Supplemental Material

Supplemental Methods

Animals

Breeders of TOW mice were obtained from the Jackson Laboratory (1, 2). Heterozygous breeding was used to generate progeny that were homozygous for the human β^S alleles ($h\beta^S/h\beta^S$) and control littermates that were homozygous for the human β^A allele ($h\beta^A/h\beta^A$). Breeders of PKCδ mice were provided by Dr. Robert O. Messing, University of Texas at Austin (3). After arriving at our laboratory, PKCδ mice were backcrossed with C57Bl/6 mice for 8 generations. Heterozygous breeding was used to generate male homozygous PKCδ-null (KO) mice and littermate wild-type (WT) control mice for the study. Unless otherwise stated, mice ages ranging from 8 to 16 weeks old were used. For all behavioral and biochemical tests, the experimenters were blinded to the genotype and treatment information. All breeding and experimental procedures were carried out in accordance with the guidelines from the International Association for the Study of Pain and the NIH Guide for the Care and Use of Laboratory Animals after approval by the Institutional Animal Care and Use Committee.

Immunohistochemistry

Mice spinal cord tissues were fixed, permeabilized, and incubated with a primary antibody for PKCδ (1:500, Santa Cruz Biotechnology), followed by another incubation with Alexa 594-labeled secondary anti-rabbit IgG antibodies (1:500, Invitrogen) (4). The antibody specificity was validated in PKCδ null mice (Supplementary Figure 11). The tissue slides were further stained with NeuN antibody (1:500, Millipore), followed by another incubation with Alexa 488-labeled anti-mouse IgG secondary antibody (1:500, Invitrogen). For VGLUT2 colabeling, PKCδ stained slides were incubated with VGLUT2 antibody (1:500, Millipore), followed by another incubation with Alexa 488-labeled anti-guinea pig IgG secondary antibody (1:500, Invitrogen). For VGAT colabeling, slides were first labeled with VGAT antibody (1:20,000; Millipore) using Tyramide Signal Amplification Kits (Invitrogen) before staining with PKCδ antibody. For consistency, only the lumbar sections were used. Images were captured by a confocal microscope (Zeiss LSM 510). The fluorescent density ratio (membrane *vs*. cytosol) was calculated from the intensity profile across each cell (representative cells and analyses were shown and indicated by dash line)

Western blotting analysis

Western blotting analysis was performed using an antibody against phosphorylated PKCδ (1:1,000; Santa Cruz Biotechnology), an antibody against PKCδ (1:1,000; Santa Cruz Biotechnology) and an antibody against GAPDH (1:1,000; Santa Cruz Biotechnology) (4). After incubation with HRP-conjugated secondary antibodies, enhanced chemiluminescence signals were captured by a ChemiDoc imaging system and analyzed using the Quantity One program (Bio-Rad).

Assessment of mechanical and thermal sensitivity

Mechanical sensitivity: Mice were placed in individual Plexiglas containers with wire mesh platform. Calibrated von Frey filaments (Stoelting) were used to press upward to the midplantar surface of the left hindpaw for 5 s or until a withdrawal response occurred. Using the “up-down” algorithm, 50% probability of paw withdrawal threshold was determined (5, 6).

Thermal sensitivity: a plantar tester (UGO BASILE Model 7372, Stoelting) (4, 6) by placing mice in clear plastic chambers sitting on a glass floor. Radiant infrared light/heat was applied to the center of the plantar surface of the left hindpaw and the latency to paw withdrawal was recorded. A cutoff time of 20s was applied to avoid tissue damage.

Conditioned place preference (CPP)

Ongoing spontaneous pain was measured using the CPP paradigm (7). Mice were exposed to the CPP apparatus (San Diego Instruments) with full access to all chambers for 3 consecutive days (30 min/day). A pre-conditioning bias test was performed to exclude mice that had a preexisting chamber bias. On conditioning day, mice first received vehicle control (5 μL saline, intrathecally or i.t.) paired with a randomly chosen chamber and, 4 h later, lidocaine (0.04 % in 5 μL saline, i.t.; Hospira) or a PKCδ inhibitor (3.0 nmole in 5 μL saline, i.t.) paired with the other chamber. The myristoylated peptide inhibitor of PKCδ (δV1-1, SFNSYELGSL) was synthesized and verified by mass spectrometry by the Protein Research Laboratory, University of Illinois at Chicago (4, 8, 9). During conditioning, mice were allowed to stay only in the paired chamber without access to other chambers immediately following saline or drug injection. On the following day, 20 h after the afternoon pairing, mice were placed in the middle chamber of the CPP box with all doors open to have free access to all chambers. Movement and duration of time each mouse spent in each chamber were recorded for 15 min for off-line analysis of chamber preference. Difference scores were calculated as (test time-preconditioning time) spent in the drug chamber.

