SUPPLEMENTARY APPENDIX

Endothelin type A receptors mediate pain in a mouse model of sickle cell disease

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Supplemental Methods

Animals

The following mice were used in this work. Townes (HbSS and HbAA) and Berkeley (BerkSS and BerkAA) (Jackson laboratories Bar Harbor, ME) , Advillin$^{Cre/+}$ mice (provided by Dr. Fan Wang, Duke University Medical Center, Durham, NC) and $ET_A^{flox/flox}$ (provided by Dr. Masashi Yanagisawa, University of Texas Southwestern Medical Center, Dallas, TX) were bred in our animal breeding facility at Rutgers New Jersey Medical School. Animals were housed under a standard 12 h light/dark cycle with water and food available *ad libitum*. After accounting for age, sex, and genotype, mice were randomly allocated to each experiment.

Hypoxia/Reoxygenation:

Mice were exposed to 8% oxygen, 92% nitrogen for 3 hr inside a hypoxic chamber (Biospherix, Winston Salem, NC). Behavior testing (beginning with mechanical and ending with cold) was then conducted immediately after a 2 hr reoxygenation period. For ET_A inhibition studies, ABT-627 was administered immediately after hypoxia exposure, at the start of reoxygenation.

Conditioned Place Preference

For ongoing pain behavior analyses, a two chamber place-preference (CPP) model (MED Associates Inc., St. Albans, VT) with a manual separating door was used to identify preference for lidocaine according to Fig. S1. Baseline preference was assessed one day prior to conditioning with lidocaine, on day 4 of ABT-627 (10 mg/kg) or vehicle (14% alcohol in sterile water) administration. During this pre-test, the door was raised and mice were allowed to roam freely between the two chambers for 15 minutes. The time spent in each chamber was then recorded as the pre-test. Conditioning occurred over a 3 day period, in which saline (intrathecal, 10 µl) was paired with one chamber and lidocaine (intrathecal, 0.4%, 10 µl) with the other. After

3 days of conditioning, testing was conducted in the same fashion as the pre-test. Time spent in each chamber was then recorded and difference scores were calculated using the following formula: test time in lidocaine chamber – pre-test time in lidocaine chamber.

Genetic Knockdown and Bone Marrow Transplantation:

Mice were given antibiotics (15 mg/kg, Sulfamethoxazole with Trimethoprim, Qualitest Pharmaceuticals, Huntsville, AL) in drinking water for 1 week prior to irradiation. 2 month old Male $ETA^{flox/flox}$ and $ETA^{cre/flox}$ mice underwent total body irradiation following baseline behavioral testing. Irradiation was performed under the guidance of Rutgers Radiation Safety Committee. Mice received a split dose of 1200 cgy: 600 cgy in the morning and 600 cgy 3 hr later. Bone marrow from BerkSS and BerkAA mice was suspended in hank's balanced saline solution (5×10^6 cells in 100 µl) and delivered via tail vein to irradiated mice. Behavioral testing was performed 2 months after bone marrow transplantation. Given that mice receiving BerkSS bone marrow only expressed human β^s globin (βSS) (S8C), the phenotypes in these recipient mice are predicted to resemble those of the SCD mice. Thus, we assume that some organs (e.g. kidney and lung) are damaged although we did not analyze them.

Isoelectric focusing of hemoglobin

Following all behavioral testing, blood was collected from anesthetized bone marrow transplanted mice and analyze for sickle β globin expression using isoelectric focusing described in supplemental methods. 10 µl of blood was collected from a perpendicular incision on the tail and diluted in 100 µl of PBS. Diluted samples were then centrifuged at 2,000 rcf for 4 minutes at 4°C. The pellet was resuspended in 100 µl of Red Blood Cell Lysis solution (5mM KH_2PO_4 and 0.5 mM EDTA) and left on ice for 15 minutes. 10 µl of 10% NaCl was added to the sample before a final centrifugation at 16,000 rcf for 4 minutes at 4°C. Isoelectric focusing was then

performed using the Resolve Hemoglobin Kit (PerkinElmer, Wallac Oy, Turku, Finland) in accordance with the manufacturer's protocol. A GE Multiphor II Electrophoresis System was used and images were taken using a standard camera. Normal human β globin, human sickle β globin, and mouse β globin standards were used as controls.

Cell Culture

DRGs were collected from 3 week-old HbAA mice, cut using fine micro-scissors into small pieces and treated with enzyme solution (5 mg/ml dispase, 1 mg/ml collagenase type I in Hank's balanced saline solution (HBSS) without Ca^{2+} and Mg^{2+} (Gibco/ThermoFisher Scientific) for 20 minutes at 37 °C, with shaking every 5 minutes. After centrifugation at 1,000 rpm for 2 minutes, the enzyme solution was replaced with fresh Neurobasal medium (Gibco/ThermoFisher Scientific) with 10% fetal bovine serum (JR Scientific, Woodland, CA), 100 units/ml Penicillin, and 100 ug/ml Streptomycin (Quality Biological, Gaithersburg, MD). The cells were then filtered through a 70 µm sterile filter. The resulting solution was then plated into six-well plates pre-coated with 50 ug/ml poly-D-lysine (Sigma, St. Louis, MO). The cells were incubated in 95% oxygen and 5% carbon dioxide at 37 °C. The following drugs were added into the medium on the second day after plating. Endothelin 1 peptide (dissolved in autoclaved water; Tocris) at a concentration of 400 nmol per well was used. PDTC (dissolved in autoclaved water) at a concentration of 1 µmol per well was used. ABT-627 (dissolved in 0.2% DMSO) at a concentration of 1 µmol per well was used. The cells were collected 24 hr (for Nav1.8 analysis) or 90 mins (for p65 analysis) after drug administration. The cell viability was assessed based on morphology and cell density.

Immunoflourescence

Immunofluorescence was performed using bilateral L3-L4 DRG. Mice were deeply anesthetized with isoflurane and then perfused with 4% paraformaldehyde. DRG Sections (20μm in thickness) were incubated with the following primary antibodies over two nights at 4°C: rabbit anti-Pre Pro ET-1 (1:1500, Bioss), rabbit anti- ET_A receptor (1:1500, Abcam), rabbit anti-Nav1.8 (1:1,500, Abcam), mouse anti-CGRP (1:500, Abcam), mouse anti-NF200 (1:800, Sigma-Aldrich), and biotinylated anti-IB4 (1:300, Sigma-Aldrich). Sections were incubated for 2 hr at room temperature in avidin-labeled with Rhodamine Red (1:200, Invitrogen), Cy2-conjugated goat anti-rabbit (1:200, Jackson ImmunoResearch, West Grove, PA), or Cy3-conjugated goat anti-mouse (1:200, Jackson ImmunoResearch, West Grove, PA). Sections were analyzed using a Leica DMI4000 Fluorescence microscope equipped with a DFC365FX camera (Leica, Germany). Labeled neurons were quantified using Image J Software.

Western blotting

Bilateral L3-L4 DRG from one mouse were collected and combined for one repeat. A 5 mm hindpaw skin punch from each hindpaw was collected and combined for each mouse. After the tissues were homogenized, Western blotting was carried out according to our previously published protocol[1]. Blots were incubated in the following antibodies overnight at 4^o C: rabbit anti-ETA receptor (1:1,000, Novus), rabbit anti-GAPDH (1:1,000, Santa Cruz Biotechnology), rabbit anti-Nav1.8 (1:1,000, Abcam), rabbit anti-NF-κB-p65 (1:1000, Cell Signaling technologies) and rabbit anti-phosphorylated (Ser 536) NF-κB - p65 (1:1000, Cell Signaling technologies), rabbit anti-ET1 (1:1,000, Abcam), mouse anti-β-actin (1:2,000; Santa-Cruz Biotechnology). Proteins were detected using horseradish peroxidase-conjugated anti-mouse or anti-rabbit secondary antibody (1:2,000 Jackson immunoresearch) and visualized with peroxide reagent and luminol/enhancer reagent (Clarity Western ECL Substrate, Bio-Rad) using a

ChemiDoc XRS System (Bio-Rad). Densitometry in Image lab software (Bio-Rad) was used to analyze blot intensity. Proteins were normalized to corresponding GAPDH or β-actin.

