EUROPEAN HEMATOLOGY ASSOCIATION
haematologica
Journal of the European Hematology Association

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Haematologica 2015 [Epub ahead of print]

Citation: Vincent L, Vang D, Nguyen J, Benson B, Lei J, and Gupta K. Cannabinoid receptor-specific mechanisms to ameliorate pain in sickle cell anemia via inhibition of mast cell activation and neurogenic inflammation. Haematologica. 2015; 100:xxx
doi:10.3324/haematol.2015.136523

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

Lucile Vincent[1], Derek Vang[1], Julia Nguyen[1], Barbara Benson[1], Jianxun Lei[1] and Kalpna Gupta[1*]

[1]Vascular Biology Center, Division of Hematology, Oncology and Transplantation, Department of Medicine, University of Minnesota, Minneapolis, MN 55455.

Running heads: Mechanisms of cannabinoid analgesia in sickle mice

*To whom correspondence should be addressed:
Kalpna Gupta, Ph.D.
Vascular Biology Center
Medicine - Hematology, Oncology and Transplantation
University of Minnesota
Mayo Mail Code 480; 420 Delaware Street SE
Minneapolis, MN, 55455, USA
Phone: international +612.6257648. Fax: international +612.6256919
E-mail: gupta014@umn.edu

Abstract

Sickle cell anaemia is a manifestation of a single point mutation in haemoglobin, but inflammation and pain are the insignia of this disease which can start in infancy and continue throughout life. Earlier studies showed that mast cell activation contributes to neurogenic inflammation and pain in sickle mice. Morphine is the common analgesic treatment but also remains a major challenge due to the side effects and ability to activate mast cells. Therefore, we examined the cannabinoid receptor-specific mechanisms to ameliorate mast cell activation, neurogenic inflammation and hyperalgesia, using HbSS-BERK sickle and cannabinoid receptor 2 deleted sickle mice. We show that cannabinoids ameliorate mast cell activation, inflammation and neurogenic inflammation in sickle mice via both cannabinoid receptors 1 and 2. Thus, cannabinoids influence systemic and neural mechanisms, ameliorating the disease pathobiology and hyperalgesia in sickle mice. This study provides a ‘proof of principle’ for the potential of cannabinoid/cannabinoid receptor-based therapeutics to treat several manifestations of sickle cell anaemia.

Introduction

Sickle-cell anaemia (SCA) is one of the most commonly inherited disorders and is associated with both unpredictable recurrent acute pain and chronic pain.[1] Opioids, specifically morphine has been the drug of choice for the treatment of severe pain associated with SCA.[1, 2] Morphine is highly histaminergic, and is known to activate mast cells.[2] We showed earlier that mast cells contribute to neurogenic inflammation and hyperalgesia in sickle mice.[3] We also found that cannabinoids ameliorate chronic and hypoxia/reoxygenation-evoked acute hyperalgesia in sickle mice.[4, 5] Cannabinoids have anti-inflammatory effects and provide protection from ischemia/reperfusion injury.[6-10] Since pain is a manifestation of complex sickle pathobiology including inflammation, vascular dysfunction and ischemia reperfusion injury, we sought to examine cannabinoid receptor-specific modulation of vascular function, inflammation and hyperalgesia.

Cannabinoid receptors (CBRs), CB1R and CB2R are expressed in the central nervous system (CNS) and non-CNS tissues, including inflammatory cells.[11-15] CB1R and CB2R activation on mast cells has been shown to inhibit degranulation and inflammation, respectively.[16] Activation of CB2R peripherally generates an antinociceptive response in inflammatory and neuropathic pain.[17] CB2R is involved with neuroinflammation and the CB2R agonist JWH-133 ameliorates stress-related neuroinflammation-dependent pathologies.[18, 19] Selective activation of peripheral CBRs is appealing because it would avoid neuropsychiatric adverse effects associated with activation of CB1R in the CNS.

Sickle mice display neurogenic inflammation and hyperalgesia via a mast-cell-dependent mechanism.[3] CBRs are important modulators of vascular function with an

anti-ischemic effect and direct anti-inflammatory effects by inhibiting mast cell degranulation.[19] Since, vascular dysfunction, ischemia/reperfusion injury and inflammation are hallmark features of SCA, we hypothesized that targeting specific CBRs may have a beneficial effect on sickle pathobiology and pain. We used transgenic HbSS-BERK mice, hereafter referred to as sickle mice, that show features of pain and inflammation similar to patients with SCA [4, 5, 20] and sickle mice deleted for CB2R, to examine the contribution of each CBR in mast cell activation, neurogenic inflammation, and pain.

Methods

Detailed procedures are described in the Online Supplementary Methods.

Animals

Sickle (HbSS-BERK) and control mice (HbAA-BERK): BERK transgenic mice are murine α and β globin knockouts that express human sickle haemoglobin (S), demonstrating severe sickle cell disease, or normal (A) haemoglobin.[4, 5, 21]

CB2R knockout (CB2R $^{-/-}$) mice: CB2R$^{-/-}$ mice (Stock # 005786; Jackson Laboratory, Bar Harbor, ME, USA) were backcrossed with 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 (Jackson Laboratory). Sickle (HbSS) and control mice (HbAA) were bred and phenotyped for sickle and normal human haemoglobin by iso-electric focussing[4] and genotyping for the knockout and haemoglobin transgenes (Transnetyx, Cordova, TN, USA). All experiments were

performed following protocols approved by the University of Minnesota's Institutional Animal Care and Use Committee.

Treatments

The cannabinoid receptor agonist, CP55,940, (Tocris Bioscience, Bio-Techne, Minneapolis, MN, USA), was prepared in 2% DMSO and 98% normal saline. Mice were treated daily with 0.3 mg/Kg CP55,940 or 2% DMSO in saline intraperitoneally in a volume of 25 µL/10 g of body weight.

To evaluate the contribution of individual CBRs, mice were treated with ACEA (Tocris Bioscience), a CB1R selective agonist (Ki = 1.4 nM), or JWH-133 (National Institute on Drug Abuse-NIDA, USA), a CB2R selective agonist (Ki = 3.4 nM).[22] Mice received 1 mg/Kg ACEA or JWH-133 prepared in 2% DMSO and 98% normal saline intraperitoneally in a volume of 25 µL/10 g of body weight.

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).[4]

Hyoxia/Reoxygenation

Mice were exposed to hypoxia with 8% O_2 and 92% N_2 for 3h followed by re-oxygenation at room air for 1h.[5]

Neurogenic inflammation

Plasma extravasation in response to vehicle (10% ethanol, 7.5% Tween in saline), capsaicin (1.6%), or substance P (100 nM) injected intradermally in the dorsal skin was assessed by the Miles assay using Evans blue dye (Sigma-Aldrich, St. Louis, MO, USA).[3]

Blood Flow measurement

Blood flow in the dorsal skin was measured with a laser Doppler blood perfusion monitor (Laserflo^{R} Model BPM 403, Vasamedics, Inc., St. Paul, MN, USA).[23]

Mast cell activation

At study endpoint, skin punch biopsies (4 mm) were incubated for indicated times and the culture medium was analyzed for cytokines (Q-Plex™ Array; Quansys Biosciences, Inc., Logan, UT, USA) and neuropeptides by ELISA.[3] Degranulating mast cells in skin sections were quantified and cultured mast cells from skin were immuno-stained for co-expression of mast cell specific c-kit/CD117 (BD Bioscience, San Jose, CA, USA), FcεR1 (eBioscience, San Diego, CA, USA) and tryptase).[3] (Santa Cruz Biotechnology, Inc.,Santa Cruz , CA, US).

Hematopathology of blood

Haematocrit, total haemoglobin, complete blood counts and red cell indices (% sickle RBCs) were determined as previously described.[3]

Statistical analysis

All data were analyzed using Prism software (v 5.0a, GraphPad Prism Inc., San Diego, CA). Repeated measures analysis of variance (ANOVA) with Bonferroni's correction was used to compare the responses between treatments. A summary of the significance analysis of ANOVA (F(DFn, DFd) values) is given in Online Supplementary Table S1. A p-value of < 0.05 was considered significant. All data are presented as mean ± SEM.

