Pain-related behaviors

All behavioral tests were conducted in a quiet room maintained at a constant temperature of 23–25°C. Mice were allowed to acclimate to the testing apparatus for a minimum of 60 min prior to each set of measurements. All mice were familiarized with each testing paradigm by performing a set of repeat measurements before collecting baseline measurements. Three measurements were recorded for each mouse in each test, or as described. Behavioral tests were performed by two individuals in a blinded fashion.

Grip force. The grip force assay was used to determine whether mice exhibited deep tissue hyperalgesia. Forepaw grip force was measured using a computerized grip force meter (SA Maier Co., Milwaukee, WI) as described earlier.[1] Mice were held by the tail and grasped a wire-mesh floor with their forepaws. Grip force increased as mice were gradually pulled by the tail, until they were pulled away from the mesh. The peak force exerted (up to 1 kg) was recorded. Deep hyperalgesia was defined as a decrease in grip force.

Mechanical hyperalgesia. Paw withdrawal threshold and the frequency of withdrawal evoked by Calibrated von Frey (Semmes-Weinstein) monofilaments (Stoetling Co., Wood Dale, IL) were used to assess mechanical sensitivity. Mice were placed on a wire mesh table under a glass container (10 x 6.5 x 6.5 cm) and were allowed to acclimate for 30 min. Then, von Frey filaments were applied to the plantar surface of each hind paw, avoiding the toes, heel and pads, for 1–2 s with an inter-stimulus interval of at least 5 s. Only vigorous withdrawal responses were counted.

Paw withdrawal threshold was determined using the up-down method.[2,3] Briefly, a series of von Frey filaments, ranging from 0.4–8.0 g, were applied to the hind paws of hBERK1 and HbA-BERK mice. Ascending or descending stimuli were applied in a consecutive fashion starting with the 1.4 g monofilament that was in the middle of the series. In the absence of a paw withdrawal to a filament, a stronger stimulus was applied whereas in the presence of paw withdrawal, the next weaker stimulus was applied. The resulting pattern of positive and negative responses was tabulated using the convention: X = withdrawal; O = no withdrawal and the 50% response threshold was interpolated using the following formula:

50% g threshold = $(10^{[Xf+\kappa\delta]})/10{,}000*9.8$; where Xf = value (log units) of the final von Frey filament used, κ = tabular value for the pattern of positive/negative responses and δ = mean difference (log units) between stimuli and 9.8 is to convert g to mN (see Hamamoto et al[4]).

We also determined the frequency of withdrawal evoked by a standard monofilament (1.0 g; 4.08 mN) applied 10 times to the plantar hind paw. This monofilament evoked withdrawal responses less than 50% of the time in normal mice.

Heat hyperalgesia. A radiant heat stimulus was applied to the plantar surface of a single hind paw from underneath a glass floor with a projector lamp bulb (CXL/CXR, 8 V, 50 W).[5] Paw withdrawal latency (PWL) to the nearest 0.1 s was automatically recorded when the mouse withdrew its paw from the stimulus. A 20-s stimulus cutoff was used to prevent tissue damage. Test trials were separated by a minimum of 5 min.

Cold hyperalgesia. Mice were placed on a cold plate maintained at 4 ± 1˚C by a BAT10 Thermometer (Physitemp Inc., Clifton, NJ) for a period of 2 min. The latency in seconds to initial lifting of either fore-paw (and keeping it suspended mid-air for 1–2 seconds) was recorded as paw withdrawal latency (PWL); and the number of times mice lifted or rubbed the forepaws together in a 2-minute period was recorded as paw withdrawal frequency (PWF).

Catalepsy
The bar test[6] was used to determine whether catalepsy was produced following cannabinoid administration. Mice were placed with its forepaws on an elevated plastic bar (diameter = 1 cm) positioned 5 cm above and parallel to the counter top. The hind paws rested on the counter top. The time (in sec) each mouse spent in this position was recorded. Catalepsy was defined as an increase in time that mice remained on the bar.

