SUPPLEMENTARY APPENDIX

Cannabinoid receptor-specific mechanisms to alleviate pain in sickle cell anemia *via* inhibition of mast cell activation and neurogenic inflammation

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Supplementary Appendix for the manuscript:

Cannabinoid receptor-specific mechanisms to ameliorate pain in sickle cell anaemia via inhibition of mast cell activation and neurogenic inflammation

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Methods

Animals

Mice were bred in AAALAC-approved housing at the University of Minnesota and as described by us.7All experiments were performed following approved protocols from the University of Minnesota's Institutional Animal Care and Use Committee and conform to the statutes of the Animal Welfare Act and the guidelines of the Public Health Service as issued in the Guide for the Care and Use of Laboratory Animals.[1]

Sickle (HbSS-BERK) and control mice (HbAA-BERK): HbSS-BERK express sickle human hemoglobin and have severe disease that simulates human sickle cell anaemia (SCA) including hemolysis, reticulocytosis, anaemia, extensive organ damage, shortened life span and pain.[2-4] Control HbAA-BERK are littermates of HbSS-BERK and therefore have the same mixed genetic background as HbSS-BERK, but exclusively express normal human hemoglobin A (human alpha and beta A globins) and no murine globins. Mice were bred and phenotyped for sickle and normal human hemoglobin by IEF.[3] Genotyping for the knockout mouse haemoglobins and human hemoglobin transgenes was done by Transnetyx (Cordova, TN).

CB2R knockout (CB2R $^{-/-}$) mice: CB2R$^{-/-}$ mice were obtained from the Jackson Laboratory (Stock # 005786; Bar Harbor, ME, USA; MGI:3604531) and backcrossed with HbSS-BERK and HbAA-BERK to obtain sickle and control mice without CB2R (HbSS/CB2R$^{-/-}$; HbAA/CB2R$^{-/-}$), and littermates with CB2R (HbSS/CB2R$^{+/+}$; HbAA/CB2R$^{+/+}$). Sickle or control mice with CB2R$^{-/-}$ or CB2R$^{+/+}$ were identified by PCR with primers specific for CB2R (Cnr2) gene were identified by PCR with primers specific for CB2R (*Cnr2*) gene as specified by the strain's genotyping protocol (Jackson Laboratory). The 3 primer sequences' IDs were moIMR0086, oIMR7552, and oIMR7565. A mixture of 1.0 μM per primer, 0.2 mM dNTP, and 0.05 U/μL Taq (Clontech Inc., Mountain View, CA) was used with modified amplification conditions consisting of an initial 5 min at 94°C followed by 30 cycles of 94°C for 30 seconds, 62°C for 20 seconds, 72°C for 45 seconds, and a final 2 min at 72°C before holding at 10°C to yield an amplification products. CB2R$^{-/-}$ and CB2R$^{+/+}$ mice were genotyped by production of a 550 bp and a 385 bp product, respectively. All sickle and control mice CB2R$^{-/-}$ or CB2R$^{+/+}$ were also genotyped and phenotyped for the different haemoglobins as described above.

Pain-related behaviors

Mice were acclimatized to each test protocol in a quiet room at constant temperature and tested for thermal- (heat and cold), mechanical-, and deep tissue-hyperalgesia (grip force), and catalepsy (Bar test) as described.[3]

Thermal hyperalgesia: A radiant heat stimulus was applied to the plantar surface of the hind paw from below with a projector lamp bulb (CXL/CXR, 8 V, 50 W). Paw withdrawal latency (PWL) to the nearest 0.1 second was recorded when the mouse withdrew its paw from the stimulus. For cold sensitivity, the latency to initial lifting of either forepaw on cold plate (3°C) and the number of times mice lifted or rubbed the forepaws together (PWF) over a period of 2 minutes were determined.