Results

Cannabinoids ameliorate chronic hyperalgesia in sickle mice

Similar to chronic pain in SCA, HbSS-BERK sickle mice demonstrate tonic hyperalgesia [4, 5, 20] compared to HbAA-BERK control mice and hypoxia/reoxygenation evoked acute hyperalgesia simulating VOC pain.[5] Earlier we showed that a single injection of CP55,940, a non-selective cannabinoid receptor agonist, at a dose of 0.3 mg/Kg ameliorated tonic deep tissue as well as CFA-induced mechanical hyperalgesia in these sickle mice.[4] Chronic pain requires repeated treatment, which can result in tolerance, therefore, we examined if chronic treatment with CP55,940 leads to a sustained analgesic effect over a period of time. Daily treatment with CP55,940 significantly reduced deep tissue-, mechanical- and thermal-hyperalgesia in sickle mice (Figure 1 A-F). The effect of CP55,940 was sustained over a period of three weeks. Due to the elaborate number of values for each test and each time point, statistical significance between vehicle and CP55,940 for each time point and for CP55,940 as compared to baseline (before treatment) are indicated in the figures and legends. Chronic treatment did not lead to catalepsy since the bar test did not show a significant difference between CP55,940 and vehicle (Figure 1G).

Cannabinoids ameliorate hyperalgesia via cannabinoid receptors

Using pharmacological and genetic approaches we analyzed if cannabinoids ameliorated chronic and acute hyperalgesia via CB1R and/or CB2R. Sickle mice were treated with either vehicle, CP55,940, CB1R agonist ACEA, or CB2R agonist JWH-133, for a week (normoxia), followed by 3 hours of hypoxia and 1 hour of reoxygenation. Deep tissue, mechanical and thermal hyperalgesia were measured before starting the treatment, at baseline, after 7 days of treatment under normoxia, and after H/R for different time periods. Under normoxic conditions 7 day treatment with CP55,940 and CB1R agonist ACEA significantly reduced deep tissue, mechanical and thermal (heat and cold) hyperalgesia as compared to baseline ($P<0.05$) or vehicle-treated sickle mice ($P<0.05$; Figure 2). However, the CB2R agonist was only able to decrease the deep tissue hyperalgesia significantly following 7 days of treatment ($P<0.05$ Vs baseline or vehicle; Figure 2A). CB2R agonist did not show a significant effect on mechanical and thermal (heat and cold) hyperalgesia (Figure 2 B-D). Therefore, under normoxic conditions representative of chronic pain in SCA, CB1R agonist as well as non-selective CBR agonist CP55,940 appear to be uniformly effective in attenuating different pain phenotypes including deep tissue, mechanical and thermal hyperalgesia in sickle mice. On the other hand, CB2R agonist only ameliorated deep tissue hyperalgesia, suggesting that CB1R agonism is critical for treating phenotypically diverse chronic pain in SCA.

Earlier we found that H/R-evoked acute deep tissue hyperalgesia in sickle mice was ameliorated by a single injection of CP55,940.[4] Here we examined if treatment with cananbinoids would prevent HR-evoked hyperalgesia. Pre-treatment of mice with

CP55,940, and CB1R agonist for 7 days decreased tonic hyperalgesia and also prevented HR-evoked deep tissue, mechanical and thermal hyperalgesia (Figure 2 A-D). However, treatment with CB2R agonist decreased tonic as well as HR-evoked deep tissue hyperalgesia (Figure 2A) but did not reduce tonic or HR-evoked mechanical or thermal (heat and cold) hyperalgesia (Figure 2 B-D).

Furthermore, to determine the contribution of either CB1R or CB2R to the analgesia provided by CP55,940, we treated CB2R deleted (HbSS $CB2R^{-/-}$) and intact CB2R (HbSS $CB2R^{+/+}$) sickle mice with a single dose of CP55,940 under normoxia (Figure 3). Control $CB2R^{-/-}$ and sickle $CB2R^{-/-}$ mice did not differ in the baseline hyperalgesia as compared to control $CB2R^{+/+}$ and sickle $CB2R^{+/+}$, respectively. An increase in grip force was observed in control $CB2R^{+/+}$ mice following CP55,940 treatment, but not in control $CB2R^{-/-}$ mice (Figure 3A). CP55,940 had no effect on mechanical or cold sensitivity in control $CB2R^{-/-}$ mice and $CB2R^{+/+}$ (Figure 3B, D). Conversely, CP55,940 increased heat sensitivity in control $CB2R^{-/-}$ mice but had no effect on control $CB2R^{+/+}$ (Figure 3C). CP55,940 treatment did not lead to catalepsy since the bar test (Figure 3E) did not show a significant difference from baseline in any group.

Sickle $CB2R^{-/-}$ and sickle $CB2R^{+/+}$ displayed similar pain behaviors at baseline and a significant decrease in hyperalgesia following CP55,940 treatment (Figure 3 A-D). However, significantly higher improvement in heat ($p < 0.001$) and deep tissue hyperalgesia ($p < 0.01$), was observed in sickle $CB2R^{-/-}$ compared to sickle $CB2R^{+/+}$ following CP55,940 treatment. Therefore, different pain phenotypes may utilize CB1R or CB2R to a variable extent to respond to cannabinoid therapy. Together, these data suggest that under both chronic and acute pain condition, activation of CB1R is critical

to ameliorate hyperalgesia, and CB2R may partly contribute to cananbinoid analgesia, perhaps by modulating inflammatory sickle pathobiology.

Cannabinoids ameliorate mast cell activation in SCA

We recently reported that mast cell activation occurs in SCA and contributes to hyperalgesia and observed a correlative increase in dorsal skin blood flow with neurogenic inflammation and mast cell activation.[3] Therefore, we analyzed if cannabinoids influence vascular flow and mast cell activation. Sickle mice treated with CP55,940 daily for 3 weeks showed a significant decrease in dorsal skin blood flow 1 hour after CP55,940 injection that persisted for the entire duration of treatment ($p<0.01$ Vs vehicle 1 hr after treatment and $p<0.001$ 1d, and 1, 2 and 3 wk; Figure 4A). Treatment with CP55,940 significantly decreased activation (degranulation) of mast cells in sickle mice compared to sickle mice treated with vehicle (Figure 4 B-D). Sickle mice treated with CP55,940 showed about 40% less activated mast cells compared to vehicle ($p<0.01$; Figure 4D). Similarly, mast cells isolated from the skin of sickle mice treated with CP55,940 exhibited lower immunoreactivity for *c-kit*, FcεRI and tryptase (Figure 4E) and released significantly less substance P (SP) and tryptase as compared to mast cells from vehicle treated mice ($p<0.05$ for both; Figure 4 F-G). Earlier we showed that mast cell activation contributes to inflammation in sickle mice by enhancing the release of several cytokines or chemokines.[3] We observed that CP55,940 treatment of sickle mice for 3 weeks significantly decreased the cytokines released from skin biopsies (IL-1α, IL-6, TNFα, MCP-1; $p < 0.01$) compared to vehicle treatment (Figure 2H). Consistent with decreased mast cell activation, treatment with CP55,940 lowered the levels of GM-CSF and RANTES, two chemokines involved in mast cell recruitment

and function[24, 25], by at least 35% ($p < 0.01$). GM-CSF plays a critical role in regulating leukocyte counts that is often elevated in SCA.[26] We have previously reported leukocytosis in sickle mice and have shown a role of mast cells in this process.[3] Treatment with CP55,940 significantly decreased WBC counts and sickle RBCs compared to vehicle under normoxia and following H/R incitement (Table 1). Thus CP55,940 treatment dampens the inflammatory response and sickling of RBCs by decreasing the activation of mast cells.

H/R-induced mast cell activation is attenuated by cannabinoids in a receptor-specific manner

Next we determined CBR-specific inhibition of mast cell activation in sickle mice under normoxia and H/R. Sickle mice showed a trend towards increase in mast cell activation following H/R injury as compared to normoxia (Figure 5 A-B). Additionally, treatment with CP55,940 for 7 days led to a significant reduction in mast cell activation, both under normoxia and following H/R compared to vehicle under respective conditions ($p<0.05$ for each condition; Figure 5B). Although, the CB1R agonist ACEA demonstrated appreciable inhibition of mast cell activation, CB2R agonist JWH-133 exhibited significant decrease in degranulating mast cells ($p<0.05$). Consistent with the inhibitory effect on mast cell activation, CP55,940 significantly reduced plasma tryptase, β-hexosaminidase and SAP after H/R injury in sickle mice compared to vehicle treatment ($p < 0.05$; Figure 5C). Serum SP level was elevated after H/R injury compared to normoxia in sickle mice ($p<0.05$; Figure 5D). CP55,940 treatment decreased SP under normoxia and following H/R injury ($p< 0.01$; Figure 5 D-E). Following H/R, CB2R agonist significantly reduced SP levels as compared to vehicle treatment ($p<0.05$; Fig. 5E). The

CB1R agonist tended to decrease serum SP but it was not statistically significant. Together, these data suggest that H/R-evoked mast cell activation leading to neuroinflammation is predominantly mediated by CB2R.

