SUPPLEMENTARY APPENDIX

Small-molecule nociceptin receptor agonist ameliorates mast cell activation and pain in sickle mice

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Supplementary file

Small-molecule nociceptin receptor agonist AT-200 ameliorates inflammation and pain in sickle mice

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Material and Methods

Somatosensory testing for pain behaviors

Behavioral measures were performed in a quiet, temperature-controlled room dedicated to behavioral testing of mice, as previously described.[47,48] All mice were acclimated to the room and to the tests before collecting data.

Mechanical paw withdrawal threshold and frequency. Mechanical allodynia was measured as paw withdrawal threshold using Von Frey filaments. Mice were placed on an elevated wire mesh table. Testing was initiated with the 5.9 mN (0.6 g) von Frey filament (Stoelting Co, Wood Dale, IL, USA), using the up-down method to stimulate the mid-plantar surface of the hind paw until the mouse withdrew its paw. If no paw withdrawal was noted, the next higher weight filament was used. If paw withdrawal was observed, the next lower weight filament was used. The resulting pattern of 6 responses was tabulated and the 50% paw withdrawal threshold was calculated as described previously by us. [47,48]

The paw withdrawal frequency (PWF) to a standard von Frey monofilament with 9.8 mN (1.0 g) applied 10 times at an inter-stimulus interval of approximately 5 seconds to the plantar surface of the hind paw was also recorded. [47,48]

Grip force to determine deep tissue/musculoskeletal pain. Peak forepaw grip force was measured using a computerized grip force meter (SA Maier Co., Milwaukee, WI) as

previously described. Mice were held by the tail and allowed to grasp a wire-mesh grid with their forepaws. Grip force increases as mice are gradually pulled away from the mesh by the tail. The peak force exerted (up to 1 kg) was recorded and presented as the mean from three trials. Deep tissue hyperalgesia was defined as a decrease in grip force. [47,48]

Withdrawal responses to heat stimuli. This was carried out using a Hargreaves apparatus. Radiant heat, from a heat source placed below, was delivered to the plantar surface of the hindpaw and paw withdrawal latency (PWL) was recorded in seconds.[48]

Withdrawal responses to cold stimuli. Mice were placed on a cold plate at 4°C. PWL and PWF responses for each hind paw over a 2 minute period were recorded. [47,48]

Balance and motor coordination. Mice were placed on a (0-72 rpm) accelerating rotarod apparatus (Model 7650, Ugo Basile, Verese, Italy). The latency to fall within a period of 5 minutes was recorded. Mice were subjected to 3 conditioning trials of 2 minutes, at intervals of 2 hours and required to remain on the bar for >60 seconds to be included in the experiment. [47]

Cutaneous Blood Flow

Cutaneous blood flow was measured non-invasively and in real time with a laser Doppler blood perfusion monitor (Laserflo^{R} Model BPM 403, Vasamedics, Inc., St. Paul, Minnesota) as described previously. Hair on the dorsal skin over the thoracolumbar region was gently shaved with electric clippers one day before blood flow measurement. The head of the Laserflo^{R} probe (model PD-434, Vasamedics, St. Paul, MN) was placed on the shaved skin and blood flow values were obtained from the midpoint of the stable portion of the recordings, as described.[49] The built-in micro-processor calculates the blood flow in ml/min/100 g tissue as the product of velocity and volume values.

Cutaneous Mast Cell Staining

Sections approximately 6 microns thick were stained with Toluidine blue. Skin sections were dipped in a solution (pH 2.3) containing 0.025 g Toluidine blue O (Allied Chemical, Morristown, NJ), 2.5 ml 70% ethanol, and 47.5 ml of 1% sodium chloride solution at

room temperature. Sections were washed with 12 changes of distilled water and allowed to air dry before mounting with DPX (Electron Microscopy Sciences, Hatfield, PA). Mast cells were counted in 12-16 reproducible and similar fields (900 x magnification) per mouse using an Olympus IX70 inverted microscope (Olympus Corporation, Center Valley, PA). Mast cells were counted and expressed as cells per square millimeter (mm^2). Degranulated mast cells with $\geq$ 8 granules outside the cell membrane were counted and quantified as a percentage of degranulating mast cells to all mast cells counted.[50]