Quantitative reverse transcription polymerase chain reaction (RT-qPCR)

Bilateral L3-L4 DRG from one mouse were combined in RNAlater (Ambion, Austin, TX) for one repeat. Total RNA was extracted using the miRNeasy kit (QIAGEN, Valencia, CA) in accordance with the manufacturer's protocol. A NanoDrop 2000 spectrophotometer (Thermo Scientific, Wilmington, DE) was used to measure RNA concentration. Qubit Fluorometric Quantitation (Invitrogen, Carlsbad, CA, USA) A260/280 nm ratios fell between 1.78 and 1.96. RNA was reverse transcribed using random hexamers, oligo (dT) primers, and ThermoScript reverse transcriptase (Invitrogen/ThermoFisher Scientific). 1 µl of template cDNA was then amplified using the primers (Integrated DNA Technologies) listed in Supplemental Table 2. Each repeat was run in triplicate, in 20 µl reactions containing the following: 250 nM forward and reverse primers, 10 µL SsoAdvanced Universal SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA), and 20 ng of amplified cDNA. PCR was performed using a BIO-RAD CFX96 real-time PCR system. Data was normalized to *α-tubulin* and the ratio of HbSS mRNA to HbAA mRNA was calculated using the ΔCt method ($2^{-\Delta\Delta Ct}$).

Single cell reverse transcription PCR (RT-PCR)

Single Cell RT-PCR was carried out as described previously[1]. Briefly, the freshly cultured DRG neurons from naïve C57/BL6 mouse (Charles River Laboratories, Wilington, MA) were prepared. Four hours after plating, a single living small DRG neuron (< 20 µm) was collected under an inverted microscope using a micromanipulator and microinjector. Reverse transcription was performed using the Superscript VILO cDNA synthesis kit (ThermoFisher) according to the manufacturer's protocol. A pre-amplification PCR was performed for *Scn10a*, *Rela*, and *Ednra*

genes using the Q5 High Fidelity DNA Polymerase kit (NEB, Ipswich, MA) and primers listed in Supplemental Table 2. Pre-amplification product and cDNA for *Gapdh* gene were then carried for standard PCR as described above using primers listed in Supplemental Table 2 followed by agarose gel-electrophoresis.

Chromatin immunoprecipitation (ChIP)

Two NF-κB binding motifs (-416 to -406 and -364 to -354 in relation to the TSS) in the *Scn10a* promoter region were identified using JASPAR database and primers flanking both motifs were designed. ChIP assays were performed using the Magna EZ ChIP-G kit (EMD Millipore, Darmstadt, Germany) according to the manufacturer's protocol using bilateral L2-L4 DRG. Immunoprecipitation was performed overnight at 4 °C using protein G magnetic beads, 2 µg of rabbit anti-NF-κB-p65 antibody (Cell signaling technologies) or 2 µg of normal mouse IgG. DNA fragments were purified and identified using RT-PCR with primers found in Supplemental Table 2.

Luciferase assay

A 593 bp fragment of the mouse *Scn10a* promoter region (including two NF-κB-binding motifs) was amplified from genomic DNA using PCR primers listed in Supplemental Table 2. The PCR product was ligated into a pGL3-Basic vector (Promega, Madison, WI) upstream of a *firefly* luciferase reporter gene using KpnI and HindIII restriction sites. Dual Luciferase assay was performed using transfected CAD cells (a mouse neuronal cell line[2]) as previously described[3]. 1 day after transfection the following drugs were added to the medium for 6 hours: PMA (20 µM, Sigma-Aldrich), PDTC (1 µM, Tocris), BIM (1 µM, Sigma-Aldrich), and vehicle (DMSO, 0.2%, Sigma-Aldrich). Drug dosages were based on previous studies[4-6].

Whole-cell patch clamp recording

To record sodium channel 1.8 current in DRG neurons, we first prepared freshly dissociated mouse DRG neurons as previously described[7-8]. Whole-cell patch clamp recording was carried out 4 to 10 h after plating. Only small DRG neurons were recorded. The electrode resistances of micropipettes ranged from 3 to 5 MΩ. Neurons were voltage-clamped with an Axon 700B amplifier (Molecular Devices, Sunnyvale, CA). The intracellular pipette solution contained (in mM) 140 CsF, 1 $MgCl_2$, 1 EGTA, 5 Na_2ATP, 10 HEPES, CsOH to adjust the pH to 7.2. The extracellular solution composed of (in mM) 32 NaCl, 20 TEA-Cl, 105 Choline chloride, 1 $MgCl_2$, 1 $CaCl_2$, 0.1 $CdCl_2$, 10 HEPES, 10 D-gluose and NaOH to adjust the pH to 7.4. Signals were filtered at 1 kHz and digitized by using a DigiData 1550 with pClamp 10.4 software (Molecular Devices). Series resistance was compensated by 60–80%. Cell membrane capacitances were acquired by reading the value for whole-cell capacitance compensation directly from the amplifier. An online P/4 leak subtraction was performed to eliminate leak current contribution. The data were stored on computer by a DigiData 1550 interface and were analyzed by the pCLAMP 10.4 software package (Molecular Devices).

To record the action potentials, we switched the recording mode into current clamp. The extracellular solution consisted of (in mM) 140 NaCl, 4 KCl, 2 $CaCl_2$, 2 $MgCl_2$, 10 HEPES and 5 glucose and pH adjusted to 7.38 by NaOH. The intracellular pipette solution contained (in mM) 135 KCl, 3 Mg-ATP, 0.5 Na_2ATP, 1.1 $CaCl_2$, 2 EGTA and 5 glucose, pH was adjusted to 7.38 with KOH and osmolarity adjusted to 300 mOsm with sucrose. The membrane potential was held at the existing resting membrane potential during the current injection. The resting membrane potential was taken 3 min after a stable recording was obtained. Depolarizing currents of 100–1,400pA (200 ms duration) were delivered in increments of 100 pA until an action

potential (AP) was evoked. The injection current threshold was defined as the minimum current required to evoke the first AP. The AP threshold was defined as the first point on the rapid rising phase of the spike at which the change in voltage exceeded 50 mV/ms. The AP amplitude was measured between the peak and the baseline. The membrane input resistance for each cell was obtained from the slope of a steady-state I–V plot in response to a series of hyperpolarizing currents, 200-ms duration delivered in steps of 100 pA from 200 pA to −2,000 pA. The after-hyperpolarization amplitude was measured between the maximum hyperpolarization and the final plateau voltage, and the AP overshoot was measured between the AP peak and 0 mV. To record spontaneous activity, neurons were placed in the chamber and perfused with extracellular solution with temperature control at 37℃ with TC-344C (Warner Instruments) and recorded 5 min in gap free protocol. The data were stored on computer by a DigiData 1550 interface and were analyzed by the pCLAMP 10.4 software package (Molecular Devices).

In Vivo Drug Administration

R-(4-methoxyphenyl)-4S-(1,3-benzodioxol-5-yl)-1-(N,N-di(n-butyl)aminocarbonyl-methyl)-pyrrolidine-3R-carboxylic acid (ABT-627) (Abbott Laboratories) or vehicle (14% alcohol in sterile water) was injected at a dose of 5 nmol/10 µl (hindpaw injection) or 10 mg/kg (intraperitoneal, i.p). For dose-dependent effects of ABT-627, ABT-627 was delivered via a single hindpaw injection at various doses between 2.5 and 5 nmol. Ammonium pyrrolidinedithiocarbamate (PDTC, Tocris) or A-803467 (Sigma) was administered via i.p at a dose of 100 mg/kg. For the effect of the Nav1.8 inhibitor on SCD pain behaviors, HbAA and HbSS mice received either vehicle (95% MEP400 and 5% DMSO) or A-803467 (100 mg/kg) intraperitoneally according to a previous study[9]. Behavior testing was conducted 30 minutes after injection.

Statistical Analysis

Results are presented as means ± SEM. Data was analyzed using a Student's t test, one way or two-way ANOVA, or one-way or two-way repeated measures ANOVA, or one-way ANOVA on Ranks. Pairwise comparisons between means were tested using the post hoc Tukey test or Dunn's test (SigmaPlot 12.5, San Jose, CA). Significance was set at $p < 0.05$, with 95% confidence intervals. Two-sided p-values are reported for student's T-tests. In cases where a repeated measures ANOVA analysis was conducted for behavioral tests, the same animals were tested across multiple timepoints/treatments. For molecular assays, at least 3 biological replicates were conducted. No corrections for multiple comparisons were made.