Cannabinoids reduce neurogenic inflammation

Earlier we found that mast cell activation contributes to neurogenic inflammation, in sickle mice.[3] Considering the H/R-induced mast cell activation observed above, we examined, the role of CBRs in ameliorating neurogenic inflammation. Evans blue (EB) leakage increased significantly in the skin of sickle mice following H/R incitement compared to normoxia ($p<0.05$; Figure 6A). CP55,940 decreased EB leakage in sickle mice under normoxia as well as following H/R ($p<0.001$; Figure 6A). Evoked leakage of EB by intradermal injection of capsaicin or SP is higher in sickle mice as compared to control mice under normoxia.[3] Treatment with CP55,940 or CB1R and CB2R agonists significantly reduced EB leakage evoked by capsaicin (CP, $p<0.001$; CB1R and CB2R $p<0.05$) and SP (CP, CB1R and CB2R $p<0.001$) as compared to vehicle (Figure 6 B-C). Thus CP55,940 reduces H/R mediated neurogenic inflammation via both CB1R and CB2R. Since neurogenic inflammation is orchestrated by peripheral nerves in conjunction with mast cell activation, it is likely that CB2R predominantly mediates the cannabinoid response on mast cells as observed above, while CB1R mediates the response on the peripheral nerves.

Discussion

Pain in SCA, may be a result of vascular dysfunction, inflammation and direct neural injury, involving multiple targets. Moreover, the unique acute pain due to “crisis” in

addition to chronic pain further adds to the complexity and heterogeneity as compared to severe pain in other conditions. Thus, it is not surprising that current pain management strategies are not always effective, requiring identification of therapeutic modalities acting on multiple targets peripherally and in the CNS. Cannabinoid receptors are unique targets because of their peripheral and central activity at a multicellular level.

Due to psychotropic effects of CB1R, the possibility to target CB2R devoid of psychotropic effects has gained attention.[27, 28] CB2R agonists and/or knockout mice provide compelling evidence that CB2R activation ameliorates neuropathic and inflammatory pain, and is protective against ischemia/reperfusion injury by decreasing the endothelial expression of adhesion molecules and secretion of chemokines [15, 29, 30], and by attenuating the leukocyte adhesion to endothelium, trans-endothelial migration, and interrelated oxidative/nitrosative damage [31, 32], all of which are consistent with the pathobiology of SCA. We show that targeting the cannabinoid receptors is effective in reducing inflammation, mast cell activation and neurogenic inflammation, which orchestrate pain.

Sickle mice exhibit spontaneous musculoskeletal pain and cutaneous hyperalgesia to mechanical, heat and cold stimuli.[4, 5] These symptoms recapitulate the pain phenotype observed in patients with SCA.[1, 20] Previously we observed that an acute dose of CP55,940, attenuated deep tissue hyperalgesia and CFA-induced mechanical hyperalgesia in sickle mice.[4, 5] Our present observations that sickle mice exhibit sustained analgesia over a 3-week chronic CP55,940 treatment, suggest that cannabinoid analgesia is devoid of tolerance.

Cannabinoids have been found to be protective against ischemia/reperfursion injury.[33] CP55,940 prevented sickling induced by H/R in sickle mice, suggesting that some of the analgesic effects of cannabinoids could be due to their effect on sickle pathobiology. Furthermore, treatment with specific CB1R (ACEA) and CB2R (JWH-133) agonists reduced deep hyperalgesia, but only CB1R agonist was able to reduce mechanical and thermal (heat and cold) hyperalgesia following H/R. Complementary to these observations, CP55,940 treatment had antihyperalgesic effect in HbSS CB2R$^{-/-}$ mice on mechanical and thermal (heat and cold) hyperalgesia but not on deep tissue hyperalgesia under normoxia. Therefore, cannabinoid analgesia is mediated through both CB1R and CB2R, which is specific to sickle pain phenotype. Thus, CBR agonists not only have an analgesic effect but also have a systemic effect on the disease pathophysiology because pre-treatment with cannabinoids for a week prevented H/R-induced hyperalgesia. Together, with our earlier studies demonstrating that CP55,940 is effective in decreasing chronic and CFA-induced hyperalgesia[4], present findings emphasize upon the analgesic potential of cannabinoids in ameliorating different pain phenotypes under normoxia (representing chronic pain) and under H/R (representing VOC). Importantly, present data support the use of both CB1R and CB2R agonists for overall analgesia, but depending upon the pain characteristic one or the other agonist may be potentially useful.

Mast cell activation contributes to sickle pathophysiology by mediating inflammation and pain.[3] Inflammatory mediators, proteases including tryptase and proinflammatory cytokines are released from mast cells upon activation and contribute to heightened inflammation in SCA. Tryptase, in addition to enhancing inflammation and neurogenic

inflammation, activates protease activated receptor 2 (PAR2) on peripheral nerve endings and promotes nociception.[3, 34, 35] Thus, sickle microenvironment favors persistent mast cell activation, consecutively causing nociceptor sensitization, which in turn aggravates hyperalgesia. Indeed our recent studies show nociceptor sensitization and activation of p38MAPK pathway in the spinal cords of sickle mice, suggestive of central sensitization.[36] Cannabinoid receptors, CB1R and CB2R are found on mast cells.[37, 38] Since, mast cells produce endocannabinoids, including anandamide, palmitoylethanolamide, and 2-arachidonylglycerol, a potential autocrine regulatory loop may exist.[39] Mast cells are tightly controlled by endocannabinoid system in the skin thereby limiting excessive activation and maturation. Human mucosal-type mast cells use CB1R mediated signaling to limit degranulation and maturation from progenitor mast cells.[37] Mast cell activation was attenuated following CP55,940 treatment with a correlative decrease in tryptase, SP and cytokines released from the skin and in cutaneous blood flow. Significantly higher acetylcholine-induced forearm blood flow has been reported in sickle patients as compared to normal subjects, and significantly increased blood flow was observed in females as compared to male sickle patients.[40] Sickle females were responsive to blood flow inhibition with nitric oxide synthase inhibitor, NG-monomethyl-L-arginine, but sickle males were not, suggestive of gender-based nitric oxide-dependent and -independent mechanisms involved. Since, CP55,940 inhibited blood flow in male mice in our study, it may be acting via NO-independent mechanisms, but may also inhibit NO-dependent blood flow in females, which requires further examination.

Mast cell activation also occurs in response to ischemia reperfusion injury.[41] Factors associated with mast cell activation were also reduced in H/R incited sickle mice following CP55,940 treatment. GM-CSF and WBC counts are elevated in SCA patients[26] and in sickle mice, which are further exaggerated upon H/R incitement.[3] Our finding that CP55,940 decreased GM-CSF levels, WBC counts and also the sickle RBCs, has important implications for improving VOC and the accompanying pain. Direct effect of CP55,940 on ameliorating sickling of RBCs is an exciting possibility, but it could also be due to an indirect effect, which warrant further examination. Importantly, the observed inhibitory effect of both CB1R and CB2R agonists on neurogenic inflammation, mast cell activation, suggests the beneficial effect of cannabinoids on complex inflammatory and vascular sickle pathobiology and associated conditions.

Several studies support the analgesic effect of cannabinoids in humans.[42, 43] Sativex, a cannabis derived oromucosal spray, containing equal proportions of THC and cannabidiol has shown to be effective in treating symptoms of multiple sclerosis, including spasticity and neuropathic pain.[44, 45] Sativex is also in two phase three trials for cancer pain and neuropathic pain.[46] Furthermore, Abrams[47] showed that using vaporized cannabis in conjunction with opioids augments the analgesic effects of opioids. Unfortunately, side effects associated with higher doses such as sedation, dizziness, blurred vision, impaired cognitive functioning and the risk for addiction limit the use of cannabinoids for therapy. However, targeting the CB1R and CB2R receptors simultaneously at the periphery would minimize the side effects and concurrently help in managing pain. Recent report by Khasabova[48] shows that the activation of peripheral CB1R and CB2R synergistically reduced tumor evoked hyperalgesia. A questionnaire-

based study evaluating the use of marijuana use in sickle patients found that 52% of patients, who indulged in marijuana, used it to reduce or prevent acute or chronic pain.[49, 50] Pain in SCA could be of mixed type including nociceptive, neuropathic and inflammatory mechanisms with the involvement of both peripheral and central nociceptor sensitization.[1] CB1R agonist was able to significantly improve hyperalgesia in the sickle mice, and CB2R agonists significantly attenuated mast cell activation and neurogenic inflammation, which may improve the condition of the systemic disease, consequently reducing pain. Since, deep hyperalgesia was ameliorated by CB2R agonist (JWH-133), therefore, targeting both CB1R and CB2R receptors simultaneously, may be of an advantage in targeting complex mixed type of pain in SCA. CP55,940 via CB2R was demonstrated to stimulate serotonin 2A receptor 5-HT(2A) activity in the pre-frontal cortex of rats, suggestive of an influence on cognitive and mood disorders.[51] Therefore, the effect of cannabinoids on neuropsychiatric conditions in SCA requires consideration.