Enzyme-linked Immunosorbent Assays

Plasma/serum were analyzed for tryptase (Antibodies-Online Inc., Atlanta, GA), calcitonin gene-related peptide (CGRP; Life Sciences Advanced Technologies, Inc., St. Petersburg, FL), substance P, serum amyloid P (SAP), interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α), and regulated upon activation, normal T cell expressed and secreted (RANTES); all from R&D systems, Minneapolis, MN, according to manufacturer instructions, using a microplate reader (Synergy HT, Biotek, Winooski, VT) and the plate reader Gen5™ 1.0 data analysis software (Biotek, Winooski, VT). All analyses and calibrations were performed in duplicate.[50]

Laser scanning confocal microscopy (LSCM) of skin

Mice were euthanized with compressed CO_2 and dorsal skin was fixed in Zamboni's solution (0.03% w/v picric acid and 2% paraformaldehyde) for 24 or 48 hours at 4°C, and then transferred to 20% sucrose with 0.05% sodium azide in PBS.[47] Approximately 6 µM thick cryosections were stained with goat anti-tryptase (1:100; Santa Cruz Biotechnology, Inc., Dallas, TX), rabbit anti-FcεR1 (1:100; eBioscience, San Diego, CA), rat anti-CD117 (1:100; BD Pharmingen, Inc., San Jose, CA) and species-specific secondary antibodies conjugated with FITC, Cy3, and Alexa649 (Jackson ImmunoResearch Laboratories, West Grove, PA) as previously described.[50] In parallel, control staining was performed with isotype-matched IgG. LSCM data sets of 2-µ each of z-stack image sets / field of view (FOV) were captured using 40X immersion oil objective with an Olympus FluoView 1000 system (Olympus Corporation, Center Valley,

PA). Co-stained images from the same FOV were overlaid as required using Adobe Photoshop (Adobe, San Jose, CA).

