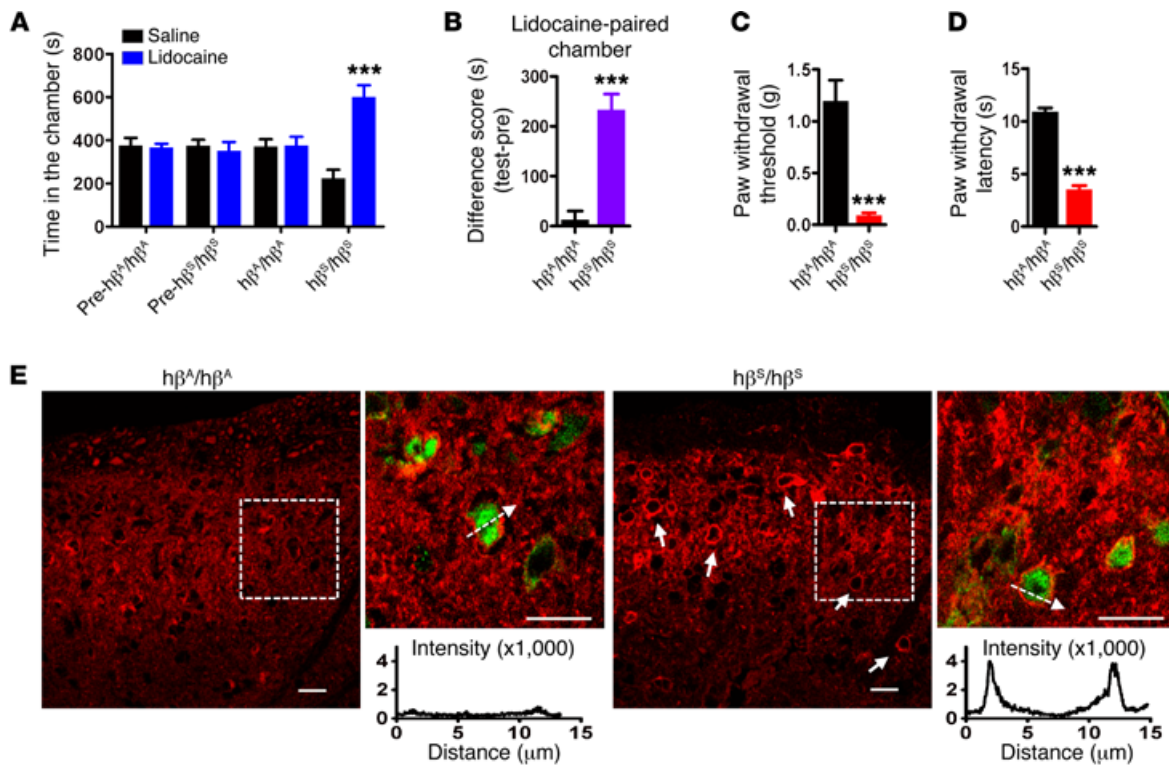
## Results and Discussion

As a prevalent manifestation in patients with SCD, ongoing or nonevoked pain is rarely investigated in preclinical settings. We have validated the use of negative reinforcement to detect ongoing spontaneous pain in mice with persistent tissue or nerve injuries, where nonrewarding analgesics (e.g., lidocaine) can elicit conditioned place preference (CPP) (11, 12). Here, we applied the CPP paradigm to investigate ongoing spontaneous pain. TOW mice spent significantly more time in lidocaine-paired chambers ( $601 \pm 54$  s) than in saline-paired chambers ( $223 \pm 40$  s) after single-trial conditioning ( $P < 0.001$ , Figure 1A). In contrast, nonsickle control  $h\beta^A/h\beta^A$  mice spent similar amounts of time in lidocaine-paired chambers ( $374 \pm 42$  s) or saline-paired chambers ( $370 \pm 34$  s, Figure 1A). The CPP difference scores (Figure 1B), indicative of chronic pain severity, were on the same order of magnitude as those in mice after spinal nerve ligation (11), which is in agreement with the clinical observation of high pain scores in SCD (2). The result was not due to