Interestingly we did not see an increase in hyperalgesia with the deletion of CB2R in either control or sickle mice. Earlier studies in the CB2R deleted C57BL/6 mice did not show an effect on baseline hyperalgesia as compared to wild type C57BL/6 in PWL to heat or mechanical allodynia using von Frey filaments or in tail withdrawal assay.[28, 52] In this study on $CB2R^{-/-}$ mice, an effect on morphine-induced antinociception was observed only in the early inflammatory phase of formalin-induced nociception, which diminished later (after 60 min). Similarly, we observed an increase in PWL in control $CB2R^{-/-}$, following CP55,940 treatment which could be due to an increase in inflammation in $CB2R^{-/-}$ and may demonstrate an anti-inflammatory effect of CP55,940

perhaps via CB1R. An increase in grip force in control mice occurred following CP55,940 treatment but not in the control CB2R$^{-/-}$, suggesting that CB2R is required for ameliorating deep hyperalgesia. Similar to our observations of no effect of CP55,940 on mechanical hyperalgesia but an increase in heat PWL in control CB2R$^{-/-}$ mice, in a previous study WIN 55,212-2 did not influence mechanical hyperalgesia but led to an increase in PWL-heat in CB2R$^{-/-}$ C57BL/6 mice in a model of neuropathic pain [28]. These data suggest a role of CB2R in the antiallodyanic effect in a neuropathic pain model. In the sickle mice we observed a uniform effect of CP55,940 on deep tissue, mechanical and thermal hyperalgesia. This shows the diverse pathobiology of sickle pain perhaps comprised of inflammation and neuropathy, which would require both CB1R and CB2R agonists for analgesia.

CB1R-mediated psychotropic effects and utilization of smoked Cannabis are major deterrants in the use of Cannabis as medicine.[53] However, recent discovery of CBR-specific agonists and delivery following vaporization provide an advantage to use cannabinoids in the medical setting following well-controlled clinical trials.[47] Societal stigma against “marijuana” also calls for the development of Cannabis derived medications in user-friendly drug-delivery systems to dignify their use. Evidence-based knowledge for Cannabis-derived medications, their dosage and side-effects need to be determined in disease-specific pre-clinical and clinical investigations, as emphasized recently.[53, 54] It is noteworthy, that in the states where medical Cannabis has been legalized in the United States, mean annual opioid overdose mortality rates between 1999 and 2010 were reduced by 24.8% (95% CI, -37.5% to -9.5%; P = 0.003).[55] Pain is associated with poor quality of life and increased morbidity in SCA and opioids remain

the mainstay of therapy.[1] Therefore, our observations in a pre-clinical setting of SCA provide a compelling rationale to examine the potential of CBR-specific agonists and cannabinoids to treat pain and ameliorate associated pathobiology in SCA.

Acknowledgments

Funding: This work was supported by NIH RO1 Grants HL68802 and 103773 and UO1 HL117664 and Institute for Engineering in Medicine grants to KG. Confocal imaging was performed using the Olympus FluoView 1000 IX2 instrument at the University of Minnesota - University Imaging Centers, http://uic.umn.edu. The authors would like to thank Stefan Kren, Katherine NH Johnson, Sugandha Rajput, Ritu Jha and Susan Thompson for breeding, genotyping, phenotyping mice and/or technical assistance; Drs. Robert P. Hebbel and David Archer for providing breeder sickle mice; Drs. David Largaespada and Anindya Bagchi for advice on mouse genetics; Dr Donald A. Simone for a critical review of the manuscript; Michael J. Franklin and Carol Taubert for editorial assistance.

Footnotes

Authorship and Disclosure: Online

Conflict of Interest: The authors declare no competing financial interests.

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Table 1. CP55,940 effect on hematological parameters in SCA.

	NORMOXIA				HYPOXIA/REOXYGENATION			
	HbAA-BERK		HbSS-BERK		HbAA-BERK		HbSS-BERK	
Parameter	**Veh**	**CP55,940**	**Veh**	**CP55,940**	**Veh**	**CP55,940**	**Veh**	**CP55,940**
Peripheral Blood								
RBC (K/µL)	11.4 ± 0.3	11.2 ± 0.1	10.0 ± 0.3	10.2 ± 0.1	11.0 ± 0.3	10.2 ± 0.1	9.1 ± 0.2	9.3 ± 0.3
Total Hb (g/dL)	12.7 ± 0.6	12.7 ± 0.5	10.2 ± 0.4*	9.8 ± 0.3*	12.9 ± 0.7	12.5 ± 0.3	9.6 ± 0.3$^{\#}$	9.9 ± 0.6
Hct (%)	45.2 ± 1.0	44.4 ± 0.9	41.6 ± 1.0**	41.5 ± 0.8	45.6 ± 1.1	43.5 ± 0.9	40.8 ± 1.1$^{\#\#}$	40.1 ± 0.9$^{\#\#}$
WBC (K/µL)	7.3 ± 0.4	7.1 ± 0.3	18.7 ± 0.5***	15.1 ± 0.4**¶	8.9 ± 0.3	7.9 ± 0.3	22.8 ± 0.8$^{¶\#\#\#}$	16.9 ± 0.9$^{\#\#Ŧ Ŧ}$
Neutrophils (K/µL)	1.7 ± 0.2	1.5 ± 0.2	7.8 ± 0.2***	6.4 ± 0.2**¶	2.1 ± 0.2	1.9 ± 0.2	9.0 ± 0.2$^{\#\#\#}$	5.6 ± 0.2$^{\# Ŧ}$
Lymphocytes (K/µL)	4.3 ± 0.4	4.3 ± 0.3	6.3 ± 0.4*	5.7 ± 0.3	5.3 ± 0.4	4.9 ± 0.3	7.5 ± 0.5$^{\#}$	6.0 ± 0.6
Monocytes (K/µL)	0.2 ± 0.1	0.3 ± 0.1	1.1 ± 0.1*	0.5 ± 0.1	0.5 ± 0.1	0.4 ± 0.1	1.4 ± 0.2$^{\#}$	0.6 ± 0.1^{Ŧ}
RBC indices								
Sickle RBC (% tot)	n/a	n/a	28.9 ± 1.8	18.4 ± 1.2¶¶	n/a	n/a	37.9 ± 1.8¶¶	23.8 ± 1.5^{¶Ŧŧŧ}

Complete blood counts were measured from whole blood after 7 days of treatment with vehicle (Veh) or CP55,940. At D7, mice were separated in 2 groups: normoxia group (control condition) or hypoxia/reoxygenation group where mice were treated with 3h of hypoxia and 1h of reoxygenation (H/R). Blood was collected on D8, (24h post end of treatment or post H/R). RBC, red blood cells; Hb, hemoglobin; Hct, hematocrit; WBC, white blood cell; n/a, not applicable. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ versus HbAA-BERK Veh Normoxia; ¶ $p < 0.05$, ¶¶ $p < 0.01$ versus HbSS- BERK Veh Normoxia; # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ versus HbAA-BERK Veh H/R; Ŧ $p < 0.05$, ŦŦ $p < 0.01$, ŦŦŦ $p < 0.001$ versus HbSS-BERK Veh H/R. *n* = 5 male mice for each group. Data are mean ± SEM (ANOVA, with Bonferroni's).

Figure Legends

Figure 1. Acute and chronic treatment with CP55,940 decreases hyperalgesia in sickle mice. Sickle mice were treated with vehicle (Veh) or CP55,940 (0.3 mg/kg/day) for 3 weeks. Pain measures were obtained before starting the drug treatments on D0 (baselines, BL), and periodically following the treatment. Measures of (**A**) deep tissue pain (grip force), (**B,C**) mechanical hyperalgesia (threshold and PWF), (**D to F**) thermal sensitivity to heat and cold, and (**G**) catalepsy are shown. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ Veh vs CP; † $p < 0.05$, †† $p < 0.01$ Vs BL of matching group (ANOVA, with Bonferroni's, see Online Supplementary Table S1 for summary of F (DFn, DFd)). Each value is the mean ± SEM from 8 male mice (~5 month old) with 3 observations per mouse. Abbreviations, PWF, paw withdrawal frequency; PWL, paw withdrawal latency; Veh, vehicle.