Locomotor function tests

A rotarod test: Mice were placed on rotarod (model series 8; IITC Life Science) and trained to remain on a fixed speed (4 RPM) for 60 s. On the following day, mice were retrained at 4 RPM for 60 s. Mice unable to achieve this threshold were removed from further study. Mice were tested 30 min later by placing them on an accelerating rotarod (4 to 40 RPM over 300 s) and the latency to fall off the rotarod was recorded (6, 10).

Open field exploration test: Mice were placed in an open field recording chamber (27” W × 8 1/8” D × 13 1/8” H) and allowed to move freely for 15 min. Movement of mice and distance traveled was monitored by 4 × 16 infrared sensors and automatically recorded in SDI software. Total distance traveled (cm) and average speed (cm/s) were calculated.

siRNA treatment

Separate groups of 8 mice were treated with PKCδ siRNA once a day for 3 consecutive days (2 μg in 5 μL, i.t.). The sequence of siRNA duplex were as following, mouse PKCδ (sense, 5′-AACUGUUUGUGAAUUUGCCdTdT -3′; antisense, 5'-GGCAAAUUCACAAACAGUUdTdT -3') (11), or scrambled negative control (sense, 5'-AUACGCGUAUUAUACGCGAUUACGAC-3'; antisense, 5'-CGUUAAUCGCGUAUAAUACGCGUAT-3'). These oligos were mixed with the RVG-9R peptide, in a molar ratio of 1:10 (siRNA:RVG) (12). RVG-9R [YTIWMPENPRPGTPCDIFTNSRGKRASNGGGGRRRRRRRRR] was synthesized and verified by mass spectrometry by the Protein Research Laboratory, University of Illinois at Chicago (13). Mechanical and thermal sensitivity tests were performed before and after

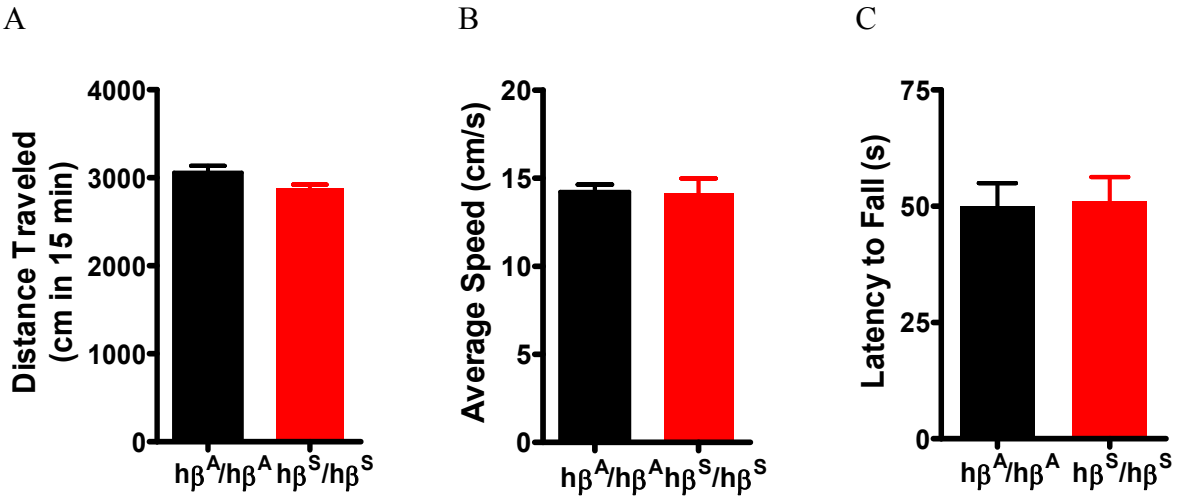
siRNA treatments. Non-evoked ongoing pain was determined on Day 4. Spinal cord samples were collected on Day 4 for immunohistochemical analysis.

Hematopoietic stem cell transplantation

Recipient mice were subjected to total body irradiation at a single dose of 10 Gy 4 - 6 h before bone marrow transplantation (14). Bone marrow was flushed out from dissected femurs and tibias of euthanized donor TOW ($h\beta^S/h\beta^S$) mice. After depleting red blood cells, bone marrow cells were suspended in PBS and delivered into recipients via retro-orbital injections ($\sim 5\times10^5$ in 0.1 mL PBS/mouse). Two weeks after transplantation, peripheral blood from recipients was collected weekly for engraftment analysis and hematological tests.