References for Supplemental Methods

1. Liang,L, Zhao J.Y, Gu X, et al. G9a inhibits CREB-triggered expression of mu opioid receptor in primary sensory neurons following peripheral nerve injury. Molecular Pain. 2016; **12**.

2. Qi,Y., Wang,J.K.T., McMillian,M, Chikaraishi,D.M. Characterization of a CNS Cell Line, CAD, in which Morphological Differentiation Is Initiated by Serum Deprivation. J. Neurosci. 1997; **17**,1217.

3. Zhao,J.Y, Liang L, Gu X, et al. DNA methyltransferase DNMT3a contributes to neuropathic pain by repressing Kcna2 in primary afferent neurons. Nat Commun. 2017;**8**, 14712.

4. Kaltschmidt,B, Linker,R.A, Deng,J, Kaltschmidt,C. Cyclooxygenase-2 is a neuronal target gene of NF-kB. BMC Mol Biol. 2002;**3**, 16.

5. Ivan,A.L.M, Campanini M.Z, Marinez R.M, et al. Pyrrolidine dithiocarbamate inhibits UVB-induced skin inflammation and oxidative stress in hairless mice and exhibits antioxidant activity in vitro. Journal of Photochemistry and Photobiology B: Biology. 2014;**138**, 124-133.

6. Bhave,G, Hu H.J, Glauner K.S, et al. Protein kinase C phosphorylation sensitizes but does not activate the capsaicin receptor transient receptor potential vanilloid 1 (TRPV1). Proceedings of the National Academy of Sciences **100**, 12480-12485 (2003).

7. Sun,L, Zhao J.Y, Gu X, et al. Nerve injury-induced epigenetic silencing of opioid receptors controlled by DNMT3a in primary afferent neurons. PAIN **158**, (2017).

8. Zhao,X, Tang Z, Zhang H, et al. A long noncoding RNA contributes to neuropathic pain by silencing Kcna2 in primary afferent neurons. Nat Neurosci **16**, 1024-1031 (2013).

9. Jarvis M.F, Honore P, Shieh C, et al. A-803467, a potent and selective Nav1.8 sodium channel blocker, attenuate neuropathic and inflammatory pain in the rat. PNAS **104**, 8520-8525 (2007).

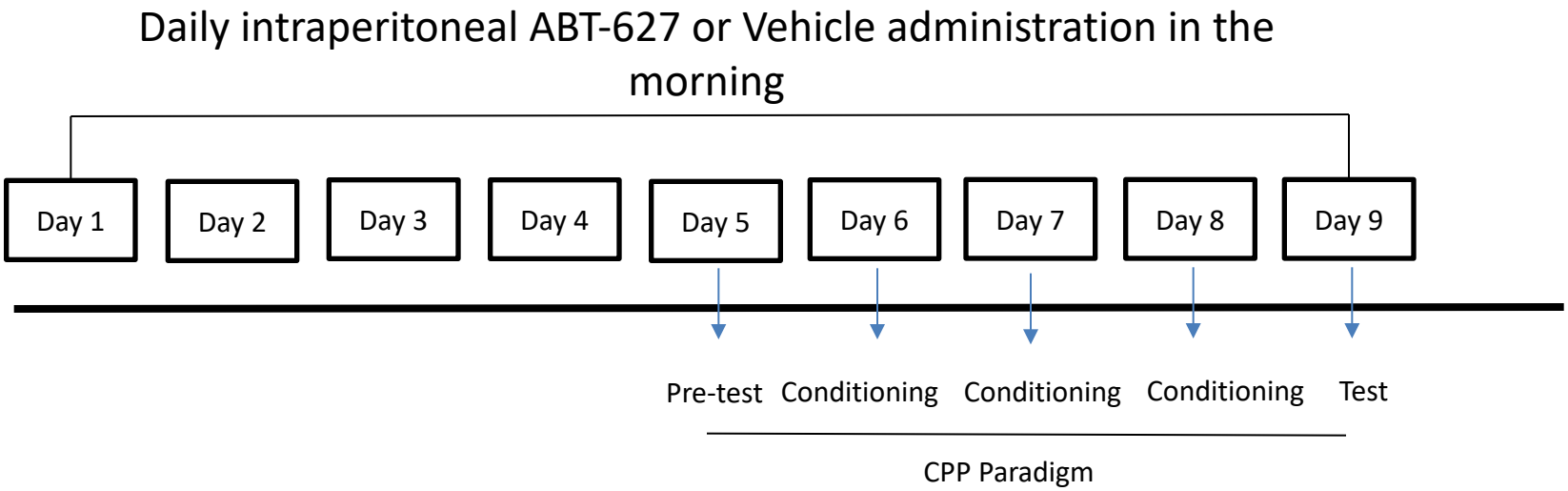
Supplemental Table 1. Action Potential Parameters of HbSS (SCD) and HbAA (Control) DRG Neurons

	Large Cell			Medium Cell			Small Cell		
	Control	SCD	t/p value	Control	SCD	t/p value	Control	SCD	t/p value
n	24cells,7mice	25cells,6mice		22cells,7mice	23cells,6mice		21cells,7mice	25cells,6mice	
Rin, MΩ	46.61±2.82	45.47±1.85	-0.342/0.734	51.99±3.02	51.41±2.75	0.141/0.888	54.65±3.08	55.69±2.91	0.246/0.807
APT, mV	-17.20±1.45	-16.71±1.19	0.256/0.799	-16.10±1.23	-15.60±1.28	-0.277/0.783	-16.88±0.99	-16.78±1.21	0.063/0.950
APO, mV	37.08±2.35	37.54±2.79	0.123/0.902	35.77±2.53	41.05±2.81	-1.390/0.172	41.00±3.37	46.08±2.99	1.130/0.264
APA, mV	97.52±2.07	98.49±2.81	0.274/0.785	95.53±2.67	90.96±2.79	1.178/0.245	99.40±3.39	96.25±2.83	-0.718/0.477
AHPA, mV	-12.11±0.91	-11.87±0.90	0.193/0.848	-17.04±1.21	-16.45±0.99	-0.380/0.706	-15.63±1.03	-15.42±1.01	0.149/0.882

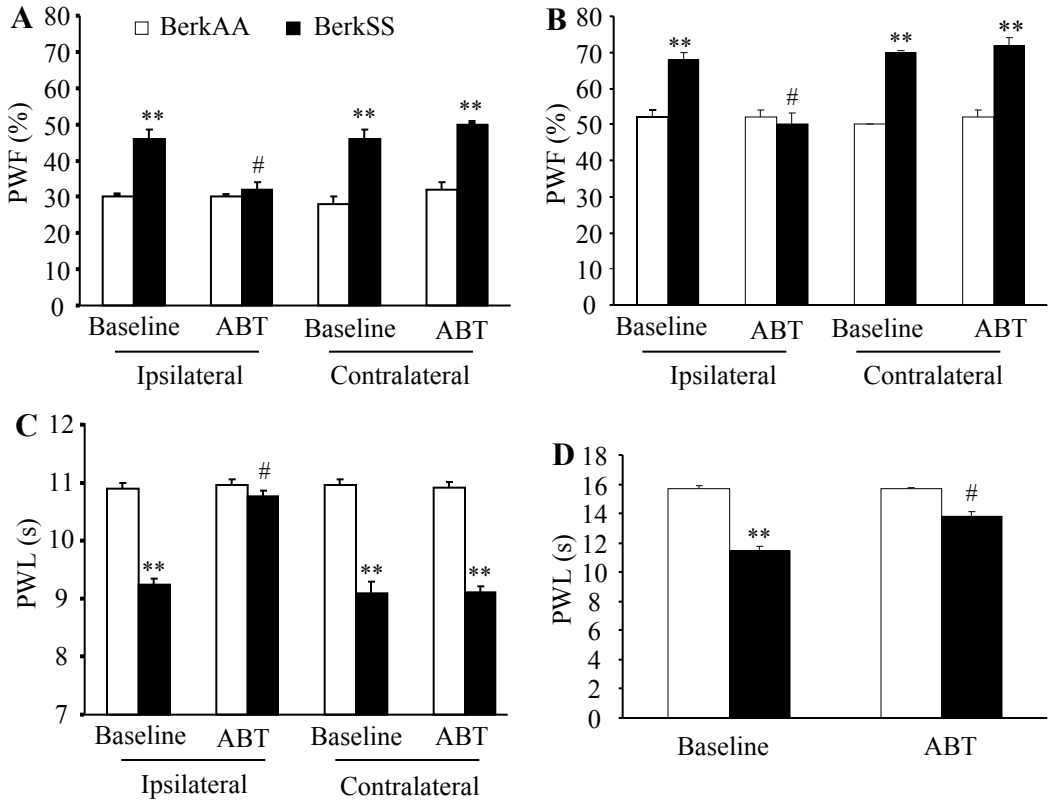
Values are Mean ± S.E.M., R_{in}: membrane input resistance. APT: action potential threshold. APA: action potential amplitude. APO: action potential overshoot. AHPA: afterhyperpolarization amplitude. All values are mean ± S.E.M. student t-test.