Figure 2. Cannabioids ameliorate hypoxia/reoxygenation-evoked hyperalgesia in a receptor specific manner. Sickle mice (HbSS) were treated with vehicle (Veh), CP55,940, CB1R agonist (ACEA) or CB2R agonist (JWH-133) for 7 days. All mice were then treated with 3h of hypoxia and 1h of reoxygenation (H/R). Pain measures were obtained before starting the drug treatments on D0 (BL) and at the conclusion of drug treatments, D7 prior to inciting H/R, immediately after H/R and periodically up to 24h after H/R. Measures of, (**A**) deep pain, (**B**) mechanical hyperalgesia and (**C-D**) thermal sensitivity to heat and cold, respectively are shown. ¶ $p < 0.05$, ¶¶ $p < 0.01$ Vs baseline (BL) of matching group; † $p < 0.05$ Vs Day 7 (D7) of matching group; * $p < 0.05$, ** $p < 0.01$ Vs Veh of matched time point. (Two-way ANOVA, with Bonferroni's, see Online Supplementary Table S1 for summary of F (DFn, DFd)). Each value is the mean ± SEM

from 5 male mice (4-5 month old) with 3 observations per mouse. Abbreviations, H/R, hypoxia reoxygenation.

Figure 3. Cannabinoid analgesia is modulated in CB2R knockout sickle mice. HbAA-CB2R$^{+/+}$, HbAA-CB2R$^{-/-}$, HbSS-CB2R$^{+/+}$ and HbSS-CB2R$^{-/-}$ mice were treated with a single injection of CP55,940 (0.3 mg/kg, i.p.). Pain measures were obtained before starting the drug treatments (BL), and periodically after the injection. Measures of, (**A**) deep pain, (**B**) mechanical hyperalgesia and (**C-D**) thermal sensitivity to heat and cold, respectively, and (**E**) catalepsy are shown. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ Vs HbAA-CB2-R+/+ of matching time point; $p < 0.05$, $p < 0.01$ Vs HbSS-CB2-R+/+ of matching time point; † $p < 0.05$, †† $p < 0.01$ Vs baseline (BL) of matching group (ANOVA, with Bonferroni's, see Online Supplementary Table S1 for summary of F (DFn, DFd). Each value is the mean ± SEM from 5 mice (3 males and 2 females, ~4.5 month old) with 3 observations per mouse.

Figure 4. CP55,940 reduces mast cell activation. Mice were treated with CP55,940 (0.3 mg/kg/day, i.p.) or vehicle for 3 weeks and analyzed as described. (**A**) Measures of cutaneous blood flow in the dorsal skin. ** $p < 0.01$, *** $p < 0.001$; † $p < 0.01$ Vs respective baseline before starting the treatments. Each value is the mean ± SEM from 8 male mice (~5 month old) with 3 observations per mouse. (**B,C**) Representative images of Toluidine blue stained dorsal skin sections of HbSS mice treated for 3 weeks with vehicle (Veh) or CP55,940 (CP). Each image representative of images from 5 mice per condition. Scale bar = 100 µm. (**D**) Ratio of degranulating/total mast cells. * $p < 0.01$; † $p < 0.05$, †† $p < 0.001$ Vs HbAA Veh. (**E**) Representative confocal images of

skin mast cells in culture stained for *c-kit*/CD117 (red), FcεRI (green), and tryptase (blue). Scale bar = 5 µm. *n=5.* (**F,G)** Substance P and tryptase in mast cell conditioned medium. *p<0.05; **p<0.01; †p<0.05 Vs HbSS Veh. (**H**) Skin biopsies were incubated in culture medium for 24 hours and cytokines released in conditioned medium were analyzed. HbSS BERK Veh and HbSS CP55,940, red and blue bars, respectively. Values are expressed as a percent of HbSS Veh. * p < 0.05, ** p <0.01 Vs HbSS Veh (ANOVA, with Bonferroni's, see Online Supplementary Table S1 for summary of F (DFn, DFd)). Each value is the mean ± SEM from 5 male mice (~5 month old).

Figure 5. CP55,940 reduces hypoxia/reoxygenation-evoked mast cell activation. Mice were treated with vehicle (Veh), CP55,940 (CP), CB1R agonist (CB1-R Ag, ACEA) or CB2R agonist (CB2-R Ag, JWH-133) for 1 week followed by normoxia (N) or hypoxia/reoxygenation (H/R) and analyzed as described. (**A**) Representative images of Toluidine blue stained dorsal skin sections of HbSS mice. Each image representative of images from 5 male mice per condition. Scale bar = 50 µm. (**B**) Ratio of degranulating/total mast cells. * p < 0.05 Vs HbSS Veh H/R (**C**) Levels of tryptase, β-hexosaminidase (β-hex) serum amyloid protein (SAP) after H/R. * p < 0.05, ** p < 0.01 Vs HbSS Veh normo, † p < 0.05 Vs HbSS Veh H/R (ANOVA, with Bonferroni's, see Online Supplementary Table S1 for summary of F (DFn, DFd)). (**D-E**) Levels of substance P in HbSS mice in normoxia or after H/R injury. Substance P expressed as the percentage of HbAA Veh in normoxia (C,E) or HbSS Veh (D). Each value is the mean ± SEM from 5 male mice, ~5 month old, in B-E.

Figure 6. CP55,940 reducec H/R injury-evoked neurogenic inflammation. (A) Spontaneous Evans blue leakage in HbSS treated with vehicle, or CP55,940 (0.3 mg/kg/day) for 1 week, under normoxia or after hypoxia/reoxygenation (H/R). **p < 0.0001; *p<0.05. **(B)** Evans blue leakage evoked by injection of saline, capsaicin, or substance P in the dorsal skin of HbSS mice treated with vehicle, CP55,940 (0.3 mg/kg), CB1R agonist (CB1-R Ag, ACEA) or CB2R agonist (CB2-R Ag, JWH-133) for 7 days followed by H/R. * p < 0.05, ** p < 0.001 Vs. Veh for each treatment; † p < 0.05 Vs CP55,940 (ANOVA, with Bonferroni's, see Online Supplementary Table S1 for summary of F (DFn, DFd)). (**C**) Images showing Evans blue leakage in the dorsal skin of sickle mice after H/R. Each image represents reproducible images from 5 male mice each; and each value is the mean ± SEM from 5 male mice, ~5 month old.