Effect of opioids and cannabinoids on deep hyperalgesia
Morphine sulfate was dissolved in normal saline and HbA-BERK, BERK and hBERK1 mice were given 10 or 20 mg/kg (i.p.) in a volume of 100 µL. Grip force was determined before and at 1, 2, 4 and 24 h after injection.

The cannabinoid receptor agonist, (−)-cis-3-[2-hydroxy-4(1,1-dimethylheptyl)phenyl]-trans -4-(3-hydroxypropyl) cyclohexanol (CP 55,940; Tocris Bioscience, Ellisville, MO) was prepared in 5% emulphor, 5% ethanol and 90% normal saline. Separate groups of HbA-BERK and hBERK1 mice received 0.3 mg/kg CP 55,940 i.p. in a volume of 0.1 ml/10 g body weight. Grip force was determined before and at 0.5, 1, 1.5, 3, 6 and 24 h after injection.

Inflammatory hyperalgesia and modulation by peripheral cannabinoid receptors
Inflammation of the left hind paw was produced in male HbA-BERK and hBERK1 mice by intraplantar injection of 10 µg (in 10 µL) Complete Freund's Adjuvant (CFA; Sigma-Aldrich, St Louis, MO). Withdrawal response frequency evoked by the application of the 4.0 g von Frey monofilament (see above) was determined before and 24 h after CFA. Mice then received an intraplantar injection of 10 µg CP 55,940 (in 10 µl) or vehicle and paw withdrawal frequencies and time spent on the bar (bar test) were determined 1, 3, and 6 h after injection.

Histology, immunofluorescent microscopy and laser scanning confocal microscopy
Skin flaps from the back of hBERK1 and HbA-BERK mice were fixed in Zamboni's solution (0.03% w/v picric acid and 2% w/v paraformaldehyde) for 48 hours at 4°C, then transferred to a 20% sucrose solution with 0.05% sodium azide in PBS.[7]
For histology, 6 µM thick sections were stained with hematoxylin and eosin. Skin histology was visualized using a light microscope (Model: BX4ITF; Olympus Optical Co. Ltd., Japan), and thickness of different layers of skin was measured using a micrometer (Olympus Optical Co. Ltd., Japan). Measurements were made at 3 different areas of each section at a magnification of X200 by substituting 1 unit for 0.5 µM as per the calibration. Photomicrographs were acquired at a magnification of X100 and X400 using an Aperio ScanScope CS digital scanner (Aperio, Vista, CA).

For Laser Scanning Confocal Microscopy, 100 μM thick sections were immunostained using following primary antibodies: Lymphatic vessels: with 1:500 goat anti-LYVE 1 (R&D, Minneapolis, MN); Blood vessels with 1:200 rat anti-CD31 (Santa Cruz Biotechnology Inc., Santa Cruz, CA) and peripheral nerves with 1:1000 rabbit anti-PGP 9.5 (Biogenisis Inc., Kingston, NH); 1:200 anti-CGRP (Abcam, Cambridge, MA) and 1:200 Substance P (Serotec, Raleigh, NC). This was followed by staining with species specific secondary antibodies labeled with Cy2, Cy3 and Cy5 (Jackson Immunoresearch, West Grove, PA). In parallel, control primary antibodies were substituted with isotype matched IgG. A montage of overlapping fields of view was prepared from the Z-stack images of 1.62 μM each, acquired using an Olympus FluoView FV1000 (Center Valley, PA).

For immunofluorescent microscopy, 6 μM thick cryosections of mouse skin were immunostained with 1:100 dilution of anti-MOR (Chemicon International, Temecula, CA), endothelial cell marker anti–CD31-PE (1:50 dilution; Pharmingen, San Diego, CA) and the nuclear stain DAPI (Molecular Probes, Eugene, OR), as described by us previously.[8] Images were acquired using a digital camera equipped with an epifluorescent microscope (Olympus, Tokyo, Japan). Isotype matched primary antibody controls included: rabbit anti-mouse IgG and rat anti-mouse IgG (Jackson Immunoresearch Labs).