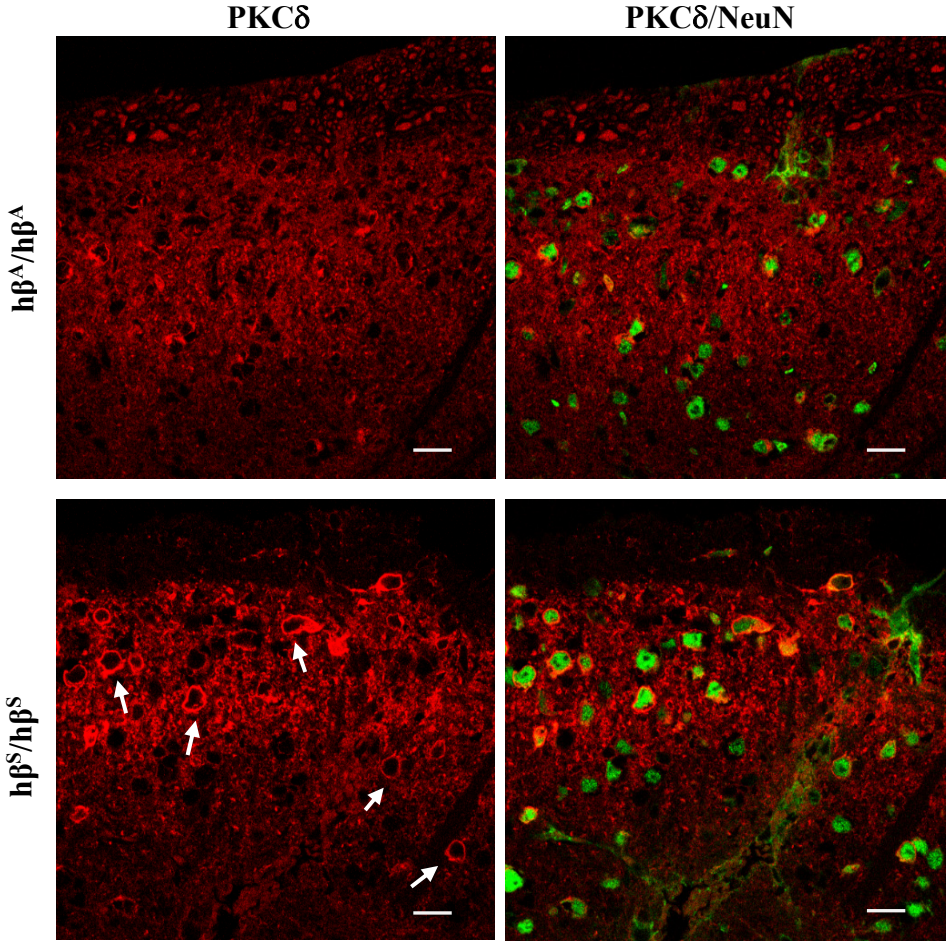
Supplemental Figures

Figure S1



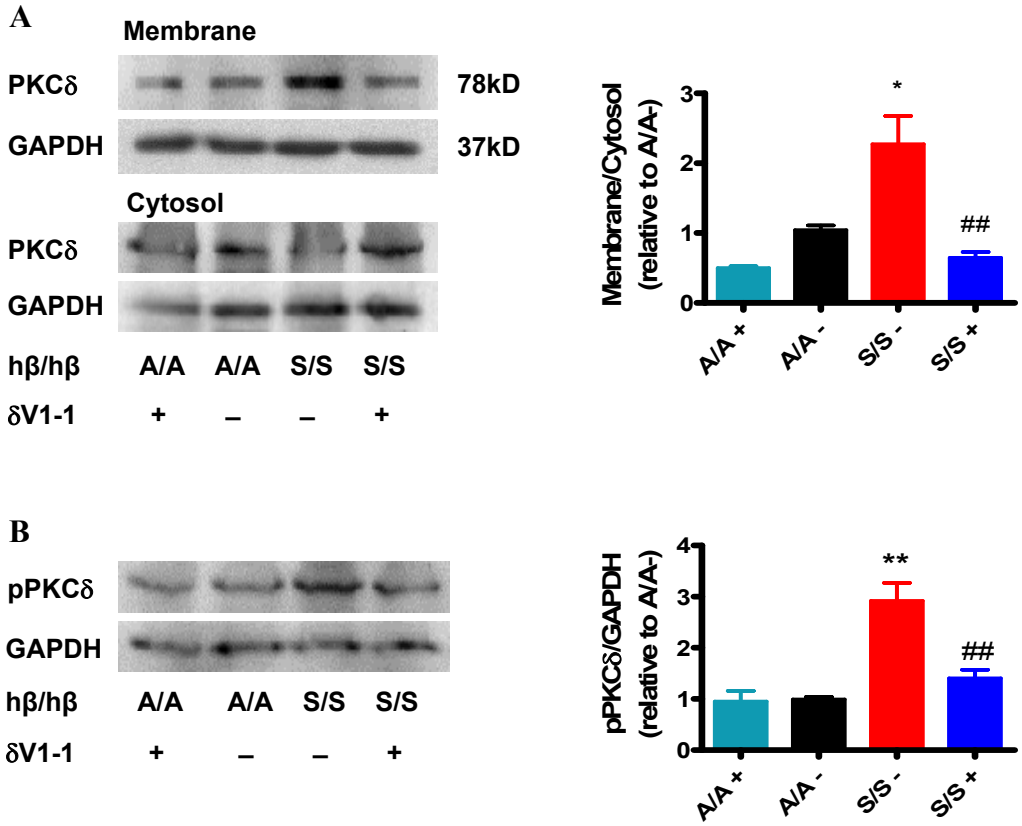
In open field exploration test (A-B), TOW sickle mice ($h\beta^S/h\beta^S$) and littermate non-sickle mice ($h\beta^A/h\beta^A$) displayed similar activity in distance and average speed traveled ($P>0.05$). Mice were monitored for 15 minutes while allowed to freely explore the chamber. Distance traveled (**A**) and average speed (**B**) were recorded as an indication of overall locomotor capability. (**C**) There was no difference in latency to fall between TOW and $h\beta^A/h\beta^A$ control mice on an accelerating rotarod test, n=8/group.