Supplemental Table 2. Primers used for molecular assays

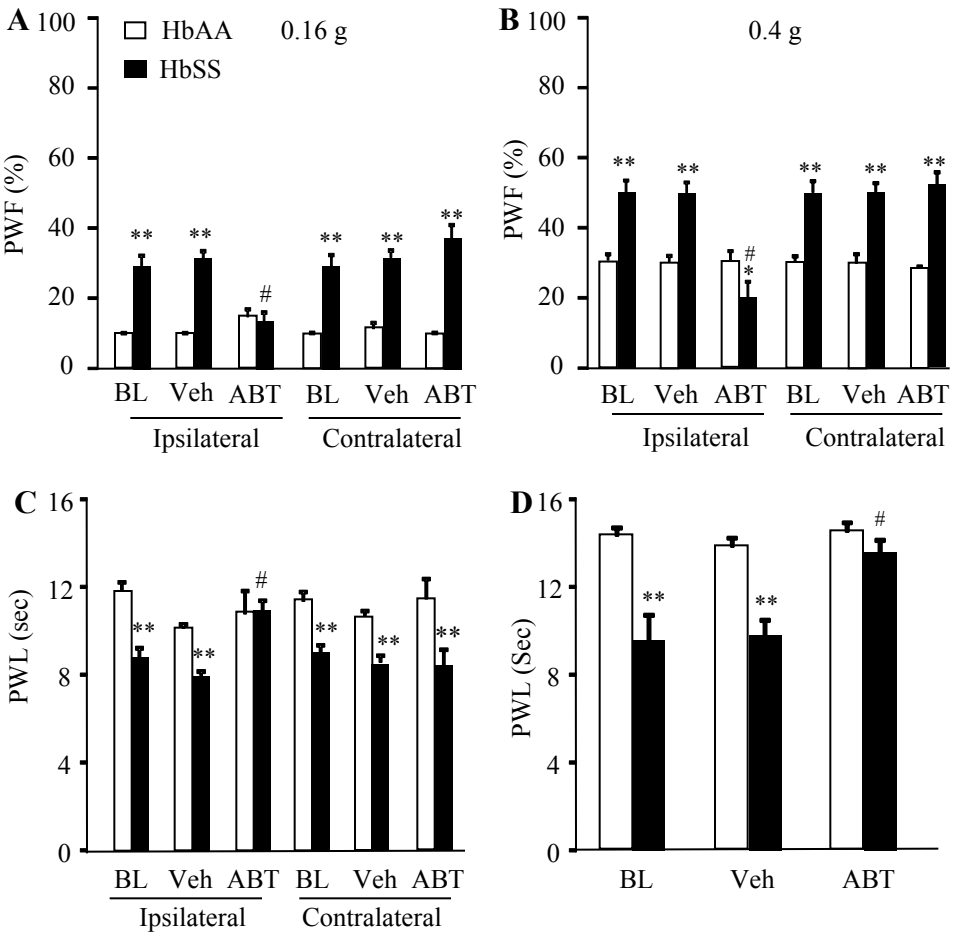
Gene	Primer Sequence	Assay
Edn1	F: 5'-TTCCCGTGATCTTCTCTCTGC-3' R: 5'-CTGCACTCCATTCTCAGCTCC-3'	RT-PCR
Ednra	F: 5'-TGGCCCTTGGAGACCTTATC-3' R: 5'-AGGTTCAAGACGGTGATGCC-3'	RT-PCR, Single Cell RT-PCR
Rela	F: 5'- TCTGCTTCCAGGTGACAGTG-3' R: 5'-ATCTTGAGCTCGGCAGTGTT-3'	RT-PCR, Single Cell RT-PCR
Scn10a	F: 5'-GTCCGTCCACTCCTGGTTCTC-3' R: 5'-TTAGACAAAACCCTCTTGCCAGT-3'	RT-PCR, Single Cell RT-PCR
Scn11a	F: 5'-ATAGCGAAGCCCCTGGAAGA-3' R: 5'-AAGACTGAATGGACAGAGATGCG-3'	RT-PCR
Ednra	F:5'-ATCAACTTTCTGGGCACCAC-3' R:5'-GGAAGCCACTGCTCTGTACC-3'	Pre-Amplification
Rela	F: 5'-GGCCTCATCCACATGAACTT-3' R: 5'-TGGTCCCGTGAAATACACCT-3'	Pre-Amplification
Scn10a	F: 5'-TACAGCACACACCGGACATT-3' R: 5'-GACCTTCAGTCCTGGGATCA-3'	Pre-Amplification
Scn10a	F: 5'-AAGCTGTAATGGGCAAGCAT-3' R: 5'-ACCCCAGTGAGAGCTGAGAA-3'	ChIP PCR
Scn10a	F: 5'-ATAGGTACCCCACCCAAATAGAGGGGATT-3' R: 5'-ATTAAGCTTCCAAGCCTGGCTCAGGCT-3'	Luciferase Assay



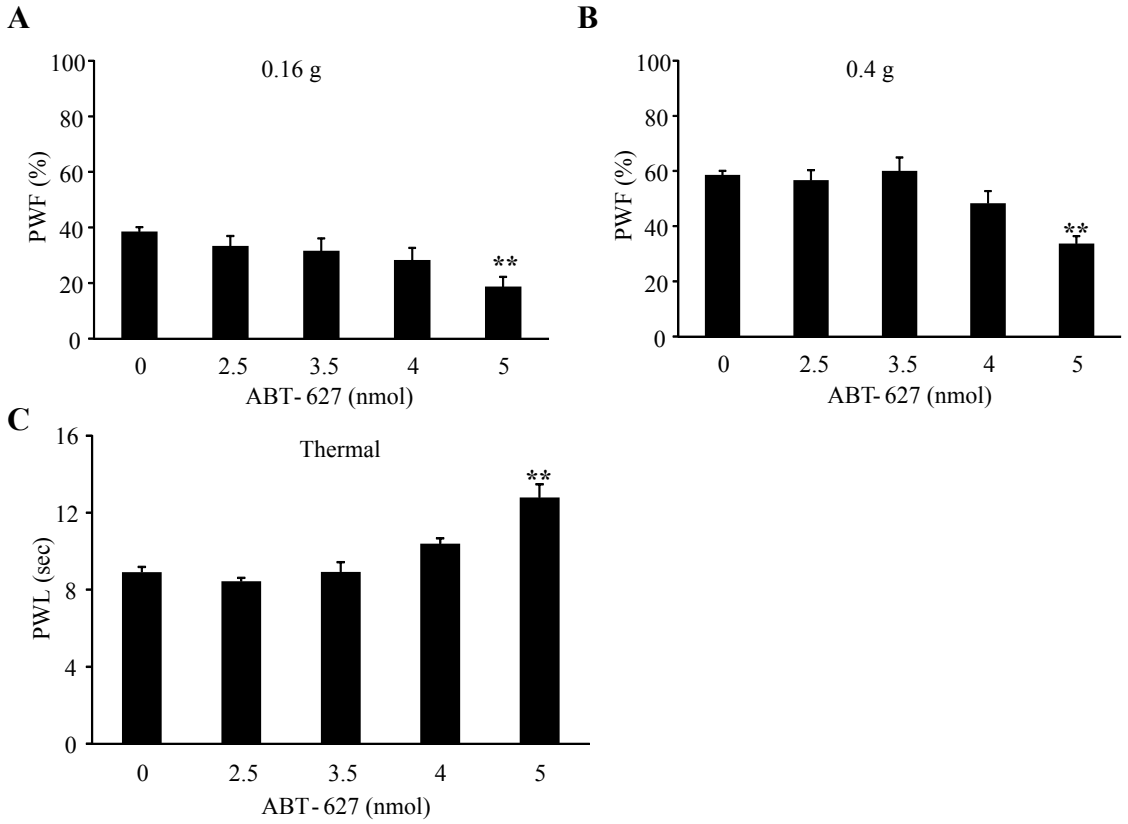
Supplemental Figure 1. CPP testing paradigm to analyze the effect of ET_A receptor inhibition on ongoing pain in HbSS and HbAA mice. HbAA and HbSS mice received daily intraperitoneal (i.p) injections of either vehicle (14% alcohol in sterile water) or ABT-627 (10 mg/kg) for 4 days prior to the start of the CPP paradigm and during the CPP paradigm. On conditioning days, mice received i.p injections of either vehicle or ABT-627 at least 1 hour before the start of conditioning.