Western blotting

Brain and whole spinal cord lysates containing 50 μg protein were resolved on a 3–15% gradient SDS-PAGE gel and transferred to a polyvinylidene difluoride membrane (Immobilon P; Millipore, Bedford, MA). For immuno-blotting we used antibodies to phospho-p38 Mitogen-Activated Protein Kinase (p38 MAPK− Thr180/Tyr182), total p38, extra-cellular signal-regulated kinases (MAPK/ERK− Thr202/Tyr204), total p44/42 MAPK/ERK; phospho-signal transducer and activator of transcription-3 (STAT3− Tyr705) and total STAT3 (79D7) (all from Cell Signaling Technology, Beverly, MA). The immuno-reactive proteins were visualized with ECF Western blotting system (Amersham Life Sciences, Buckinghamshire, United Kingdom), and chemi-luminescent signals were acquired using a Storm 860 PhosphoImager (Molecular Dynamics, Sunnyvale, CA).[9] Densitometric analysis of the protein bands was performed using Molecular Analyst Software (Molecular Bioscience group, Hercules, CA).

RT-PCR

Total RNA was isolated from the whole spinal cords and brains of the mice using Trizol reagent and 5 μg RNA was reverse transcribed using first-strand synthesis system (Invitrogen, Carlsbad, CA).[10] PCRs were performed using Taq DNA polymerase (Continental Lab. Products, San Diego, CA). The sequences of primers were:

Cyclo-oxygenase-2 (COX-2): (GenBank acc. # NM_011198.3): Sense: 5′-ACT CAC TCA GTT TGT TGA GTC ATT-3′, Anti-sense: 5′-TTTGATTAGTACTGTAGGGTTAAT-3′

MOR-1: (GenBank acc. # U26915.1): Sense: 5′-AGA GGA AGA GGC TGG GGC G-3′, Anti-sense: 5′-CAT ACA TGA CCA GGA AGT TTC CAA AG-3′

Interleukin-6 (IL-6): (Genbank acc. # NM 031168.1): Sense: 5′-ATG AAG TTC CTC TCT GCA AG-3′, Anti-sense: 5′-CTG TAT CTC TCT GAA GGA CT-3′

Toll-like receptor 4 (TLR-4): (GenBank acc. # NM_021297.2): Sense: 5′-GGT GGC TGT GGA GAC AAA AT-3′, Anti-sense: 5′-AAT TCC CTG AAA GGC TTG GT-3′

GAPDH (GenBank acc. # NM_008084.2): Sense: 5′-AAC TTT GGC ATT GTG GAA GGG CTC-3′, Anti-sense: 5′-AGA TGC CTG CTT CAC CAC CTT CTT-3′

Amplification was performed for 30 cycles at 94°C for 50 s, 56°C for 50 s and 72°C for 50 s with a final extension cycle for 10 min at 72°C in a PTC-100 thermocycler (MJ Research, Waltham, MA). PCR products were resolved on a 1% agarose gel and visualized using ethidium bromide. Gels were scanned using a gel dock imaging system (Alpha Innotech, San Leonardo, CA), and densitometric analysis of the DNA bands was performed using Molecular Analyst Software (Molecular Bioscience group, Hercules, CA). The PCR products were sequenced at the Microchemical facility at the University of Minnesota to verify that they matched the expected DNA sequences.

Cytokine assays

Sera from hBERK1 and HbA-BERK mice were analyzed using the Magnetic Bead based Bio-Plex Assays (Bio-Rad Laboratories, Inc., Hercules, CA) for inflammatory cytokines including Interleukin 1α (IL-1α), Interleukin 6 (IL-6), Regulated upon Activation, Normal T cell Expressed and Secreted/Chemokine (C-C motif) Ligand 5 (RANTES/CCL5) and Tumor Necrosis Factor α (TNFα). The magnetic bead-based Bio-Plex assay platform uses a series of color-coded magnetic beads each coupled to a unique antibody specific for markers analyzed. Each magnetic bead was dyed with two fluorophores (classification dyes) that absorbed maximally at 635 nm and emitted at two distinct wavelengths. The capture antibody-coupled beads served as solid phases for the capture of analytes, followed by binding of a second biotinylated antibody in a sandwich-like assay. Quantitation was performed using the reporter dye, streptavidin-phycoerythrin, a fluorophore that absorbed maximally at 532 nm and emitted at a third distinct wavelength. The detector unit consisted of a flow cell that enabled magnetic beads to travel in a single file (laminar flow) through a region illuminated by a pair of lasers.