Results

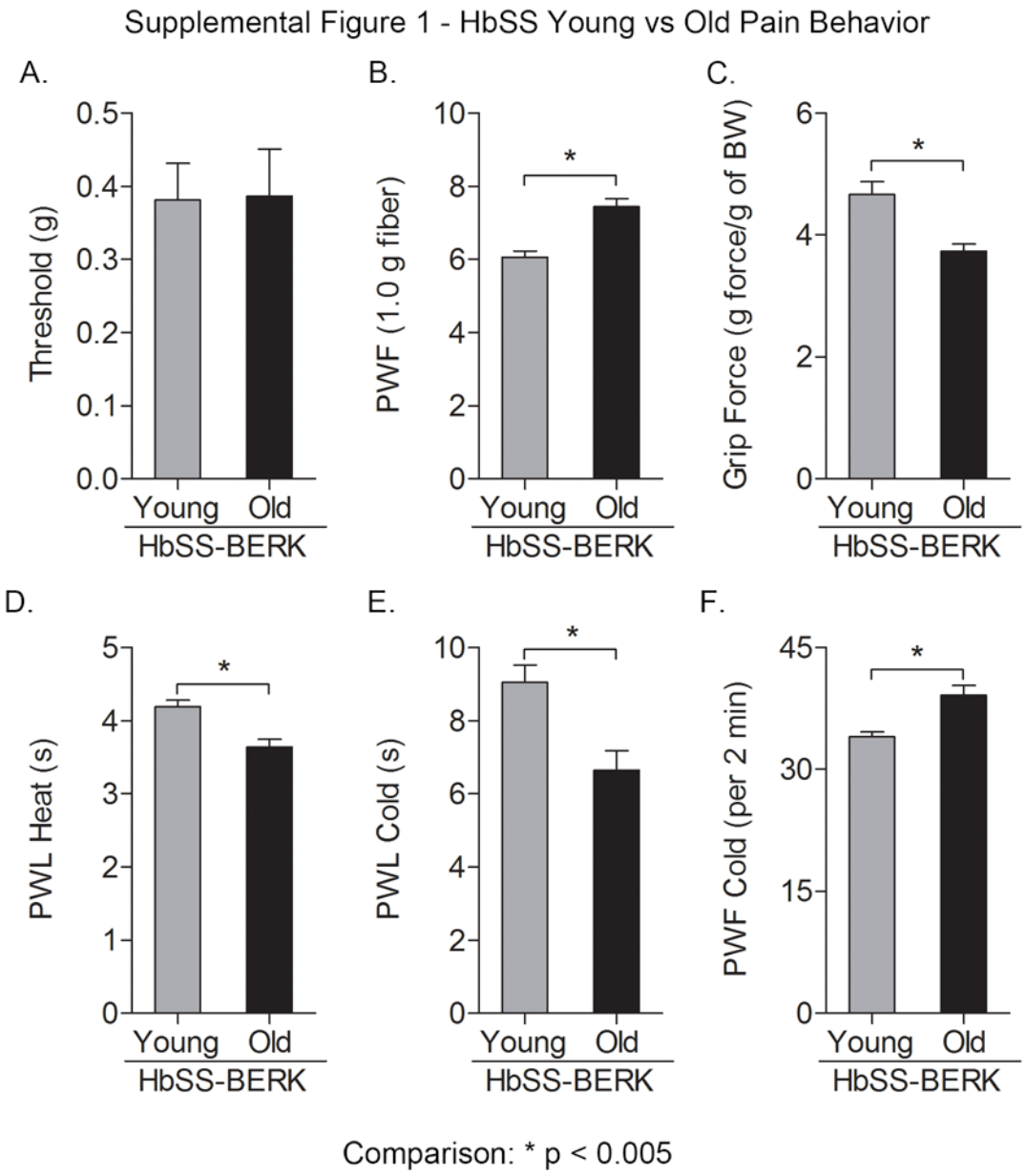

Figure S1. Pain behaviors in young and old control (HbAA-BERK) and sickle HbSS-BERK mice. (A) Mechanical withdrawal threshold using von Frey monofilaments. Lower thresholds are indicative of increased sensitivity to cutaneous nociception. (B) Paw withdrawal frequency (PWF) in response to 10 applications of a 9.8 mN (1.0 g) von Frey monofilament in the same mice. (C) Grip Force measurements normalized for body weights indicating deep tissue/musculoskeletal pain are shown. PWL to heat (D), to cold (E) and PWF on a cold plate (F) in the same young and old mice. Hollow white

bars represent young HbAA-BERK mice and hollow white bars with black horizontal lines represent old HbAA-BERK mice. Solid black bars represent young HbSS-BERK mice and black bars with white horizontal lines represent old HbSS-BERK mice. Data are shown as mean ± SEM from 10-12 mice in HbAA-BERK and from 16 mice in HbSS-BERK per age group. Statistical significance is denoted by * $P < 0.05$ and ** $P < 0.005$. Mean age of mice ± SEM in months were, young HbAA-BERK, 4.05 ± 0.2, old HbAA-BERK, 18.5 ± 1.1, young HbSS-BERK, 4.4 ± 0.3, and old HbSS-BERK, 20.9 ± 0.2.