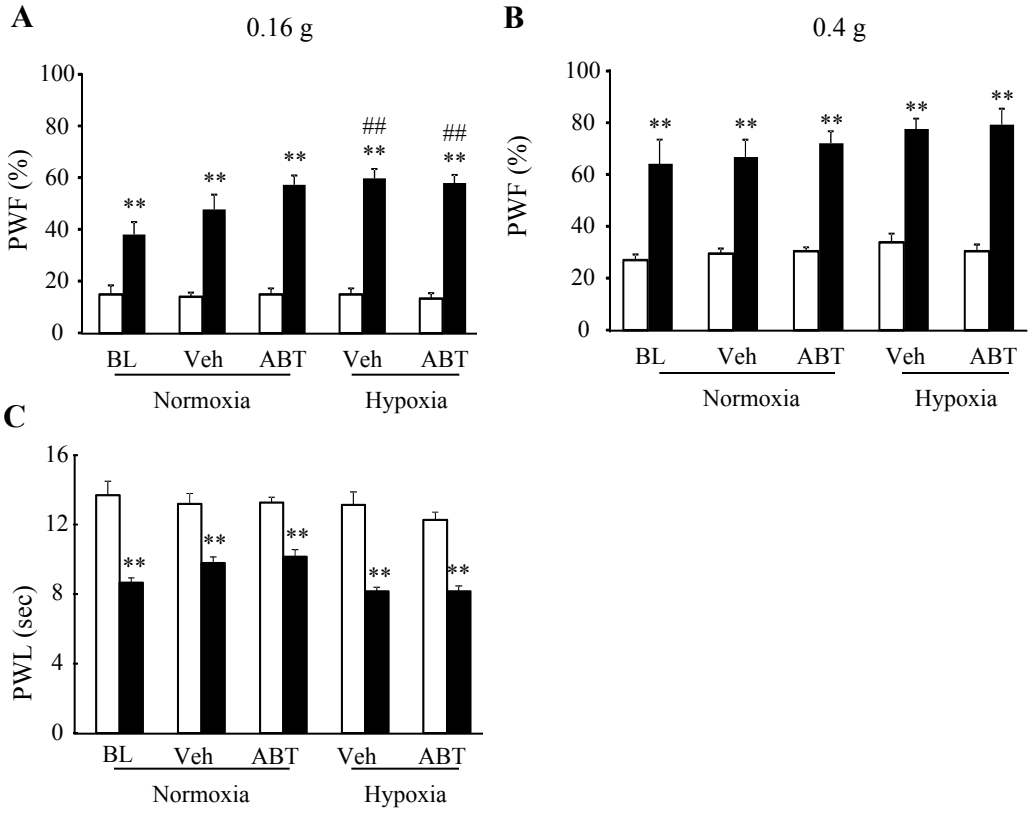
Supplemental Figure 2. Hind paw injection of ABT-627 attenuates pain hypersensitivity in male BerkSS mice. 6 month old male BerkSS mice display increased paw withdrawal frequencies (PWF) to a 0.16 g low force (A) and a 0.4 g medium force (B) von Frey filament and decreased paw withdrawal latencies (PWL) to thermal (C) and cold (D) stimuli. A single unilateral hindpaw injection of ABT-627 (ABT) alleviates pain hypersensitivity on the ipsilateral side of BerkSS mice (A-D). ABT-627 administration to BerkAA mice does not affect basal responses (A-D). n = 5 mice/genotype, two-way repeated measures ANOVA with post-hoc Tukey test, $^{**}p < 0.01$ vs the corresponding baseline of BerkAA mice. $\#p < 0.05$ vs the corresponding baseline of the BerkSS mice.



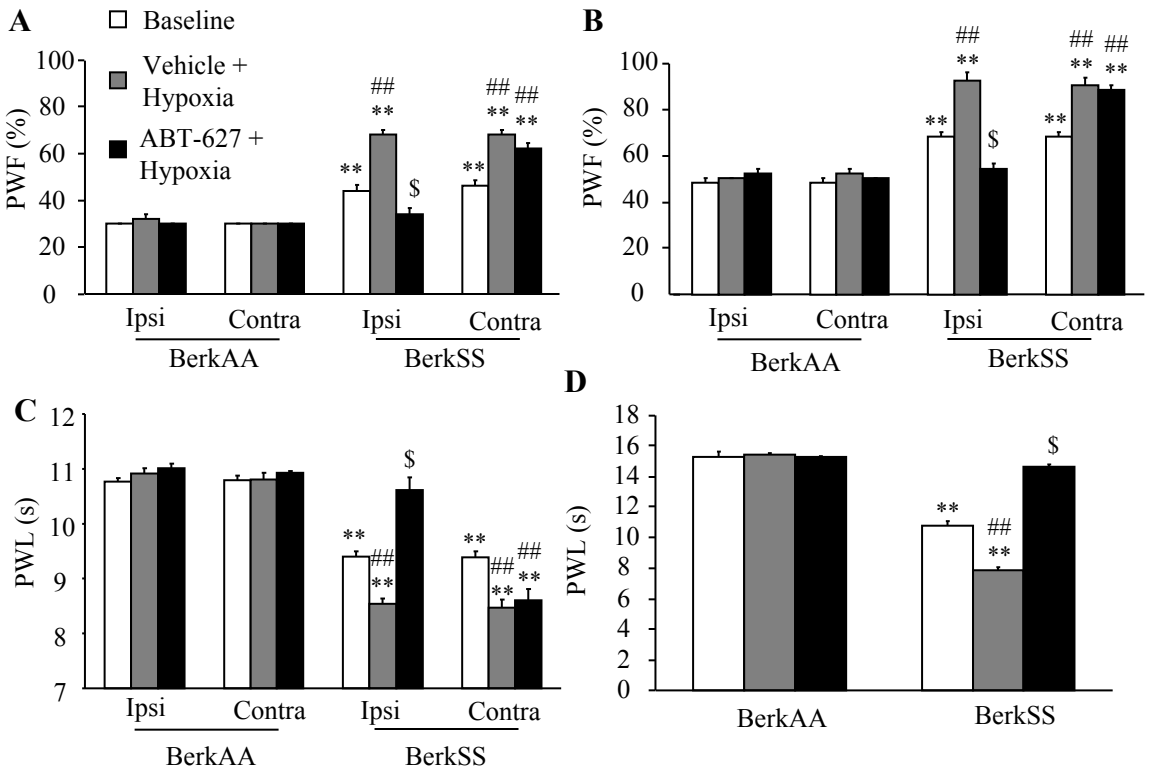
Supplemental Figure 3. Hind paw injection of ABT-627 attenuates pain hypersensitivity in female Townes HbSS mice. 4 month old female Townes HbSS mice possess increased paw withdrawal frequencies (PWF) to a 0.16 g low force (A) and a 0.4 g medium force (B) von Frey filament and decreased paw withdrawal latencies (PWL) to thermal (C), and cold (D) stimuli. A single unilateral hindpaw injection of ABT-627 (ABT) alleviates pain hypersensitivity on the ipsilateral side of HbSS mice (A-D). ABT-627 administration to HbAA mice does not affect basal responses (A-D). BL: baseline. Veh: Vehicle. ABT: ABT-627. n = 6 mice/genotype, two-way repeated measures ANOVA with post-hoc Tukey test. $^{**}p < 0.01$ and $^{*}p < 0.05$ vs the corresponding baseline of HbAA mice. $\#p < 0.05$ vs the corresponding baseline of HbSS mice.