**Conflict of interest:** The authors have declared that no conflict of interest exists.

**Submitted:** December 21, 2015; **Accepted:** May 5, 2016.

**Reference information:** *J Clin Invest.* 2016;126(8):3053–3057. doi:10.1172/JCI86165.



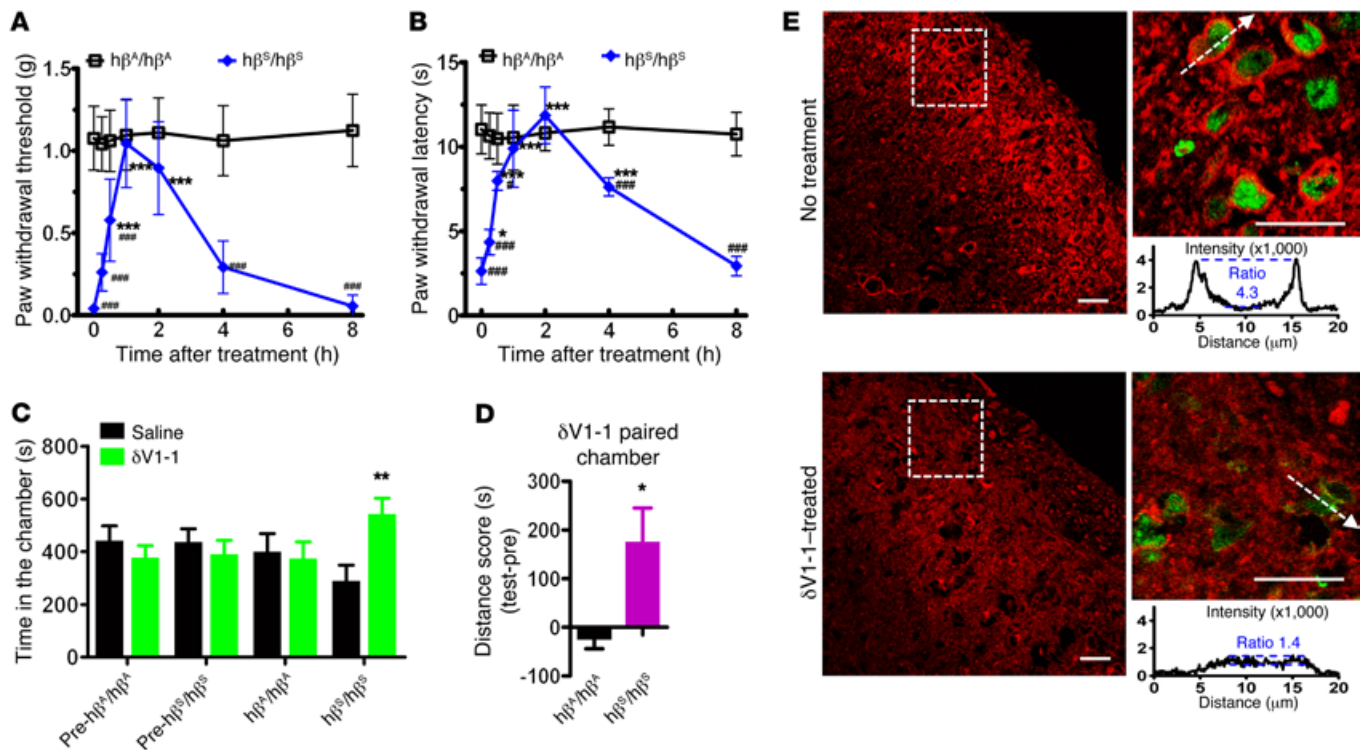
**Figure 1. TOW mice exhibited ongoing spontaneous pain, evoked pain, and activation of spinal PKC $\delta$ .** (A) Lidocaine (0.04%, i.t.) induced CPP in TOW ( $h\beta^S/h\beta^S$ ) SCD mice. TOW mice spent significantly more time in lidocaine-paired chambers, whereas nonsickle control mice showed no chamber preference. (B) Difference scores between test time and preconditioning (pre) time confirmed that  $h\beta^S/h\beta^S$ , but not  $h\beta^A/h\beta^A$ , mice developed lidocaine CPP. (C) TOW mice displayed significantly reduced paw withdrawal threshold to von Frey filaments when compared with  $h\beta^A/h\beta^A$  mice. (D) In response to noxious radiant heat, TOW mice showed significantly shorter paw withdrawal latency compared with  $h\beta^A/h\beta^A$  mice. Data were analyzed by ANOVA followed by Dunnett's *t* test. Two-way ANOVA (pairing vs. treatment) and post hoc Bonferroni's test were used to analyze CPP data. Difference scores were analyzed by 2-tailed paired *t* test. \*\*\* $P < 0.001$  vs.  $h\beta^A/h\beta^A$ .  $n = 8$ /group. (E) Immunohistochemistry showed plasma membrane translocation of PKC $\delta$  (indicated by solid arrows) in the superficial lamina region of the dorsal spinal cord in TOW, but not  $h\beta^A/h\beta^A$ , mice. PKC $\delta$  fluorescent intensity across representative cells (indicated by dashed arrows) is shown. Red, PKC $\delta$ ; green, NeuN. Scale bars: 20  $\mu$ m.  $n = 15$  slices from 3 mice.