Figure S2



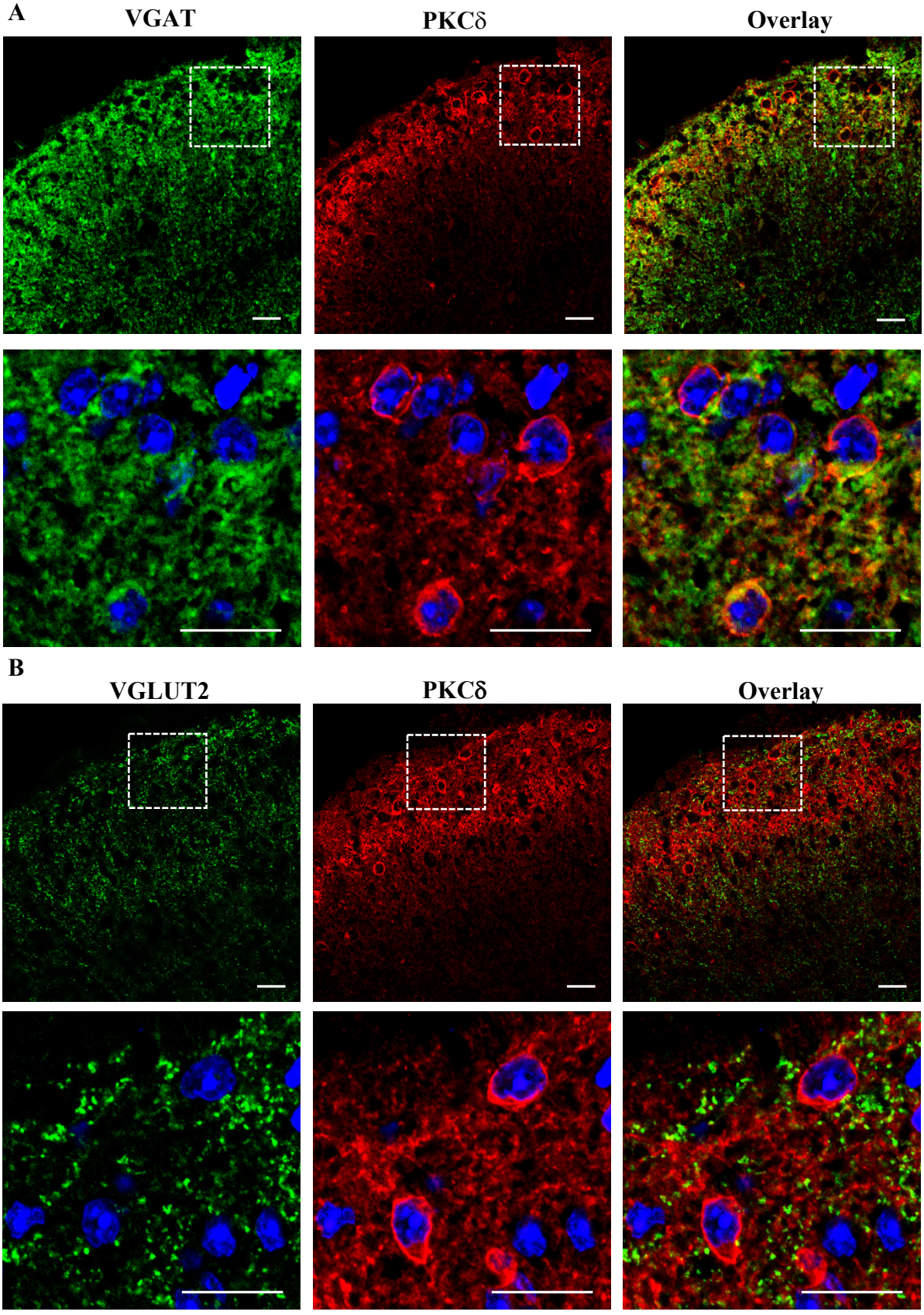
Immunohistochemical analysis showed cells with PKCδ translocation are positive for NeuN immunoreactivity in the superficial lamina region of the dorsal spinal cord in TOW ($h\beta^S/h\beta^S$) mice, with non-sickle littermates mice ($h\beta^A/h\beta^A$) as control that did not exhibit PKCδ translocation. Red: PKCδ, Green: NeuN, Scale bar: 20 μm, n=15 slices from 3 mice.