Mechanical hyperalgesia: Paw withdrawal threshold was determined using the up-down method.[76,77] Briefly, a series of von Frey filaments, ranging from 0.4 - 8.0 g, were applied to the hind paws of mice The resulting pattern of responses was tabulated using the convention: X = withdrawal; O = no withdrawal and the 50% response threshold was

calculated using the following formula: 50% g threshold = $(10^{[Xf+\kappa\delta]})/10{,}000$; where Xf = value (log units) of the final von Frey filament used, κ = tabular value for the pattern of positive/negative responses, δ = mean difference (log units) between stimuli.

The paw withdrawal frequency (PWF) evoked by evoked by 10 consecutive applications of a 1.0 g (4.08 mN) von Frey (Semmes-Weinstein) monofilament (Stoelting Co., Wood Dale, IL, USA) to the plantar surface of each hind paw, for 1-2 seconds with a force sufficient to bend the filament. An inter-stimulus interval of at least 5 seconds was observed. Only vigorous withdrawal responses were counted.

Grip force: To assess deep tissue hyperalgesia, peak forepaw grip force was measured using a computerized grip force meter (SA Maier Co., Milwaukee, WI, USA). Mice held by the tail were made to pull on a wire-mesh gauge with their forepaws. As they were gradually pulled by the tail, the peak force (in g) exerted was recorded. Deep tissue hyperalgesia was defined as a decrease in the grip force.

Bar test: Mice were placed with their forepaws on a horizontal metal bar that was kept parallel and 5 cm above the counter-top where the hind-paws rested. The time (in seconds) spent in this position was recorded. Catalepsy was defined as an increase in the time spent in this position.

Neurogenic inflammation

Dorsal skin of mice was shaved 24 hrs before plasma extravasation was assessed using Miles assay [5]. Evans blue dye (Sigma-Aldrich, St. Louis, MO, USA) at a concentration of 50 mg/kg was injected intravenously into the tail vein 5 minutes before 50 µL of vehicle (10% ethanol, 7.5% Tween 80 in Saline), capsaicin (1.6%), or substance P (100 nM) were injected intradermally to the dorsal skin. Each site was randomized and spaced approximately 20 mm apart. Mice were euthanized 30 minutes after intradermal injections. Blue lesions at application sites were measured for diameter and excised. Skins were weighed, incubated in formamide for 24h at 56°C, and Evans blue content was measured spectrophotometrically at 620 nm.

Blood Flow measurement

Blood flow in the dorsal skin was measured non-invasively and in real time with a laser Doppler blood perfusion monitory (Laserflo^R Model BPM 403, Vasamedics, Inc., St. Paul, MN, USA). Electric clippers were used to gently shave hair on the dorsal skin over the thoracolumbar region one day before blood flow measurement. The head of the Laserflo^R probe (model PD-434, Vasamedics) was placed on the shaved skin and blood flow values were obtained from the midpoint of the stable portion of the recordings, as described.[6] Skin was illuminated for 120 seconds with a low-power, solid-state laser diode that generates a beam of infrared light ($\lambda = 780\pm20$). The small change in the wavelength the light (Doppler shift) through moving blood cells of is produced. The built-in micro-processor calculates the blood flow in ml/min/100 g tissue and is expressed in arbitrary units (Perfusion Units, PU) by the microprocessor as the product of velocity and volume values.

Cytokine and neuropeptide release

At study endpoint, skin punch biopsies (4 mm) were incubated in DMEM plus antibiotics with 2 mM L-glutamine and 10 mM HEPES for indicated times at 37°C in a 5% CO_2 chamber. The culture medium was analyzed for cytokines and neuropeptides.[5]

Skin supernatants were analyzed using a microplate-based array using Q-Plex™ Array Technology through sample testing services of Quansys Biosciences, Inc., (Logan, UT, USA) for cytokines: tumor necrosis factor-α (TNF-α), granulocyte macrophage colony stimulating factor (GM-CSF), interferon-γ (IFN-γ), interleukin-10 (IL-10), interleukin-1α (IL-1α), interleukin-1β (IL-1β), interleukin-4 (IL-4), and interleukin-6 (IL-6) were tested; and the chemokines monocyte chemotactic protein-1 (MCP-1/CCL2), macrophage inflammatory protein-1α (MIP-1α), and regulated upon activation normal T-cell expressed and secreted (RANTES).