Figure 1

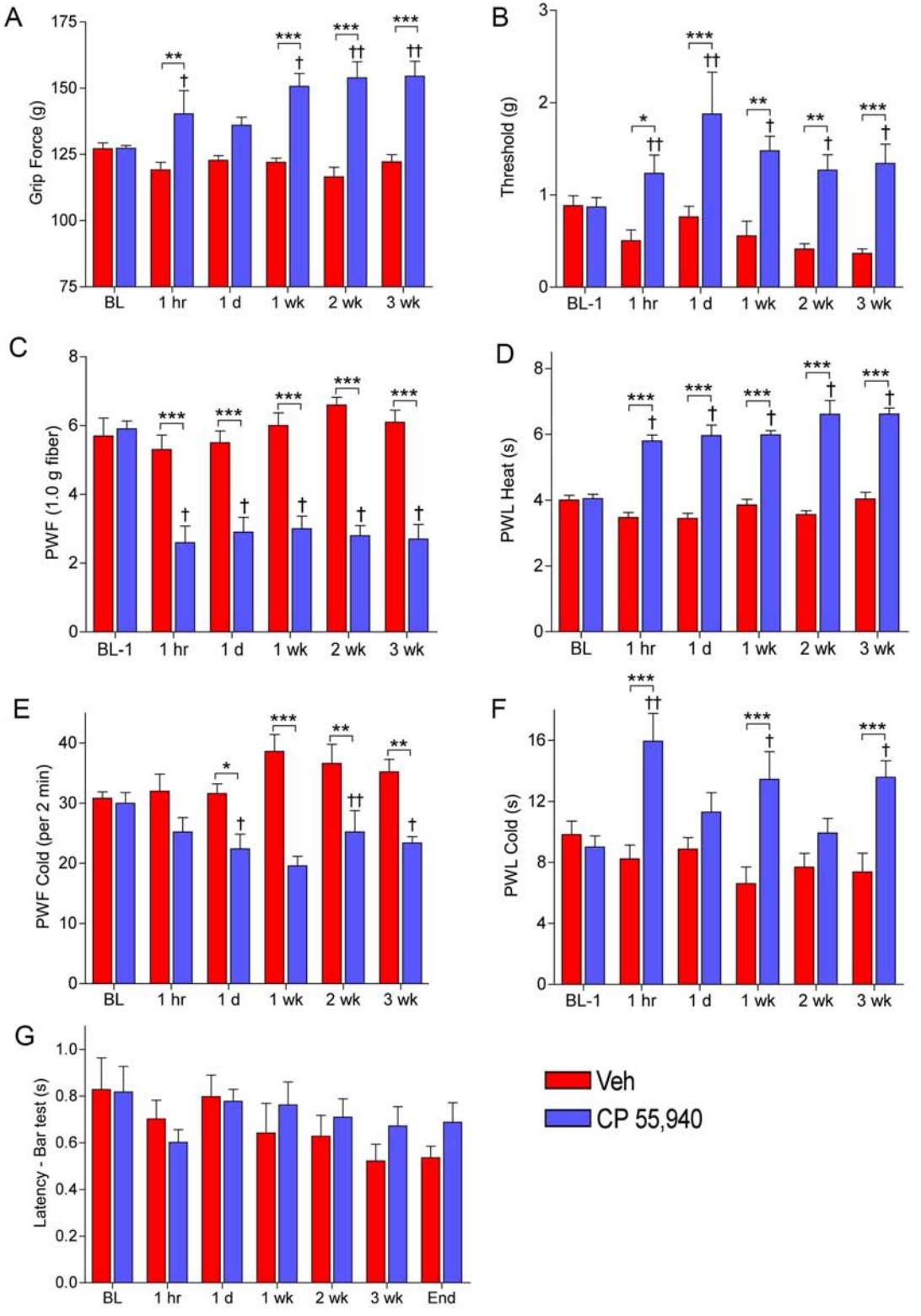

A
Grip Force (g)
175
150
125
100
75
BL
1 hr
1 d
1 wk
2 wk
3 wk
B
Threshold (g)
3
2
1
0
BL-1
1 hr
1 d
1 wk
2 wk
3 wk
C
PWF (1.0 g fiber)
8
6
4
2
0
BL-1
1 hr
1 d
1 wk
2 wk
3 wk
D
PWL Heat (s)
8
6
4
2
0
BL
1 hr
1 d
1 wk
2 wk
3 wk
E
PWF Cold (per 2 min)
40
30
20
10
0
BL
1 hr
1 d
1 wk
2 wk
3 wk
F
PWL Cold (s)
16
12
8
4
0
BL-1
1 hr
1 d
1 wk
2 wk
3 wk
G
Latency - Bar test (s)
1.0
0.8
0.6
0.4
0.2
0.0
BL
1 hr
1 d
1 wk
2 wk
3 wk
End
Veh
CP 55,940

Figure 2

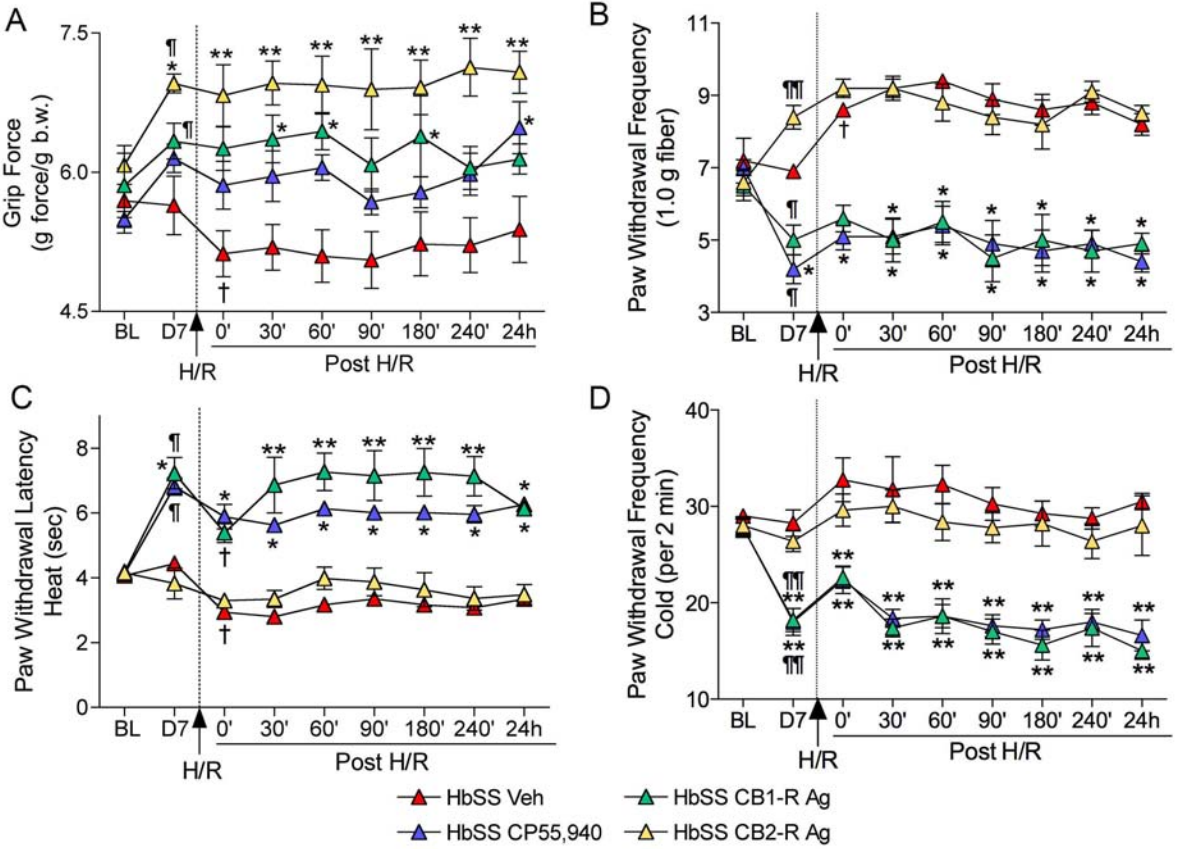

A
Grip Force
(g force/g b.w.)
7.5
6.0
4.5
BL
D7
H/R
0'
30'
60'
90'
180'
240'
24h
Post H/R
B
Paw Withdrawal Frequency
(1.0 g fiber)
11
9
7
5
3
C
Paw Withdrawal Latency
Heat (sec)
8
6
4
2
0
D
Paw Withdrawal Frequency
Cold (per 2 min)
40
30
20
10
HbSS Veh
HbSS CB1-R Ag
HbSS CP55,940
HbSS CB2-R Ag