Figure S3



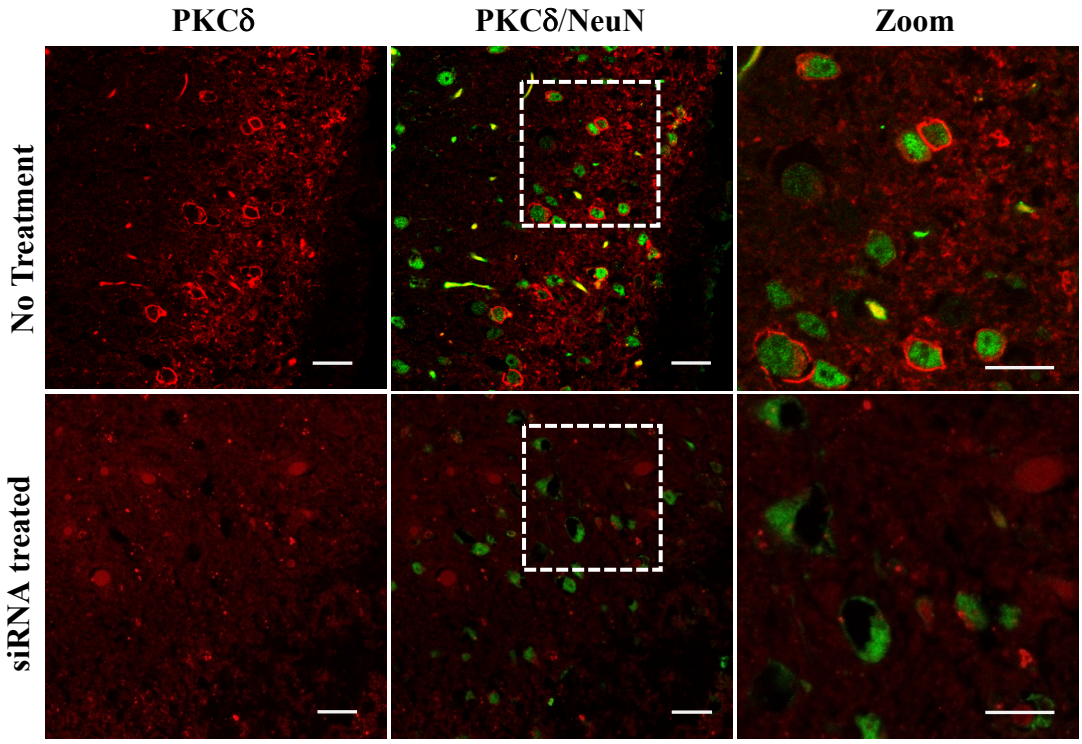
(**A**) Spinal PKCδ translocation from cytosol to plasma membrane was determined by subcellular fractionation followed by western blotting analysis. PKCδ translocation was suppressed by δV1-1. (**B**) Elevated level of phosphorylated PKCδ (pPKCδ) in $h\beta^S/h\beta^S$ mice was attenuated by δV1-1. **P*<0.05, ***P*<0.01 *vs*. the "$h\beta^A/h\beta^A$" group, ##*P*<0.01 *vs*. the "$h\beta^S/h\beta^S$" group, n=3.

Figure S4



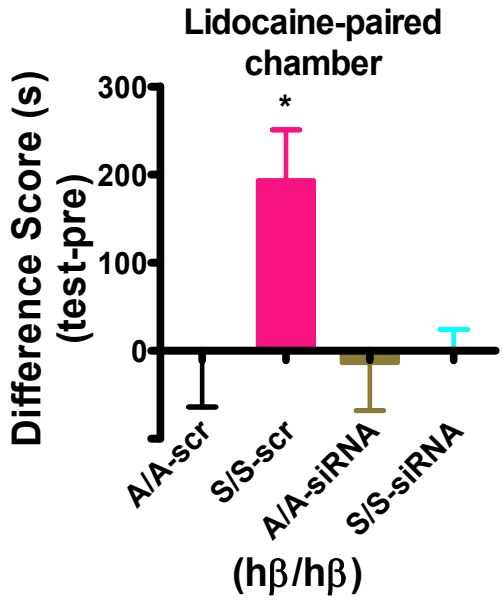
(**A**) Doubled-stained images of vesicular GABA transporter (VGAT, green) and PKCδ (red) in the spinal cord. (**B**) Doubled-stained images of vesicular glutamate transporter 2 (VGLUT2, green) and PKCδ (red) in the spinal cord. Scale bar: 20 μm, n=15 slices from 3 mice. In TOW ($h\beta^S/h\beta^S$) mice, cells with PKCδ translocation in the spinal cord are VGAT(+) GABAergic inhibitory neurons.