Tryptase (American Research Products, Inc., Waltham, MA, USA), β-Hexosaminidase (Cedarlane Labs, Burlington, NC, USA), Substance P (SP), serum amyloid P (SAP; all from R&D Systems, Minneapolis, MN, USA) levels in the skin supernatants were measured and read accordingly to manufacturer instructions with a microplate reader

(Synergy HT, BioTek, Winooski, VT, USA). Assays were calculated with the plate reader Gen5™ 1.0 data analysis software (BioTek). All analyses and calibrations were performed in duplicate.

Mast cell analysis

Six micron thick dorsal skin sections were stained with Toluidine blue and allowed to air dry before mounting with DPX (Electron Microscopy Sciences, Hatfield, PA). Mast cells were counted in 20 reproducible and similar fields (600 X magnification) per mouse using an Olympus IX70 inverted microscope (Olympus Corporation, Center Valley, PA, USA). Mast cells were counted as cells per mm^2. 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.[7]

Mast cell isolation and culture

Mast cells from the skin of mice were isolated and enriched by positive selection using anti-CD117 antibodies.[5, 8] Cytospins of cultured mast cells were co-stained for mast cell specific c-kit/CD117 (BD Bioscience, San Jose, CA, USA), FcεR1 (eBioscience, San Diego, CA, USA) and tryptase (Santa Cruz Biotechnology, Inc., Santa Cruz , CA, USA) antibodies. Species-specific fluorophor-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) were used for detection. In parallel, primary antibodies were substituted with control isotype-matched IgG. Confocal images were captured using a 60X immersion oil objective with a FluoView 1000 system (Olympus Corporation). All co-stained images from the same FOV were overlaid as shown in results were overlaid using Adobe Photoshop (Adobe, San Jose, CA).

Hematopathology of blood

Blood was collected at study endpoint by cardiac puncture for the determination of haematocrit, total haemoglobin, and complete blood counts. For WBC counts, the RBCs were immediately lysed by diluting whole blood 20-fold in 2% acetic acid containing 30

μg/mL ethylenediaminetetraacetic acid (EDTA). For sickle RBC counts, blood was immediately mixed (1:1) with 2% sodium metabisulfite (Sigma Aldrich) and incubated at room temperature for 15 min followed by fixation with formalin buffer (1:1; Thermo Fisher Scientific, Waltham, MA, USA). Sickle RBCs were calculated using an Olympus IX70 inverted microscope (Olympus Corporation) in 10 fields per sample at 600X magnification and expressed as a percentage of total RBC.

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Supplementary Table S1.

Results of ANOVA analysis of the significance of cannabinoid receptor agonist modulation of the behavior of sickle mice.