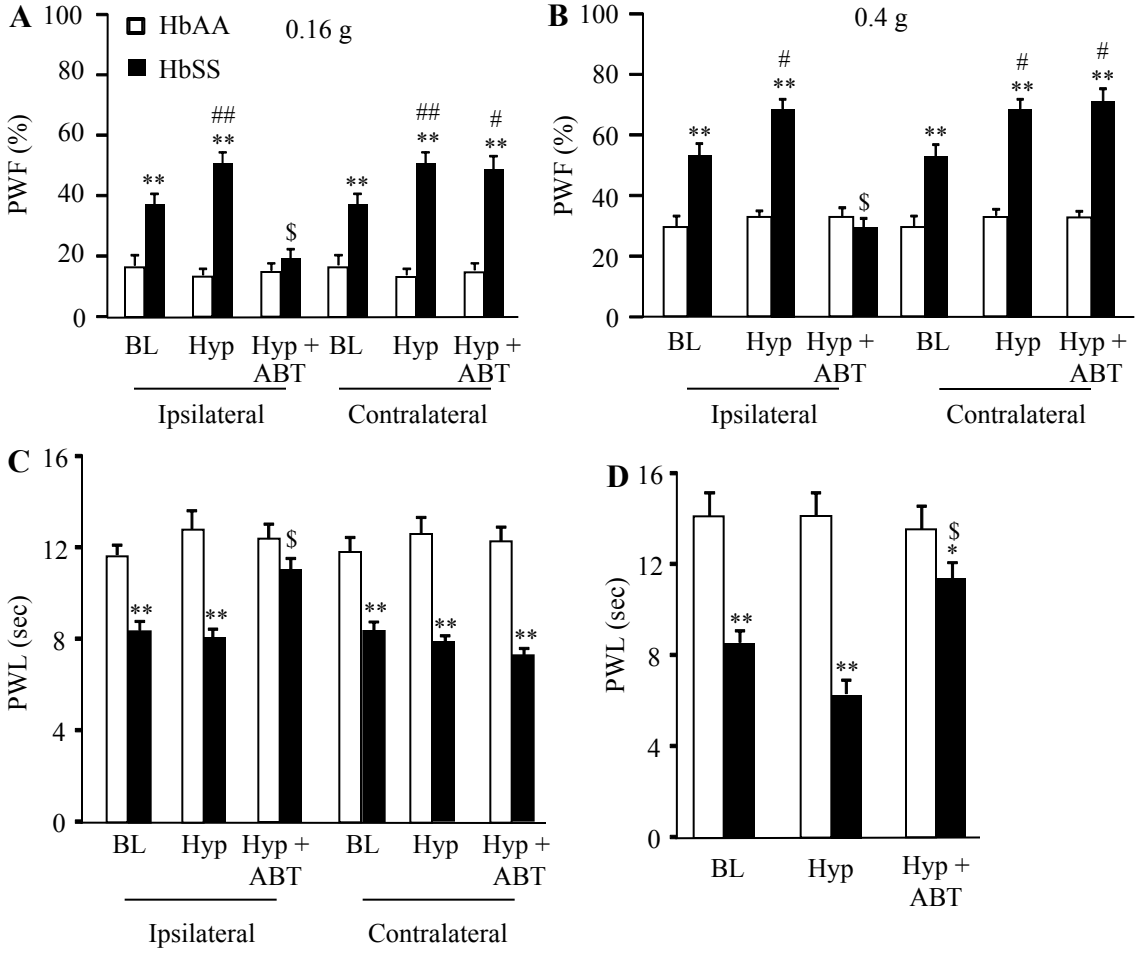
Supplemental Figure 4. Dose-dependent relief of pain hypersensitivities in HbSS mice. Mechanical, 0.16 g (A) and 0.4 g (B), and heat (C) evoked pain was measured in male 4 month old HbSS mice at various doses of ABT-627 delivered subcutaneously to the hindpaw. Significant attenuation of mechanical and heat pain hypersensitivity was achieved with a single dose of 5 nmol ABT-627. n = 5 mice/dose/test. One-way repeated measures ANOVA followed by post-hoc tukey test. $F_{dose}(4,20) = 6.25$ for 0.16 g, 11.684 for 0.4g, and 65.036 for thermal. $^{**}p < 0.01$ *vs* the corresponding control (0).



Supplemental Figure 5. Hindpaw administration of ABT-627 did not alleviate pain hypersensitivity in the contralateral paw of HbSS mice. Mechanical paw withdrawal frequencies (PWF) in response to a 0.16g (A) and a 0.4 g (B) von Frey filament on the contralateral side remained significantly elevated in the HbSS mice after ABT-627 hindpaw administration under both normoxic and hypoxic conditions. Thermal paw withdrawal latencies (PWL) were significantly lower in the HbSS mice for the contralateral side after ABT-627 hindpaw administration under both normoxic and hypoxic conditions. BL: baseline. Veh: vehicle. ABT: ABT-627. n = 6 mice/genotype/treatment. Two-way repeated measures ANOVA, *post-hoc* Tukey test. *$p < 0.05$, **$p < 0.01$ *vs* the corresponding HbAA group. ##$p < 0.01$ *vs* the HbSS baseline value. \$$p < 0.05$ *vs* the corresponding vehicle-treated HbSS group.

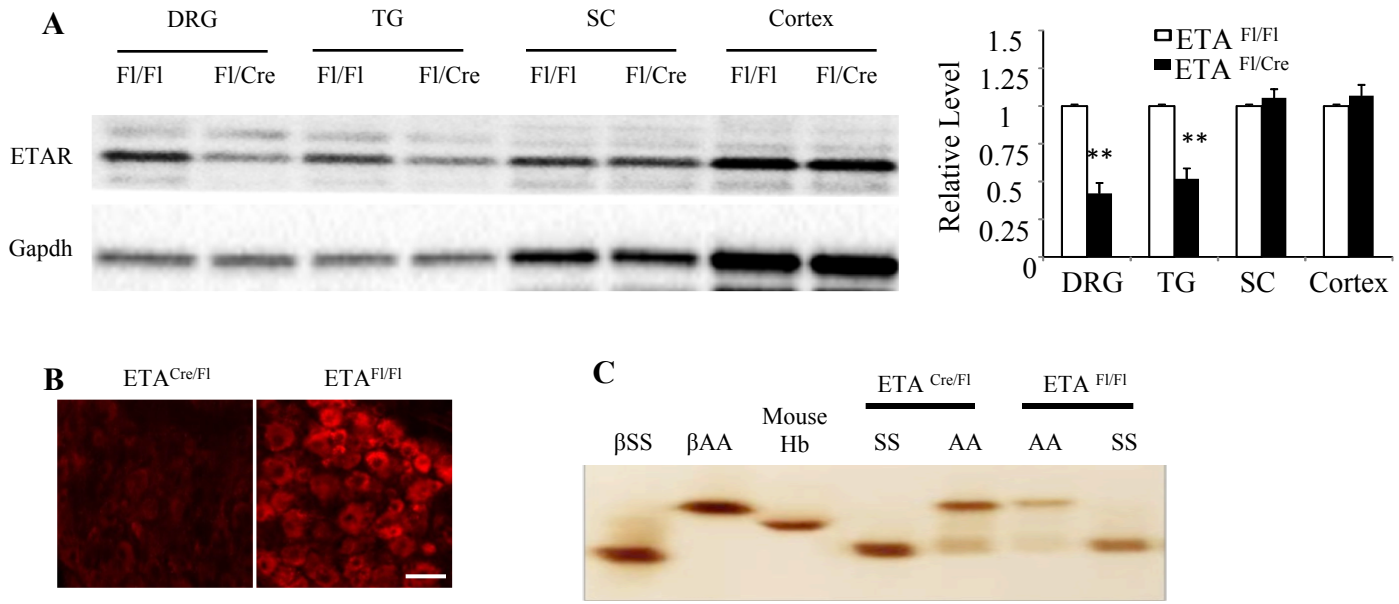


Supplemental Figure 6. Hind paw injection of ABT-627 attenuates hypoxia/reoxygenation-exacerbation in pain hypersensitivity in male BerkSS mice.
Hypoxia/reoxygenation exacerbates pain hypersensitivity to a 0.16 g low force (A) and a 0.4 g medium force (B) von Frey filament, thermal (C), and cold (D) stimuli. A single unilateral hindpaw injection of ABT-627 immediately following hypoxia exposure attenuates these pain hypersensitivities on the ipsilateral side of BerkSS mice (A-D). ABT-627 administration to BerkAA mice does not affect basal responses (A-D). Ipsi: ipsilateral. Contra: contralateral. PWF: paw withdrawal frequency. PWL: paw withdrawal latency. n = 5 mice/genotype. Two-way repeated measures ANOVA with post-hoc Tukey test. $^{**}p < 0.01$ vs the BerkAA baseline on the corresponding side. $^{\#\#}p < 0.01$ vs the BerkSS baseline on the corresponding side. $\$p < 0.05$ vs the corresponding BerkSS mice treated with the vehicle plus hypoxia.