Figure S5



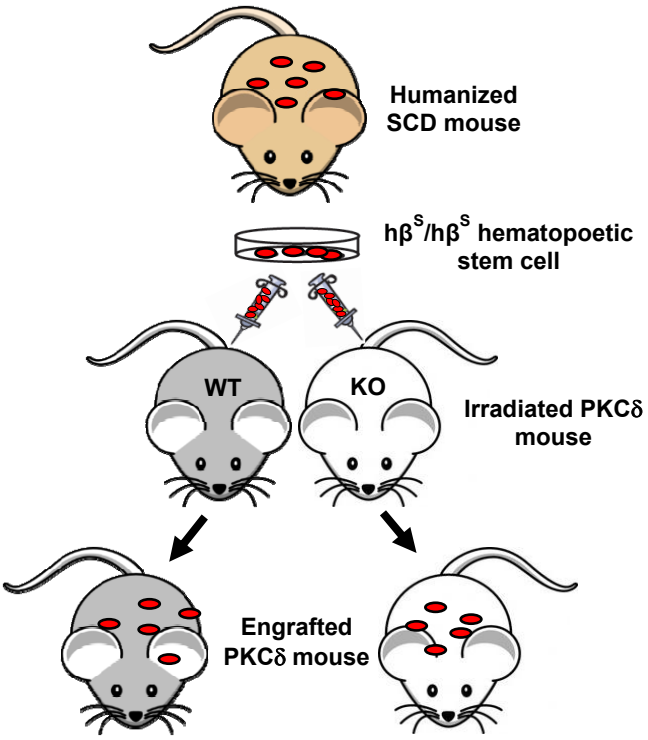
Neuronal specific knockdown of PKCδ was achieved by spinal delivery of RVG/PKCδ siRNA (2 μg, i.t., once per day, 3 day). Red: PKCδ, Green: NeuN. Scale bar: 20 μm, n=15 slices from 3 mice.

Figure S6



Difference score confirmed the absence of chamber preference in RVG/PKCδ siRNA treated TOW mice. TOW mice treated with RVG/scrambled (scr) siRNA exhibited significant difference scores. *P<0.05, n=6.

Figure S7

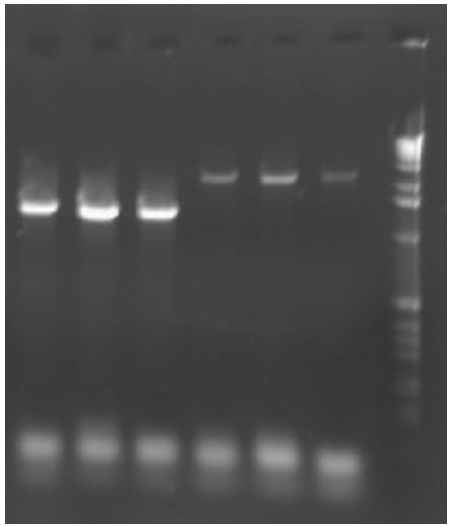


Hematopoietic stem cell transplant in PKCδ WT and KO mice.
PKCδ WT or KO mice received bone marrow transplant with donor hematopoietic stem cells from TOW ($h\beta^S/h\beta^S$) mice.