changes in locomotor function, as we found no differences in open field activity or rotarod performance between TOW and control mice (Supplemental Figure 1; supplemental material available online with this article; doi:10.1172/JCI86165DS1). This is the first direct evidence, to our knowledge, that SCD is accompanied by nonevoked spontaneous pain in mice.

We next determined the nociceptive responses of TOW mice toward evoked mechanical and thermal stimuli. As compared with control mice ( $1.20 \pm 0.20$  g), TOW mice displayed significantly reduced pain threshold ( $0.09 \pm 0.03$  g) to normally innocuous von Frey filament probing ( $P < 0.001$ , Figure 1C), indicative of the presence of tactile allodynia. In addition, TOW mice exhibited shortened response latency to noxious heat stimuli applied to the hind paw ( $3.52 \pm 0.38$  s in TOW mice vs.  $10.91 \pm 0.36$  s in control mice,  $P < 0.001$ , Figure 1D), indicating the presence of thermal hyperalgesia.

To investigate the underlying mechanisms of chronic pain in SCD, we examined PKC-mediated nociceptive signaling. We have identified PKC $\delta$  as a dual mediator of both spontaneous pain and evoked pain in peripheral neuropathy produced by paclitaxel (13). Therefore, it is compelling to investigate whether PKC $\delta$ -dependent mechanisms are relevant for chronic pain in SCD. First, we evaluated spinal PKC $\delta$  activity in TOW

mice by analyzing PKC $\delta$  plasma membrane translocation. The immunoreactivity of PKC $\delta$  was primarily found in the superficial laminae of the spinal cord dorsal horn (Figure 1E). In control mice, PKC $\delta$  distributed evenly across plasma membrane and cytosol (Figure 1E). In contrast, prominent membrane enrichment of PKC $\delta$ , indicative of its activation, was observed in the superficial laminae of the spinal cord dorsal horn in TOW mice (Figure 1E). All cells with PKC $\delta$  translocation are positive for NeuN immunoreactivity (Supplemental Figure 2). On the other hand, 83%  $\pm$  7% of NeuN-positive cells had substantial PKC $\delta$  translocation (total 450 cells were imaged from 3 mice, 5 slides/mouse). Western blotting analysis also showed significant membrane translocation of PKC $\delta$  from cytosol and increased levels of phosphorylated PKC $\delta$  (p-PKC $\delta$ ) in TOW mice (Supplemental Figure 3), which are consistent with hyperactivation of spinal PKC $\delta$  in TOW mice, correlating with the persistent pain state. Furthermore, PKC $\delta$  translocation occurred in GABAergic inhibitory neurons (VGAT $^+$ ), but not in glutamatergic excitatory ones (VGLUT2 $^+$ ) (Supplemental Figure 4). Activation of PKC decreases GABA transport activity by reducing the latter's cell surface expression (14) or catalytic efficiency (15), which suggests PKC $\delta$  may promote chronic pain in SCD through deactivation of inhibitory GABAergic mechanisms.