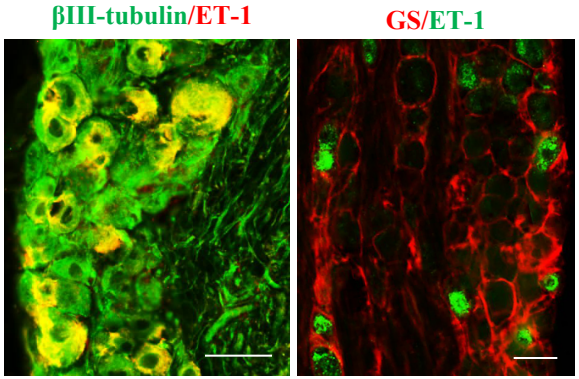


Supplemental Figure 7. Hind paw injection of ABT-627 attenuates pain hypersensitivity following hypoxia/reoxygenation in female Townes HbSS mice.

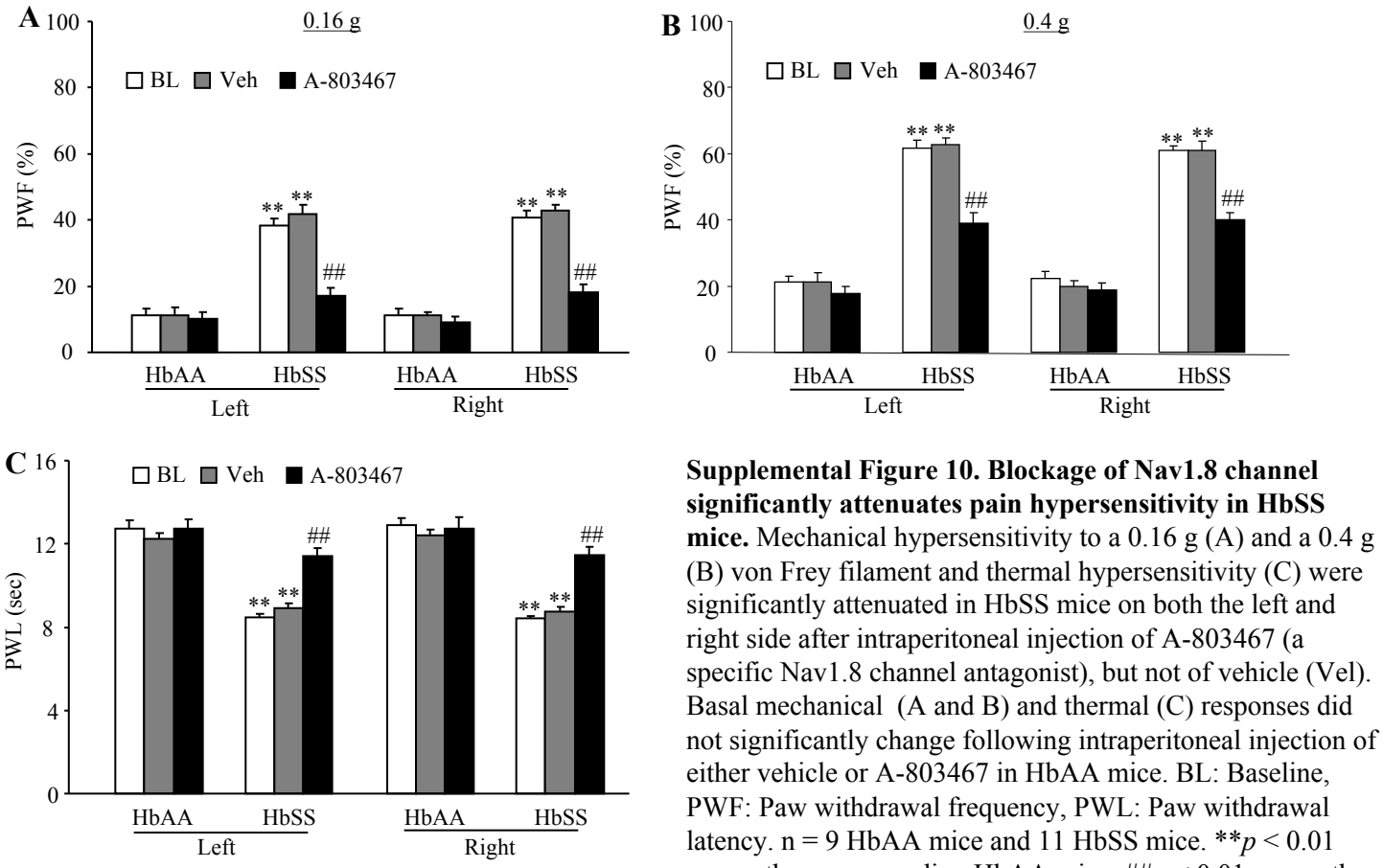
4 month old female Townes HbSS display pain hypersensitivity to a 0.16 g low force (A) and a 0.4 g medium force (B) von Frey filament, thermal (C), and cold (D) stimuli at baseline (BL) levels. Hypoxia/reoxygenation (Hyp) only exacerbates increased paw withdrawal frequencies (PWF) to a 0.16 g low force (A) and a 0.4 g medium force (B) von Frey filament in female HbSS mice. A single unilateral hindpaw injection of ABT-627 (ABT) immediately following hypoxia exposure attenuates these pain hypersensitivities on the ipsilateral side of HbSS mice (A-D). ABT-627 administration to female HbAA mice does not affect basal responses (A-D). PWL: paw withdrawal latency. n = 6 mice/genotype. Two-way repeated measures ANOVA with post-hoc Tukey test. $^{**}p < 0.01$ vs the HbAA baseline on the corresponding side. $\#p < 0.05$, $\#\#p < 0.01$ vs the HbSS baseline on the corresponding side. $\$p < 0.05$ vs the corresponding HbSS mice following hypoxia/reoxygenation.