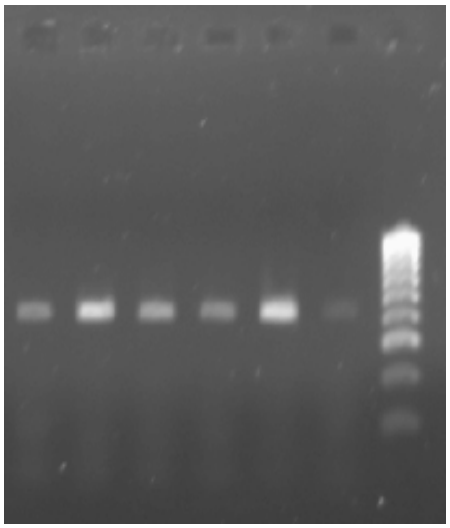
Figure S8

Pre-transplant

A. PKCδ

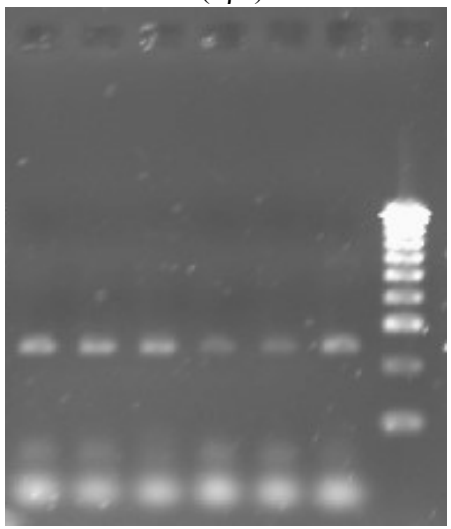


B. Mouse Hb ($m\beta^A$)

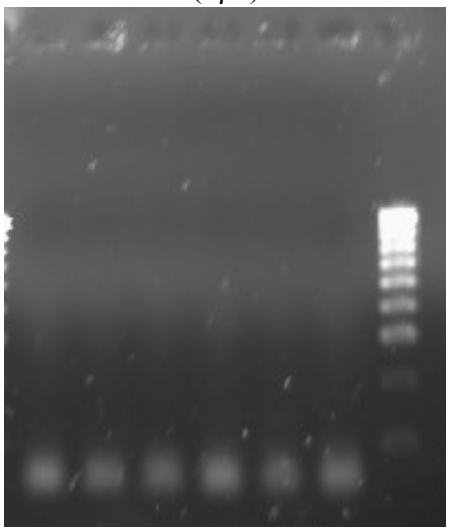


Post-transplant

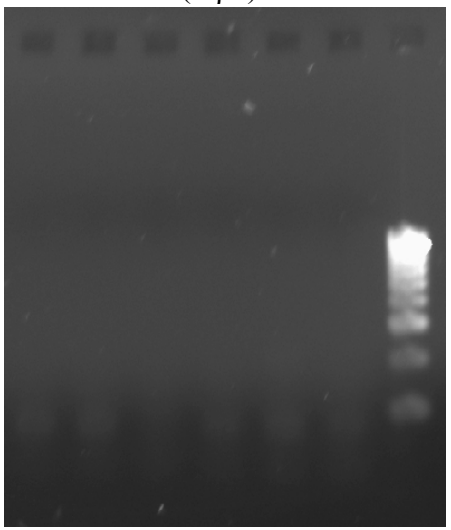
C. Human Hb ($h\beta^S$)



D. Human Hb ($h\beta^A$)



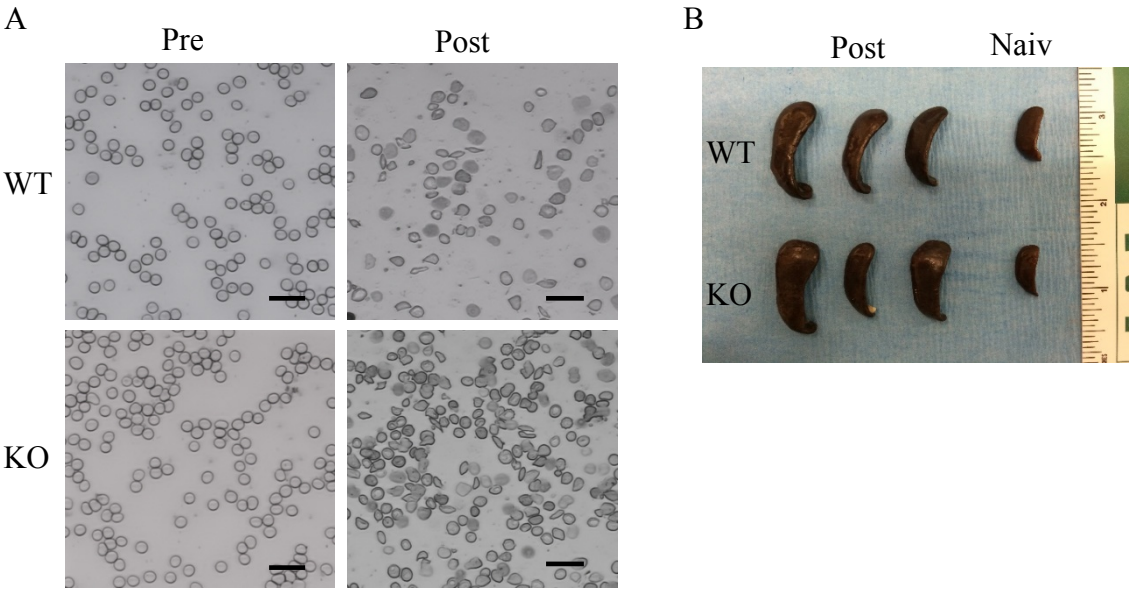
E. Mouse Hb ($m\beta^A$)



Replacement of the mouse β-globin gene ($m\beta^A$) with human sickle β-globin gene ($h\beta^S$) in PKCδ WT and KO mice after bone marrow transplantation