**Figure 2. PKC $\delta$  inhibition transiently attenuated chronic pain in TOW mice.** Mechanical (A) and thermal (B) sensitivities before (0) and after the injection of  $\delta$ V1-1 (3 nmole, i.t.). \* $P < 0.05$ , \*\*\* $P < 0.001$  vs. 0; #### $P < 0.001$  vs.  $h\beta^A/h\beta^A$ .  $n = 8$ /group. (C) TOW mice spent significantly more time in  $\delta$ V1-1- than saline-paired chambers, whereas  $h\beta^A/h\beta^A$  mice spent similar amounts of time in both chambers. (D) Difference scores confirmed the presence of chamber preference to  $\delta$ V1-1 in TOW but not  $h\beta^A/h\beta^A$  mice. \* $P < 0.05$ ; \*\* $P < 0.01$ .  $n = 8$ /group. (E) In TOW mice, plasma membrane translocation of PKC $\delta$  (indicated by dashed arrows across representative cells and corresponding fluorescent intensity plots) was abolished 30 minutes after  $\delta$ V1-1 injection. Red, PKC $\delta$ ; green, NeuN. Scale bars: 20  $\mu$ m.  $n = 15$  slices from 3 mice. Data were analyzed by ANOVA followed by Dunnett's  $t$  test. Two-way ANOVA (pairing vs. treatment) and post hoc Bonferroni's test were used to analyze CPP data. Difference scores were analyzed by 2-tailed paired  $t$  test.

To identify the functional relevance of PKC $\delta$  in sickle cell pain, we directly targeted PKC $\delta$  in TOW and control mice by several complementary approaches. Corresponding to the amino acids 8–17 in the C2 domain of PKC $\delta$ ,  $\delta$ V1-1 peptide is a highly selective inhibitor of PKC $\delta$ , which acts by competing for binding to its anchoring protein RACK $\delta$  (16). Mechanical hypersensitivity in TOW mice was transiently but effectively suppressed by a single intrathecal (i.t.) administration of  $\delta$ V1-1 (3 nmole). The peak anti-allodynic effect was observed at 1 hour when the paw withdrawal threshold in TOW mice was restored to a level that was indistinguishable from that in control mice (Figure 2A).  $\delta$ V1-1 also significantly attenuated thermal hyperalgesia in TOW mice, with a quick onset in 15 minutes and peak antihyperalgesic effect at 2 hours, which lasted for at least 4 hours (Figure 2B). On the other hand,  $\delta$ V1-1 did not alter mechanical or thermal sensitivity in control mice, confirming a previous report that PKC $\delta$  does not participate in acute pain signaling (17).