Statistical analysis

All data were analyzed using Prism software (v 5.0a, GraphPad Prism Inc., San Diego, CA). Students unpaired *t*-test was used to compare behavioral responses of HbA-BERK and hBERK1 mice in the grip force test, paw withdrawal threshold and frequency of withdrawal using von Frey monofilaments, withdrawal latency to radiant heat, latency and frequency of withdrawal responses to cold, time spent on the bar (bar test), and all morphometric and densitometric analyses. For comparing the von Frey responses of HbA-BERK and hBERK1 mice at 5, 8 and 15 mo, the one-way ANOVA with Bonferroni correction was used. For testing the effect of morphine and CP 55,940 on tonic hyperalgesia and effect of CP 55,940 on CFA induced inflammatory pain, each dependent measure was compared between treatment groups (morphine or CP 55,940 or vehicle) and across test times (including baseline) using a two-way repeated measures ANOVA with Bonferroni correction. A p-value of $< .05$ was considered significant. All data are presented as mean ± SEM.

Results

Effect of morphine and CP 55,940 on grip force in HbA-BERK mice: Both 10 and 20 mg/Kg dose of morphine had no effect on grip force in HbA-BERK (Fig. S1A). Similarly, 0.3 mg/kg CP 55, 940 showed no effect on grip force in HbA-BERK mice (Fig S1B). These mice do not have hyperalgesia and therefore both the analgesics had any effect on the grip force.

Effect of morphine CP 55,940 on catalepsy: The ip dose of 0.3 mg/Kg as well as local administration of 10 microg CP 55, 940, did not produce catalepsy in either HbA-BERK, BERK or hBERK1 (Figs S1C and D; and S2A and B), suggesting that these doses did not produce motor effects.

Epidermal and dermal thinning in sickle mice: The H & E staining of skin sections shows thinner epidermal and dermal layers in hBERK1 (S3 B and E) and BERK (S3 C and F) as compared to HbA-BERK (S3 A and D). Differences in thickness are more appreciable at higher magnification in Fig D–F.