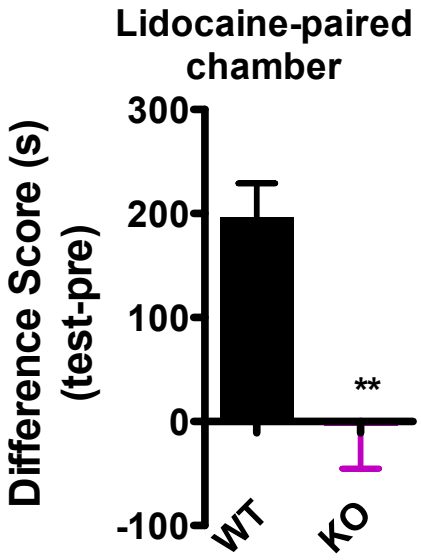
(**A**) PCR analysis of the DNA from peripheral blood of individual PKCδ WT and KO mice before bone marrow transplantation, (**B**) Both PKCδ WT and KO mice expressed mouse β-globin gene before transplantation. (**C**) Two weeks after the transplantation, mice engrafted with sickle hematopoietic stem cells from donor TOW $h\beta^S/h\beta^S$ mice, expressed human sickle β-globin gene. (**D**) Normal human β-globin gene ($h\beta^A$) was not present in the receipt mice. (**E**) Mouse β-globin gene ($m\beta^A$) was no longer expressed in the recipient mice.

Figure S9



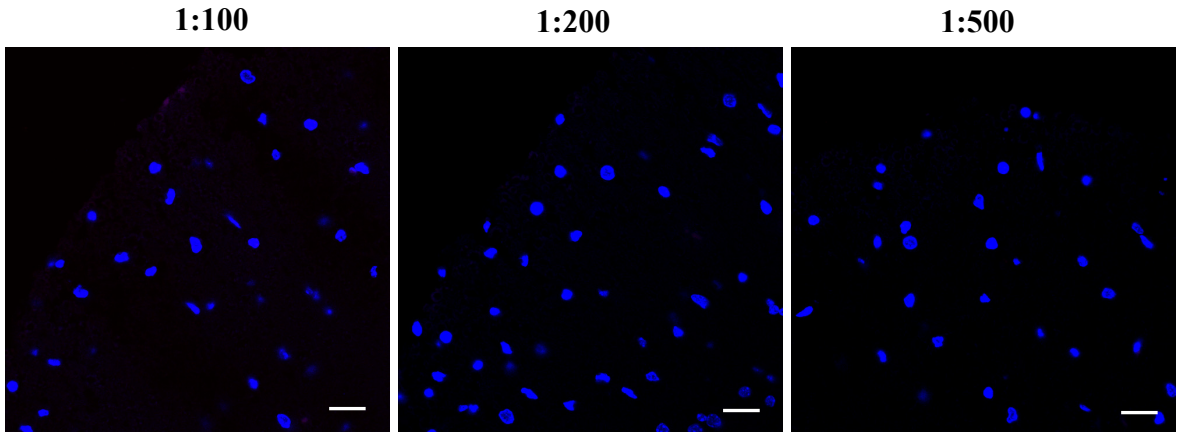
(**A**) Representative blood smears of PKCδ WT and KO mice before (Pre) and 4 weeks after transplantation (Post), n=3. (**B**) Splenomegaly was observed in both PKCδ WT and KO mice 4 weeks after transplantation.

Figure S10



Difference score confirmed the absence of chamber preference in PKCδ KO mice 4 weeks after transplantation by CPP paradigm. In comparison, PKCδ WT mice showed significant difference scores after bone marrow transplant with donor hematopoietic stem cells from TOW ($h\beta^S/h\beta^S$) mice. $^{**}P<0.01$, n=6.

Figure S11



Lack of immunoreactivity by the PKCδ antibody in the spinal cord of PKCδ-null mice.

These data helped to validate the specificity of the PKCδ antibody. Red: PKCδ, Blue: DAPI. Scale bar: 20 µm, n=15 slices from 3 mice.

Supplemental Table

Table S1. Hematological parameters of PKCδ null and wildtype mice before and 4 weeks after bone marrow transplantation

Mouse group (n=3)	HGB (g/dl)	RBC (10^6/µL)	Reticulocytes (%)	RDW (%)	MCV (fl)	PLT (10^6/µL)
WT Pre	12.25±1.05	9.70±0.56	3.00±0.22	12.35±0.15	42.95±0.85	1245.50±119.50
WT Post	6.30±0.90*	5.94±0.80*	40.75±2.98***	26.85±1.75***	53.30±1.00*	559.50±109.50**
KO Pre	12.90±1.50	8.99±0.73	3.27±1.78	13.35±0.55	43.45±2.05	1044.00±64.00
KO Post	6.55±0.55*	5.87±0.21*	46.64±3.82***	24.15±0.35***	52.40±2.80*	539.00±49.00*

Values represent the mean ± SD. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared with the "Pre" group.
HGB: Hemoglobin concentration; RBC: red blood cell count; RDW, red cell distribution width; MCV: mean corpuscular volume; PLT: Platelet count.

Reference

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