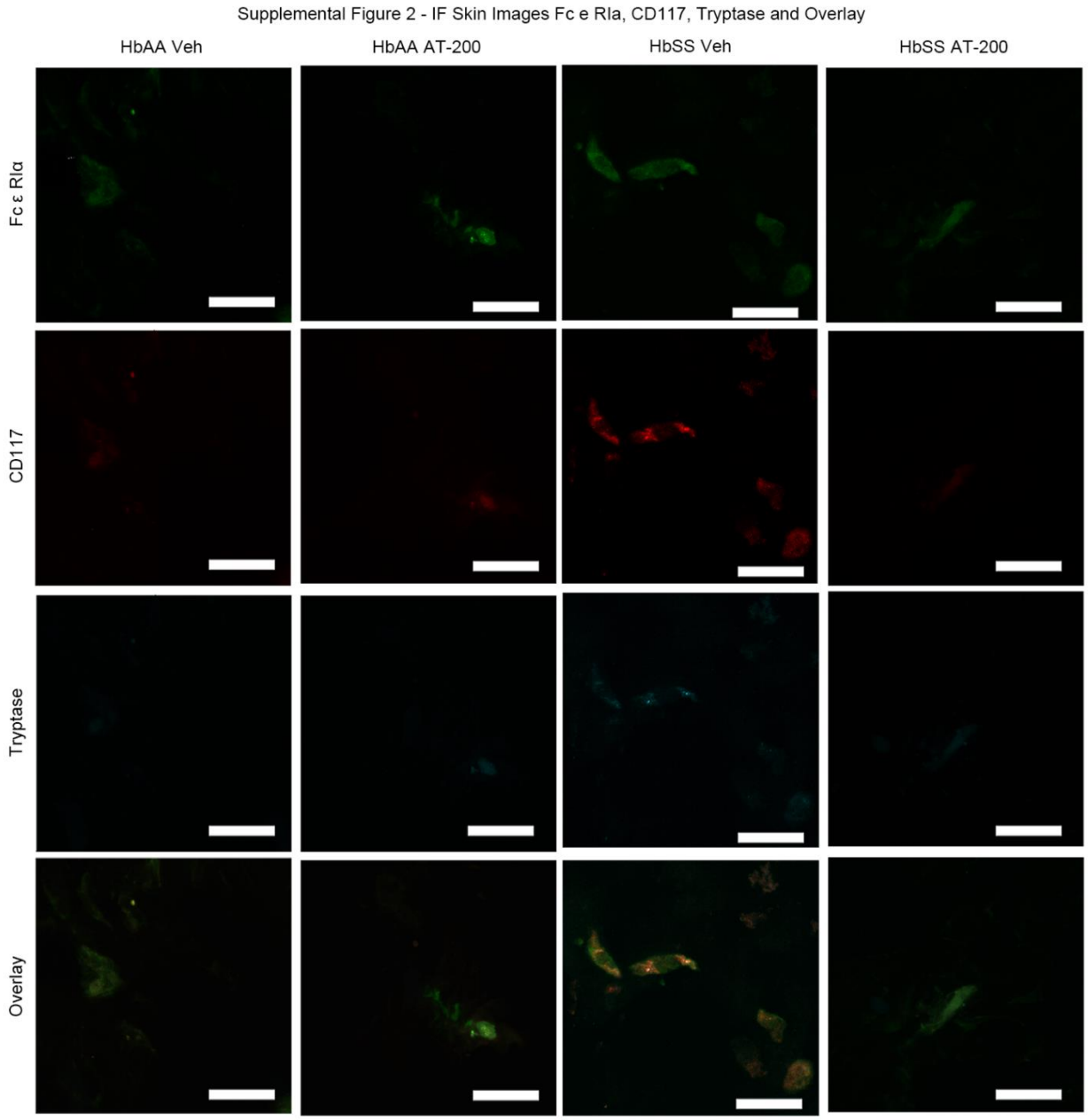

Figure S2. Mast cell activation markers in the skin. A panel of LSCM images of MCs from sickle and control mice skin treated with vehicle or AT-200. Images show FCεRI- (first row, pseudo-colored green), *c-kit*/CD117- (second row, pseudo-colored red), and tryptase-immunoreactive (third row, pseudo-colored turquoise) mast cells. The fourth row is an overlay of the three colors. HbSS-BERK vehicle-treated mice (third column)

showed increased immunoreactivity as compared to MCs of control mice (first column) and AT-200 treated HbSS-BERK mice (fourth column). Original magnification X900; white scale bar represents 20 μm. Each figure is representative of images from 3 sections of skins of 10-12 different mice per treatment group. Image acquisition information: Fluoview FV1000 Laser Scanning Confocal BX61 Microscope (Olympus), 60X/1.42 oil objective lens, In-built image acquisition system, Adobe Photoshop. Mean age of mice ± SEM in months were, HbAA-BERK Vehicle, 4.06 ± 0.29, HbAA-BERK AT-200, 4.04 ± 0.29, HbSS-BERK Vehicle, 4.38 ± 0.32, and HbSS-BERK AT-200, 4.41 ± 0.34.

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