Figure 3

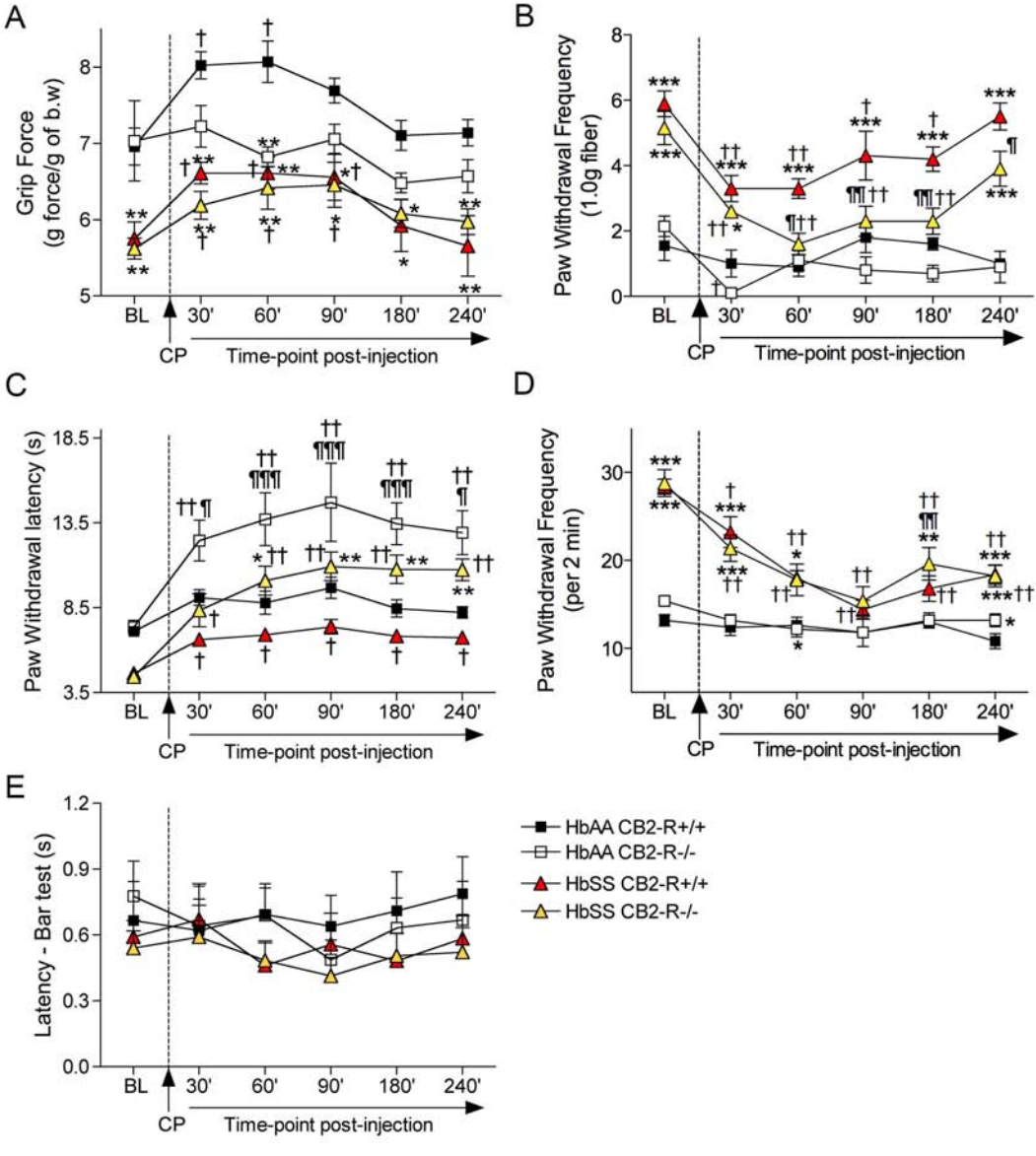

A
Grip Force
(g force/g of b.w)
8
7
6
5
BL
CP
30'
60'
90'
180'
240'
Time-point post-injection
B
Paw Withdrawal Frequency
(1.0g fiber)
8
6
4
2
0
BL
CP
30'
60'
90'
180'
240'
Time-point post-injection
C
Paw Withdrawal latency (s)
18.5
13.5
8.5
3.5
BL
CP
30'
60'
90'
180'
240'
Time-point post-injection
D
Paw Withdrawal Frequency
(per 2 min)
30
20
10
BL
CP
30'
60'
90'
180'
240'
Time-point post-injection
E
Latency - Bar test (s)
1.2
0.9
0.6
0.3
0.0
BL
CP
30'
60'
90'
180'
240'
Time-point post-injection
HbAA CB2-R+/+
HbAA CB2-R-/-
HbSS CB2-R+/+
HbSS CB2-R-/-

Figure 4

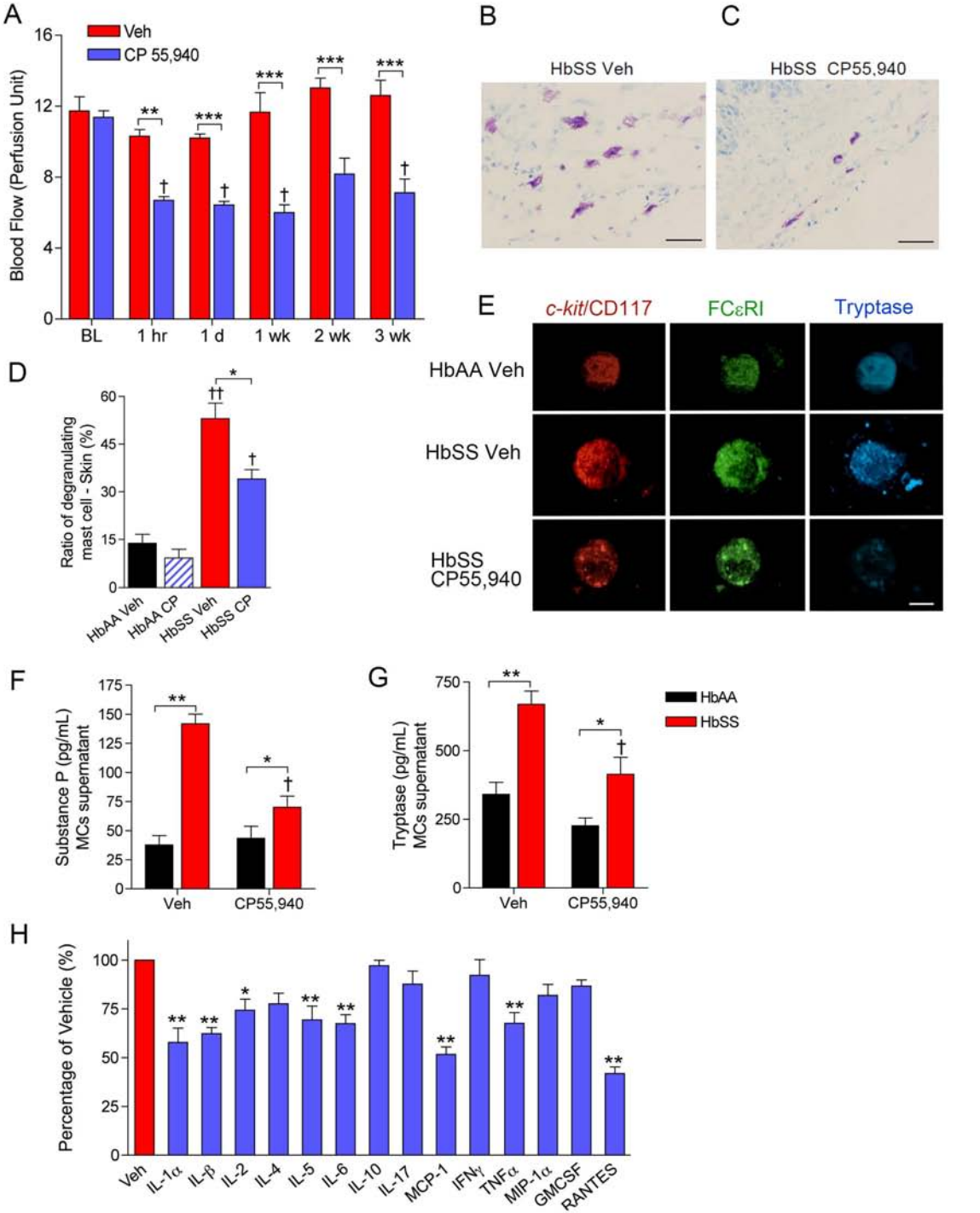

A
Veh
CP 55,940
Blood Flow (Perfusion Unit)
16
12
8
4
0
BL
1 hr
1 d
1 wk
2 wk
3 wk
B
HbSS Veh
C
HbSS CP55,940
D
Ratio of degranulating mast cell - Skin (%)
60
45
30
15
0
HbAA Veh
HbAA CP
HbSS Veh
HbSS CP
E
c-kit/CD117
FCεRI
Tryptase
HbAA Veh
HbSS Veh
HbSS CP55,940
F
Substance P (pg/mL) MCs supernatant
175
150
125
100
75
50
25
0
Veh
CP55,940
G
Tryptase (pg/mL) MCs supernatant
750
500
250
0
HbAA
HbSS
Veh
CP55,940
H
Percentage of Vehicle (%)
100
75
50
25
0
Veh
IL-1α
IL-β
IL-2
IL-4
IL-5
IL-6
IL-10
IL-17
MCP-1
IFNγ
TNFα
MIP-1α
GMCSF
RANTES