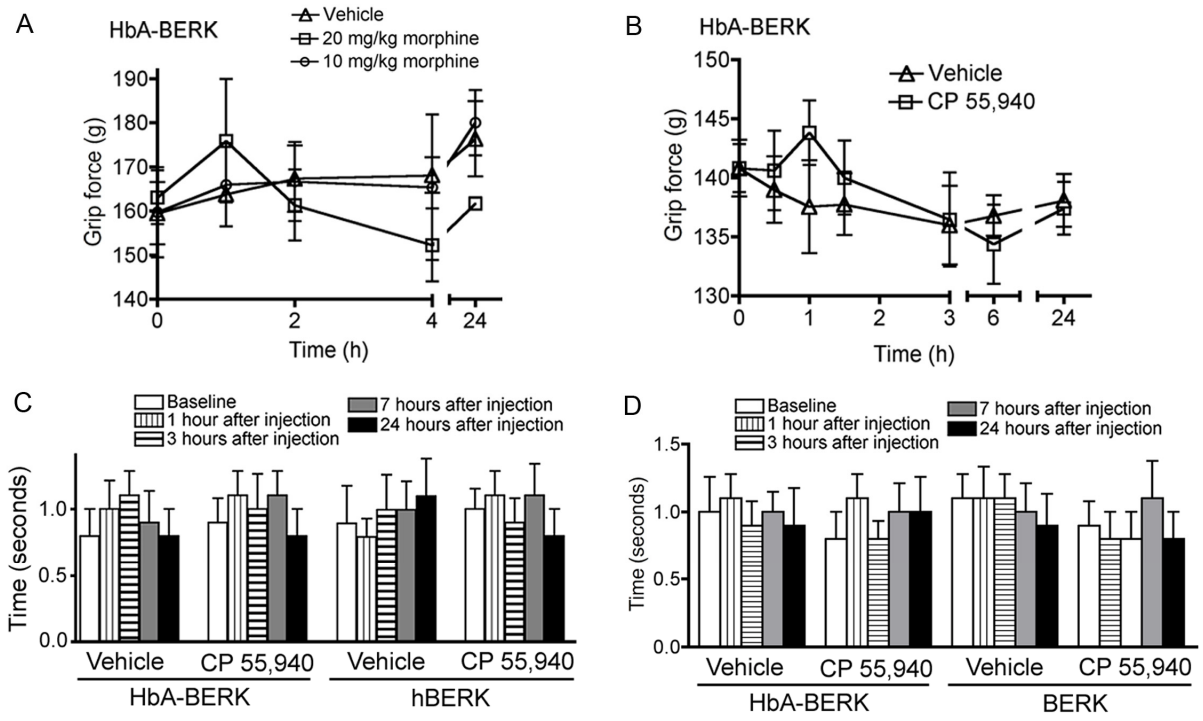

Figure S1. Effect of morphine and CP 55,940 on grip force, and evaluation for catalepsy
(A) In 8 month old HbA-BERK mice treated with morphine or vehicle. (B) In 8 month old HbA-BERK mice treated with CP 55, 940 or vehicle. (C and D) The bar test was used to assess for catalepsy in hBERK1 (C) and BERK (D) and age-matched HbA-BERK mice treated with CP 55,940 or vehicle (the cohort of mice in Fig. 3). All data are shown as mean ± SEM from 4–6 mice with 3 observations per mouse.

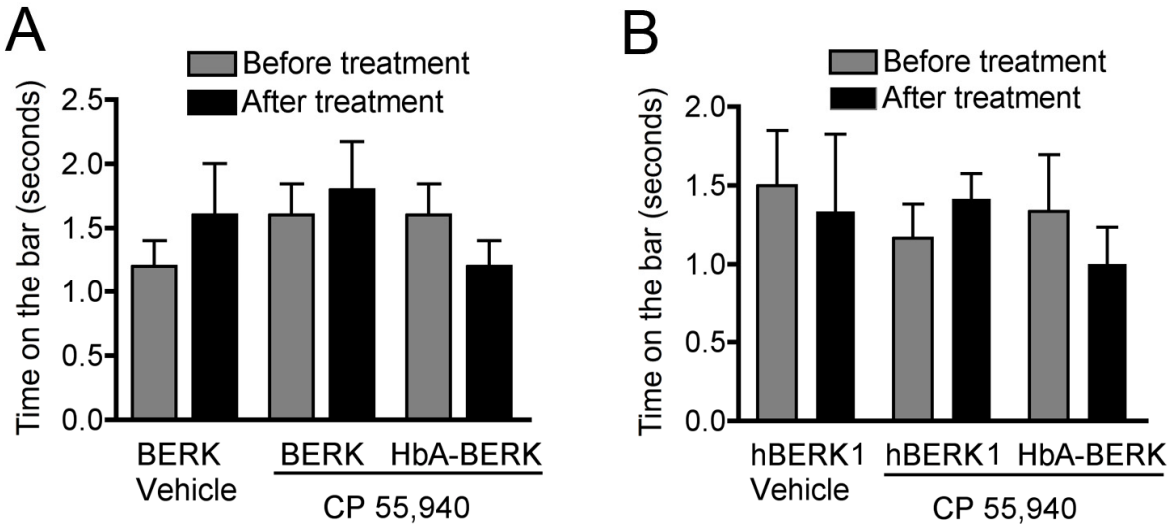

Figure S2. Evaluation for catalepsy

The bar test was used to assess catalepsy in mice injected with CFA and then treated with vehicle or CP 55,940. Catalepsy was measured 24 h after CP 55,940 or vehicle treatment, in the cohort of mice in Fig. 4. Data are shown as mean ± SEM from 4 mice with 3 observations per mouse.