Figure	Interaction	Time (Row Factor)	Treatment (Column Factor)
1A	$F_{5, 48} = 5.179$, $p = 0.0007$	$F_{5, 48} = 2.232$, $p = 0.0662$	$F_{1, 48} = 81.09$, $p < 0.0001$
1B	$F_{5, 108} = 2.516$, $p = 0.0339$	$F_{5, 108} = 2.247$, $p = 0.0548$	$F_{1, 108} = 54.26$, $p = < 0.0001$
1C	$F_{5, 108} = 6.979$, $p < 0.0001$	$F_{5, 108} = 5.769$, $p < 0.0001$	$F_{1, 108} = 135.1$, $p < 0.0001$
1D	$F_{5, 108} = 12.80$, $p < 0.0001$	$F_{5, 108} = 9.241$, $p < 0.0001$	$F_{1, 108} = 305.8$, $p < 0.0001$
1E	$F_{5, 48} = 3.340$, $p = 0.0114$	$F_{5, 48} = 0.7027$, $p = 0.6241$	$F_{1, 48} = 53.37$, $p < 0.0001$
1F	$F_{5, 48} = 5.179$, $p = 0.0039$	$F_{5, 48} = 1.802$, $p = 0.1303$	$F_{1, 48} = 36.55$, $p < 0.0001$
1G	$F_{6, 56} = 0.5962$, $p = 0.7321$	$F_{6, 56} = 1.834$, $p = 0.1089$	$F_{1, 56} = 1.249$, $p = 0.2686$
2A	$F_{24, 143} = 0.7829$, $p = 0.7531$	$F_{8, 143} = 1.323$, $p = 0.2367$	$F_{3, 143} = 50.04$, $p < 0.0001$
2B	$F_{24, 144} = 3.208$, $p < 0.0001$	$F_{8, 144} = 2.657$, $p = 0.0095$	$F_{3, 144} = 170.2$, $p < 0.0001$
2C	$F_{24, 143} = 07829$, $p = 0.7531$	$F_{8, 143} = 07829$, $p = 0.2367$	$F_{3, 143} = 50.04$, $p < 0.0001$
2D	$F_{24, 135} = 2.090$, $p = 0.0044$	$F_{8, 135} = 6.741$, $p < 0.0001$	$F_{3, 135} = 122.0$, $p < 0.0001$
3A	$F_{15, 110} = 0.8820$, $p = 0.5857$	$F_{5, 110} = 6.792$, $p < 0.0001$	$F_{3, 110} = 34.39$, $p < 0.0001$
3B	$F_{15, 100} = 2.653$, $p = 0.0020$	$F_{5, 100} = 14.95$, $p < 0.0001$	$F_{3, 100} = 98.70$, $p < 0.0001$
3C	$F_{15, 98} = 2.099$, $p = 0.0159$	$F_{5, 98} = 21.13$, $p < 0.0001$	$F_{3, 98} = 59.80$, $p < 0.0001$
3D	$F_{15, 96} = 4.258$, $p < 0.0001$	$F_{5, 96} = 22.64$, $p < 0.0001$	$F_{3, 96} = 76.38$, $p < 0.0001$
3E	$F_{15, 216} = 02461$, $p = 0.9984$	$F_{5, 216} = 0.4846$, $p = 0.7875$	$F_{3, 216} = 2.204$, $p = 0.886$
4A	$F_{5, 48} = 4.669$, $p = 0.0014$	$F_{5, 48} = 8.169$, $p < 0.0001$	$F_{1, 48} = 115.5$, $p < 0.0001$
4D	n/a	n/a	$F_{3, 16} = 33.77$, $p < 0.0001$
4H	n/a	n/a	$F_{3, 144} =$, $p < 0.0001$
5B	n/a	n/a	$F_{5, 24} = 7.157$, $p = 0.0003$
5C	n/a	$F_{4, 36} = 0.6644$, $p = 0.6208$	$F_{9, 36} = 13.58$, $p < 0.0001$
5D	n/a	n/a	$F_{3, 16} = 20.71$, $p < 0.0001$
5E	n/a	n/a	$F_{5, 24} = 10.79$, $p < 0.0001$
6A	$F_{1,16} = 4.530$ $p = 0.0492$	$F_{1,16} = 8.792$, $p = 0.0091$	$F_{1,16} = 259.6$, $p < 0.0001$
6B	$F_{6,48} = 3.306$, $p = 0.0084$	$F_{2, 48} = 378.0$, $p < 0.0001$	$F_{3, 48} = 37.80$, $p < 0.0001$

Comparisons of comparisons of baseline levels with levels at other time points and vehicle against cannabinoid receptor agonist at individual time points or multiple parameters analyzed were based on a 2-way ANOVA with Bonferroni's multiple comparisons. (Fig 1-3, Fig 4A, Fig 5C and Fig 6A-B). A 1-way ANOVA is used to determine the effect of treatment between groups for a single parameter (Fig 4D, and Fig 5B,D,E). $p < 0.05$ was considered significant, $p < 0.01$ was considered very significant and $p < 0.0001$ was considered extremely significant.