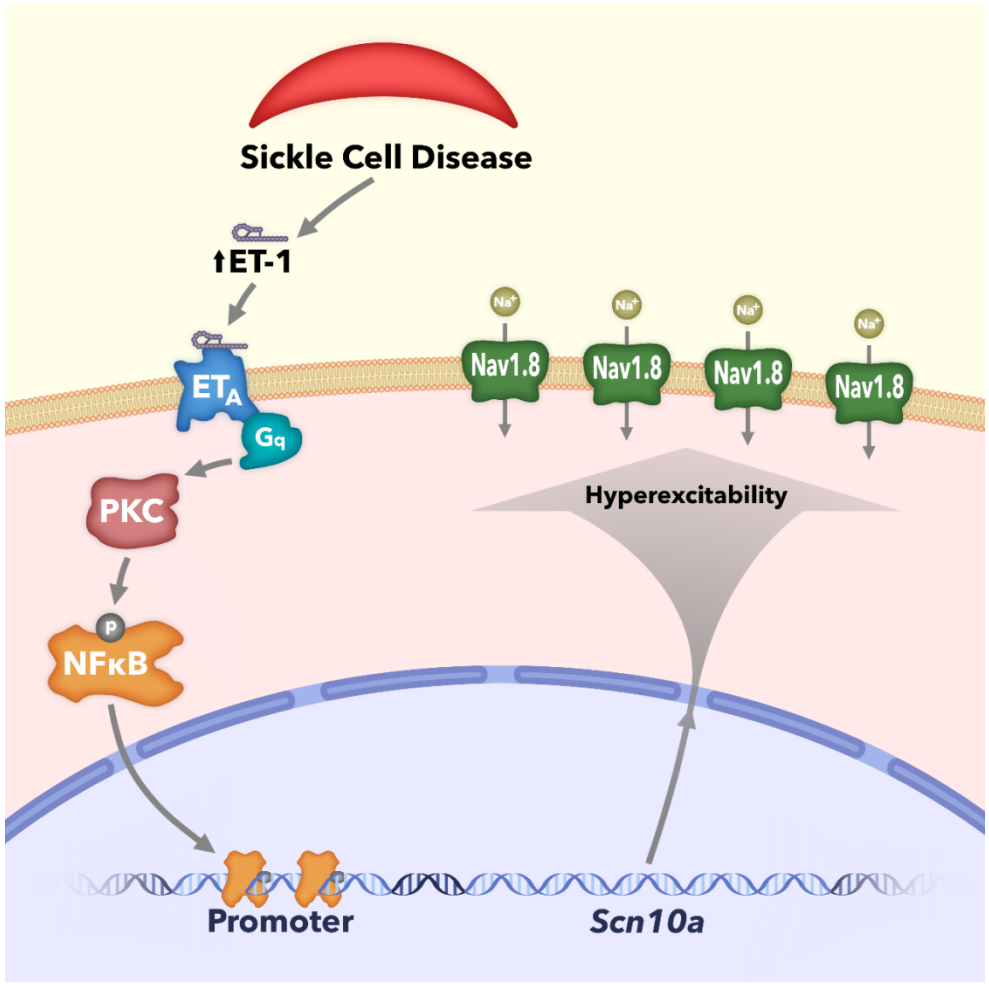
Supplemental Figure 8. Selective and specific knockdown of ET_A receptors in the dorsal root ganglia (DRG) and trigeminal ganglia(TG) of $ET_A^{cre/fl}$ mice and the identification of the exclusive expression of human globins in $ET_A^{cre/fl}$ mice and $ET_A^{fl/fl}$ mice transplanted with bone marrow.
(A) Western blotting analysis shows exclusive knockdown of ETA receptors (ETAR) in the DRG and trigeminal ganglia (TG) of $ETA^{cre/fl}$ mice, while ETA receptor expression remains intact in the spinal cord (SC) and cortex. Left panel: representative Western blots; Right panel: summary of densitometric analysis. n = 3 mice/genotype. $^{**}p < 0.01$ student's t-test. (B) Immunostaining confirms successful knockdown of ET_A receptors in the L4 DRG of $ET_A^{cre/fl}$ mice. Scale bar: 50 µm. n = 3 mice/genotype. (C) Following bone marrow transplantation, blood samples were collected from recipient mice and hemoglobin expression was analyzed using isoelectric focusing. A representative image shows that mice transplanted with BerkSS (SS) bone marrow express only human βSS (sickle β globin), while mice transplanted with the BerkAA (AA) bone marrow express only human βAA. None of the bone marrow recipients expressed mouse hemoglobin (Hb).



Supplemental Figure 9. Pre-pro ET-1 is predominantly expressed in DRG neurons. Double-immunohistochemistry reveled the colocalization of Pre-pro ET-1 (ET-1) with βIII-tubulin, but not with glutamine synthetase (GS) in HbSS DRG. Scale bars: 50 μm



Supplemental Figure 10. Blockage of Nav1.8 channel significantly attenuates pain hypersensitivity in HbSS mice. Mechanical hypersensitivity to a 0.16 g (A) and a 0.4 g (B) von Frey filament and thermal hypersensitivity (C) were significantly attenuated in HbSS mice on both the left and right side after intraperitoneal injection of A-803467 (a specific Nav1.8 channel antagonist), but not of vehicle (Vel). Basal mechanical (A and B) and thermal (C) responses did not significantly change following intraperitoneal injection of either vehicle or A-803467 in HbAA mice. BL: Baseline, PWF: Paw withdrawal frequency, PWL: Paw withdrawal latency. n = 9 HbAA mice and 11 HbSS mice. $^{**}p < 0.01$ versus the corresponding HbAA mice. $^{\#\#}p < 0.01$ versus the corresponding baseline in HbSS mice.



Supplemental Figure 11. Proposed model for an ET_A receptor-dependent mechanism of SCD-associated pain. The elevated ET-1 in the DRG of SCD mice activate ET_A receptors located in peripheral terminals and soma of DRG neurons. Downstream of ET_A receptor activation leads to the PKC-triggered translocation of NF-κB. The latter binds to the promoter region of *Scn10a* gene and enhances its transcriptional activity, resulting in upregulation of Nav1.8 channels at the cell membrane of DRG neurons and leading to DRG neuronal hyperexcitability and pain.

Supplemental Figure 12. Full western blot images.

Figure 5 B

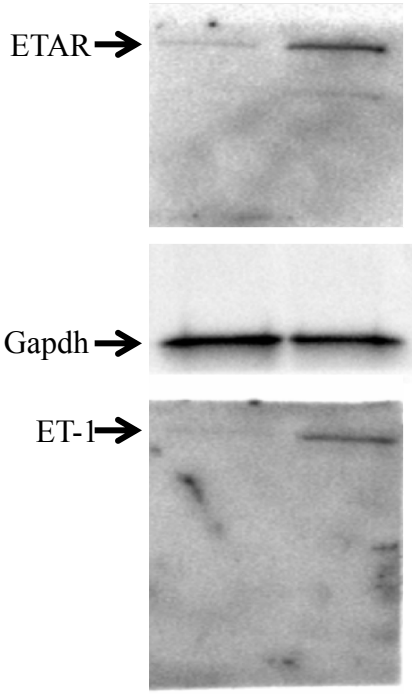


Figure 5 E

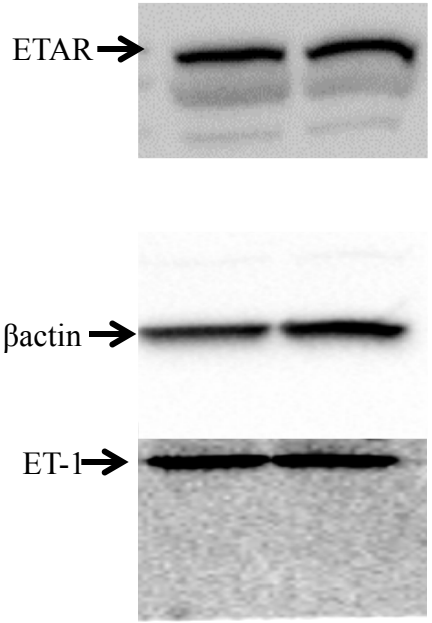


Figure 6 B

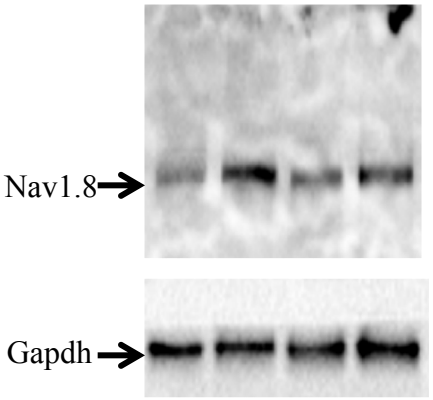
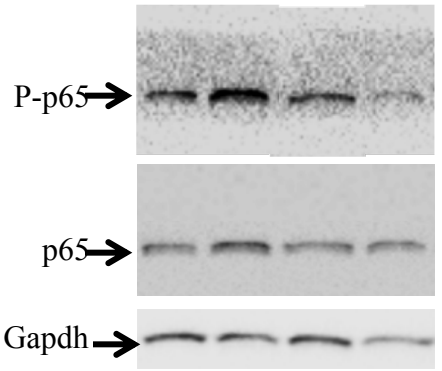


Figure 9C



Supplemental Figure 5 A

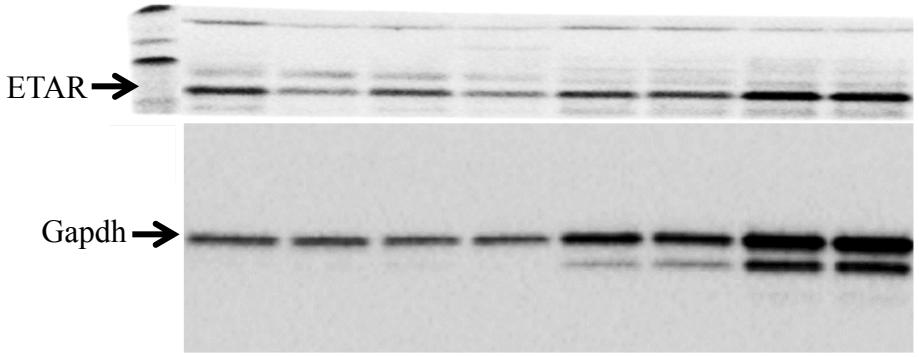


Figure 8B