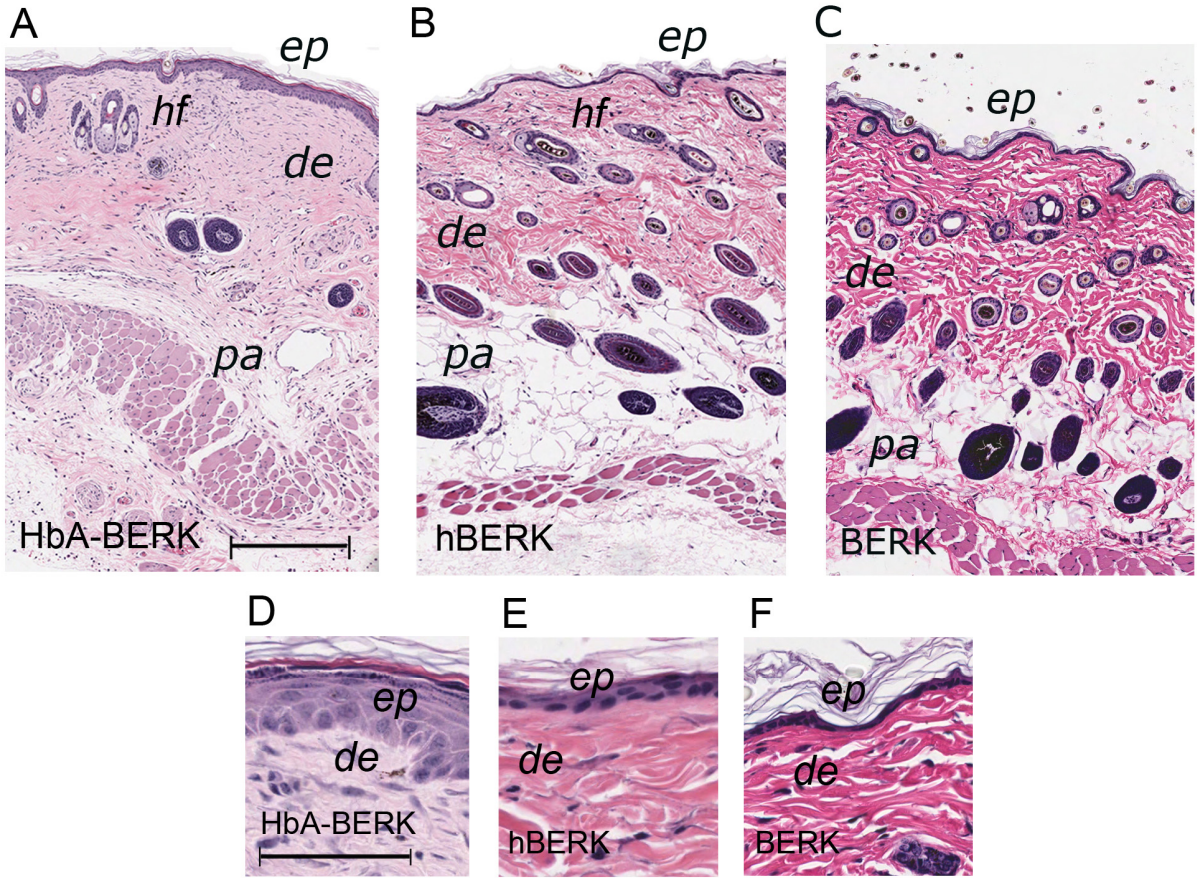

Figure S3. Epidermal and dermal architecture in BERK and hBERK1 mice
Photomicrographs of 6 µM thick skin sections from 5 month old male BERK and hBERK1 mice and age and sex matched HbA-BERK mice stained with hematoxylin and eosin are shown at magnifications of X100 (A–C) and X400 (D–F). hf: hair follicle, ep: epidermis, de: dermis, pa: panniculus. Scale bar: A–C, 100 µM and D–F, 50 µM.

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