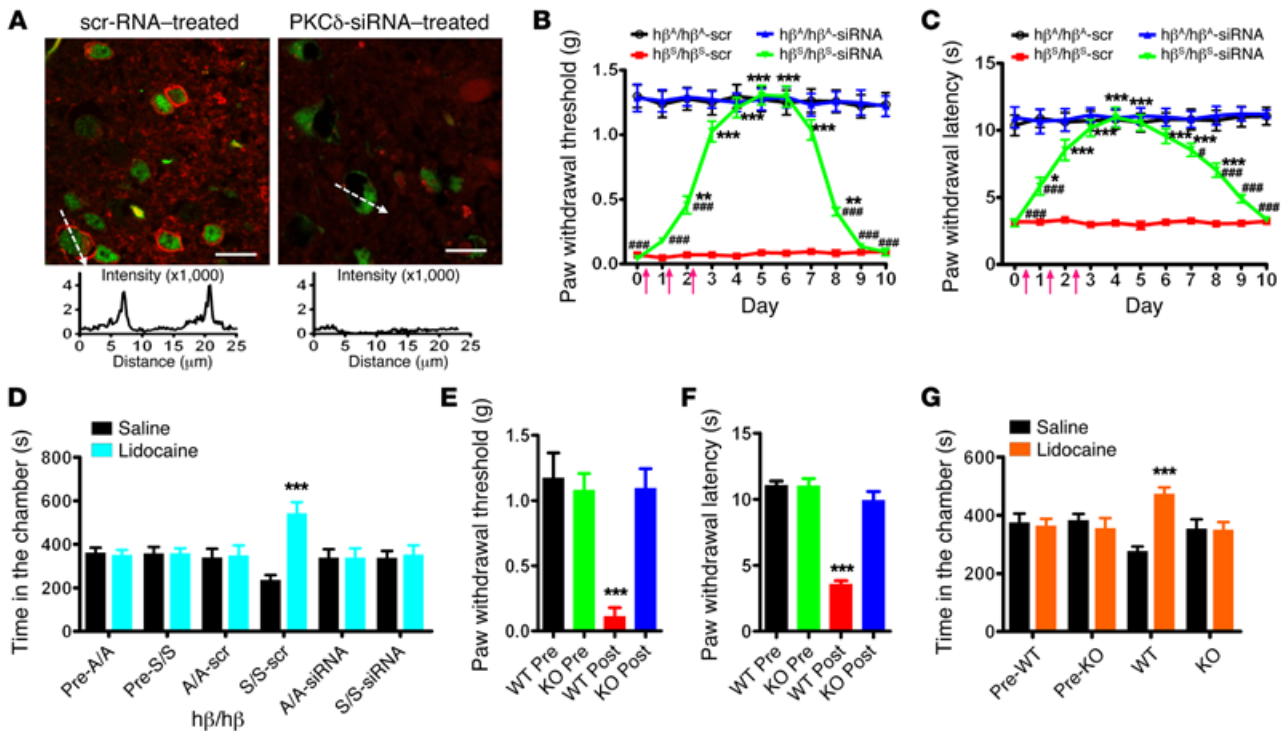
We next determined the participation of spinal PKC $\delta$  in ongoing pain. If the latter is mediated by PKC $\delta$ , it is expected that PKC $\delta$  inhibition would suppress ongoing pain and produce CPP in TOW mice. After being paired with  $\delta$ V1-1 for 30 minutes, TOW mice showed a strong preference for  $\delta$ V1-1-paired (543  $\pm$  60 s) over saline-paired chambers (289  $\pm$  60 s,  $P < 0.01$ , Figure 2C), illustrating that  $\delta$ V1-1 induced CPP in TOW mice. In contrast, control mice spent similar amounts of time in saline chambers (399  $\pm$  69 s)

and  $\delta$ V1-1 chambers (375  $\pm$  62 s). The significant difference score ( $P < 0.05$ ) generated in TOW, not control, mice confirmed the involvement of PKC $\delta$  in ongoing spontaneous pain in SCD (Figure 2D).

Enhanced spinal PKC $\delta$  translocation in TOW mice (membrane/cytoplasmic intensity ratio: 4.1  $\pm$  1.2) was suppressed by  $\delta$ V1-1 (1.8  $\pm$  0.7,  $P < 0.001$ ) 30 minutes after injection (Figure 2E; 450 NeuN[+] cells from 3 mice/group, 5 slides/mouse). The effective inhibition of PKC $\delta$  by  $\delta$ V1-1 in vivo was also confirmed by Western blotting analysis, where both PKC $\delta$  translocation and p-PKC $\delta$  were reduced by  $\delta$ V1-1 (Supplemental Figure 3). These biochemical data confirmed PKC $\delta$  inhibition by  $\delta$ V1-1, suggesting a causative role of PKC $\delta$  in evoked and ongoing pain associated with SCD.

To ascertain the type of cells with PKC $\delta$  translocation, we selectively targeted neuronal PKC $\delta$  by delivering PKC $\delta$  siRNA to TOW mice in complex with RVG-9R, a chimeric peptide derived from rabies virus glycoprotein. RVG-9R enables siRNA selective binding to neurons and enhances knockdown efficiency of siRNA (18, 19). Neuronal-specific knockdown of PKC $\delta$  was observed in the spinal cord dorsal horn of TOW mice treated with RVG/siRNA for 3 days (Figure 3A and Supplemental Figure 5). Meanwhile, significant reversal of mechanical allodynia and heat hyperalgesia in TOW mice was achieved and lasted for 8 days (Figure 3, B and C). Ongoing spontaneous pain was assessed on day 4 when lidocaine elicited CPP in TOW mice treated with RVG/scrambled RNA, but not in TOW mice treated with RVG/siRNA (Figure 3D). Neuronal-





**Figure 3. Sickle cell pain behaviors in mice after neuronal PKC $\delta$  silencing and in PKC $\delta$ -KO mice.** (A) Spinal delivery of RVG-9R/PKC $\delta$ -siRNA (2  $\mu$ g/d for 3 days, i.t.) specifically knocked down PKC $\delta$  (red) in NeuN-positive (green) cells. Scale bars: 20  $\mu$ m.  $n = 15$  slices from 3 mice. PKC $\delta$  fluorescent intensity across representative cells (indicated by dashed arrows) is illustrated in the chart. Mechanical (B) and thermal (C) sensitivities were tested before and after siRNA treatments. Arrows denote siRNA injections. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$  vs. h $\beta^{\Delta}$ /h $\beta^{\Delta}$ -scr; # $P < 0.05$ , ### $P < 0.001$  vs. h $\beta^{\Delta}$ /h $\beta^{\Delta}$ -siRNA.  $n = 8$ /group. (D) When tested on day 4, lidocaine produced CPP in TOW mice treated with RVG-9R/scrambled (scr) RNA duplex, not RVG-9R/PKC $\delta$ -siRNA. \*\*\* $P < 0.001$ .  $n = 6$ /group. (E) Mechanical allodynia and (F) thermal hyperalgesia developed in PKC $\delta$ -WT mice, not PKC $\delta$ -KO mice, after hematopoietic stem cell transplant from donor TOW mice. \*\*\* $P < 0.001$  vs. pre.  $n = 6$ /group. (G) Lidocaine produced CPP paradigm in PKC $\delta$ -WT mice, not PKC $\delta$ -KO mice, 4 weeks after transplant. \*\*\* $P < 0.001$ .  $n = 6$ /group. Data were analyzed by ANOVA followed by Dunnett's  $t$  test. Two-way ANOVA (pairing vs. treatment) and post hoc Bonferroni's test were used to analyze CPP data

specific PKC $\delta$  silencing by RVG/siRNA abolished CPP difference scores in TOW mice (Supplemental Figure 6). These data validated the essential role of neuronal PKC $\delta$  in mediating evoked and ongoing spontaneous pain in SCD.

To rule out the possibility of nonspecific or incomplete targeting of PKC $\delta$ , we took another innovative approach to generating sickle cell anemia in *Pkcd*<sup>-/-</sup> (PKC $\delta$ -KO) mice by hematopoietic stem cell transplantation. After being conditioned with a lethal dose of total body irradiation, PKC $\delta$ -KO mice or WT littermates received bone marrow cells harvested from TOW mice. The donor hematopoietic stem cells carrying the human sickle  $\beta$ -globin gene were able to reconstitute the hematopoietic system of recipient mice and produce sickle cell anemia (Supplemental Figure 7). Stable engraftment was observed as early as 2 weeks after the transplant by PCR analysis (Supplemental Figure 8). Typical hematologic defects and systematic signs of SCD were established in recipient PKC $\delta$ -KO and WT mice 4 weeks after the transplant when the recipient PKC $\delta$ -KO or WT mice no longer produced mouse hematocytes, but expressed human sickle hemoglobin (Supplemental Figure 9 and Supplemental Table 1). This manipulation provided a unique approach to superimposing a TOW SCD model on a PKC $\delta$ -KO background, allowing complete deletion of PKC $\delta$  in the central nervous system and a separation neuronal mechanism of pain from that of the disease itself.

Before transplant, baseline mechanical and thermal sensitivities were not different between PKC $\delta$ -WT and PKC $\delta$ -KO mice (Figure 3, E and F). When tested 4 weeks after the transplant, recipient WT mice exhibited significantly decreased thresholds to von Frey filament probing (Figure 3E) and shortened withdrawal latencies to radiant heat stimulus (Figure 3F), which were consistent with the expression of evoked pain phenotype in SCD. In contrast, transplanted PKC $\delta$ -KO mice did not show changes in either mechanical or thermal sensitivity when compared with pretransplant baseline (Figure 3, E and F), although SCD phenotypes were fully established at that time (Supplemental Figure 9 and Supplemental Table 1).

When PKC $\delta$ -KO and WT mice were subject to the CPP test 4 weeks after transplant, recipient WT mice spent significantly more time in lidocaine-paired chambers (474  $\pm$  23 s vs. 277  $\pm$  15 s,  $P < 0.001$ , Figure 3G), indicating the presence of ongoing pain. In contrast, transplanted PKC $\delta$ -KO mice showed no preference for saline-paired (354  $\pm$  32 s) or lidocaine-paired (350  $\pm$  26 s) chambers. The difference score confirmed the existence of ongoing spontaneous pain in transplanted PKC $\delta$ -WT, but not PKC $\delta$ -KO, mice (Supplemental Figure 10). These data indicated that PKC $\delta$  is required for the initiation/development of sickle cell pain, since ongoing and evoked pain behaviors were no longer associated with SCD in transplanted PKC $\delta$ -KO mice.

In summary, we have characterized ongoing spontaneous pain, mechanical allodynia, and thermal hyperalgesia in TOW mice that closely mimic SCD in humans (8). This is the first report, to our knowledge, of the presence of ongoing spontaneous pain in a preclinical sickle cell model. Moreover, we found that spinal PKC $\delta$ , specifically that in GABAergic inhibitory neurons, is a critical mechanism for the generation and maintenance of ongoing and evoked pain in TOW mice.

While we specifically investigated PKC $\delta$  in sickle cell pain, additional studies will be necessary to determine the role of other kinases that may function alone or in concert with PKC $\delta$  for pain in SCD. For example, p38 mitogen-activated protein kinase and PKC $\epsilon$  have been identified as mechanisms for neuropathic pain development and hyperalgesic priming (13, 20, 21). Equally important is identifying downstream effectors for PKC $\delta$ . As cold allodynia is an integral pain subtype in SCD with great clinical relevance (6), it is of great interest to investigate the regulation of cold sensors, such as transient receptor potential melastatin 8 (TRPM8) and transient receptor potential cation channel A1 (TRPA1), by spinal PKC $\delta$  in future studies (22).

Hematopoietic stem cell transplant in transgenic mice employed here may be a particularly powerful approach to elucidating central mechanisms in sickle cell pain. In this case, the vital role of CNS PKC $\delta$  in sickle cell pain was revealed. The technique can be readily applied to studying other genes. Our findings offer insights into sickle cell pain mechanisms and may ultimately lead to translational research targeting PKC $\delta$  for alleviating chronic pain in SCD.

## Methods

Detailed information is available in the Supplemental Methods.

**Statistics.** Data are expressed as mean  $\pm$  SEM. Statistical comparisons among multiple groups were analyzed by ANOVA followed by Dunnett's *t* test. Two-way ANOVA (pairing vs. treatment) was applied followed by Bonferroni's test to analyze CPP data. Difference scores and pre/posttransplant data were analyzed using 2-tailed, paired *t* test. A *P* value of less than 0.05 was considered statistically significant.

**Study approval.** All in vivo studies were carried out in accordance with the NIH *Guide for the Care and Use of Laboratory Animals* (National Academies Press, 2011.) after approval by the University of Illinois at Chicago IACUC.

## Author contributions

YH and ZJW designed research, analyzed data, and wrote the paper. YH, JN, and RW performed experiments. REM, ROM, DJW, and JD offered advice and contributed to manuscript editing.

## Acknowledgments

The study was supported by a grant from the National Heart, Lung, and Blood Institute (NHLBI) (R01HL098141). Y. He is a Sickle Cell Scholar supported by grant U01HL117658 from the NHLBI.

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