Figure 5

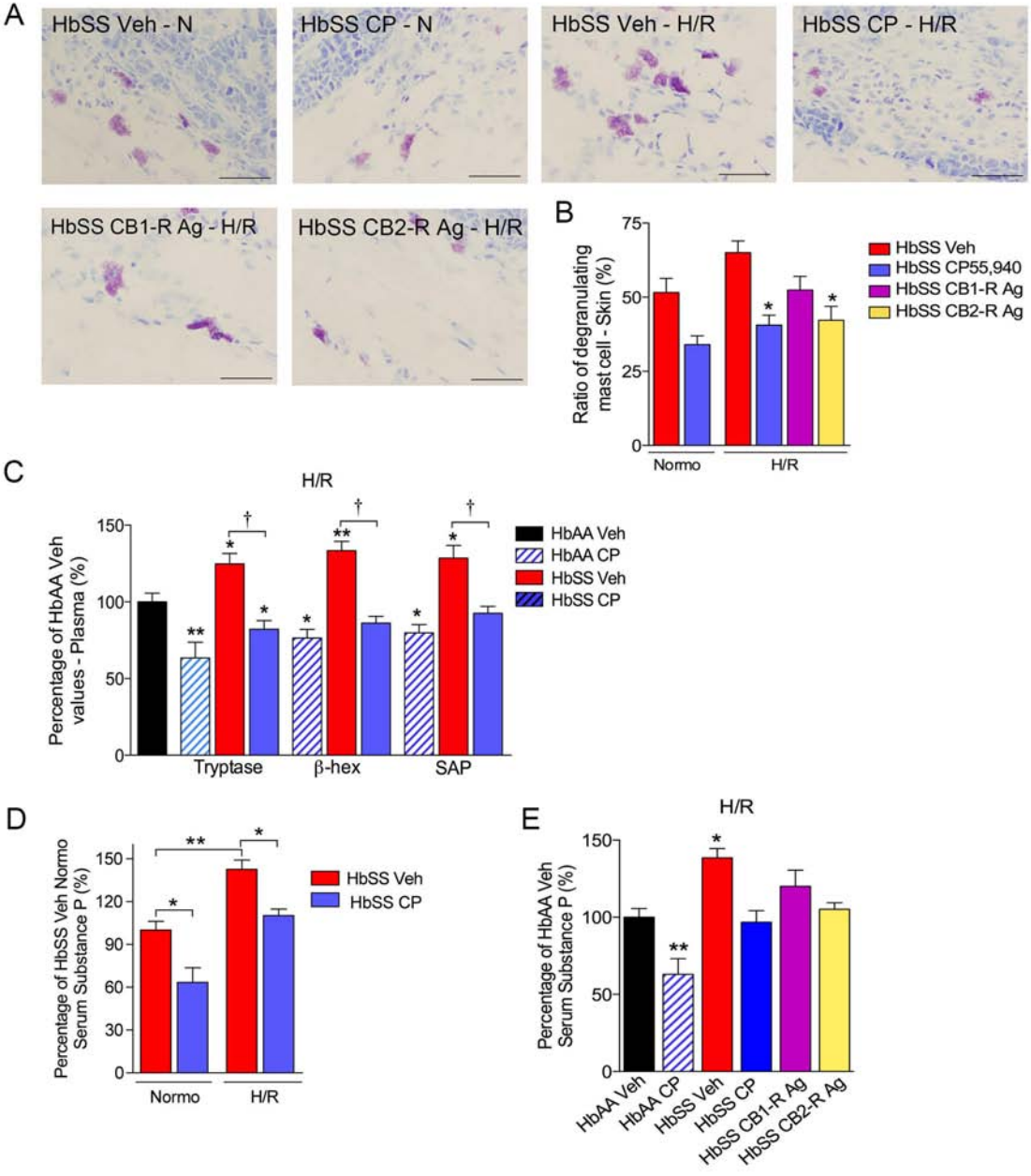
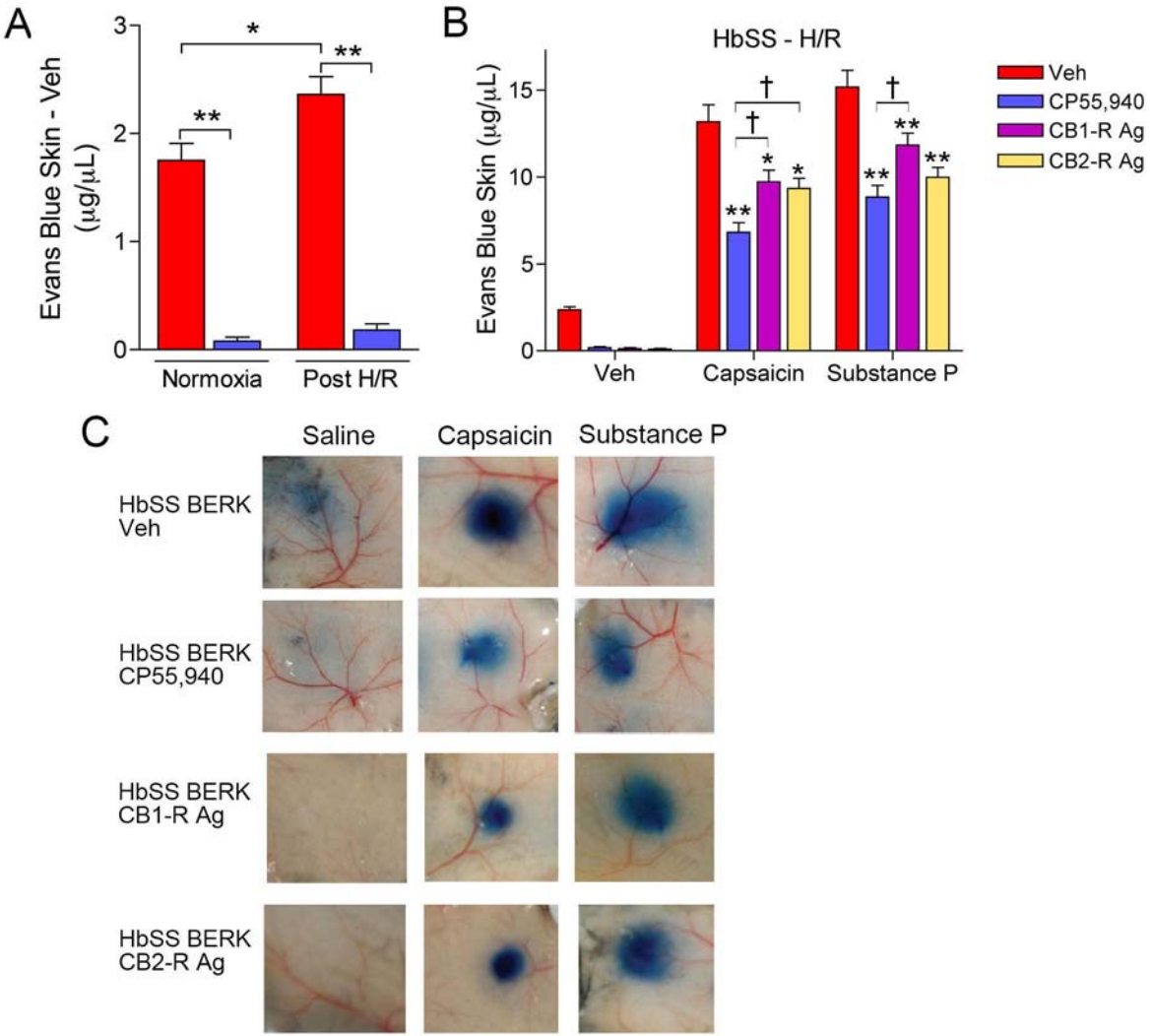

A
HbSS Veh - N
HbSS CP - N
HbSS Veh - H/R
HbSS CP - H/R
HbSS CB1-R Ag - H/R
HbSS CB2-R Ag - H/R
B
Ratio of degranulating mast cell - Skin (%)
75
50
25
0
Normo
H/R
HbSS Veh
HbSS CP55,940
HbSS CB1-R Ag
HbSS CB2-R Ag
C
H/R
Percentage of HbAA Veh values - Plasma (%)
150
100
50
0
Tryptase
β-hex
SAP
HbAA Veh
HbAA CP
HbSS Veh
HbSS CP
D
Percentage of HbSS Veh Normo Serum Substance P (%)
150
120
90
60
30
0
Normo
H/R
HbSS Veh
HbSS CP
E
H/R
Percentage of HbAA Veh Serum Substance P (%)
150
100
50
0
HbAA Veh
HbAA CP
HbSS Veh
HbSS CP
HbSS CB1-R Ag
HbSS CB2-R Ag

Figure 6


A
Evans Blue Skin - Veh (μg/μL)
Normoxia
Post H/R
B
HbSS - H/R
Evans Blue Skin (μg/μL)
Veh
Capsaicin
Substance P
Veh
CP55,940
CB1-R Ag
CB2-R Ag
C
Saline
Capsaicin
Substance P
HbSS BERK Veh
HbSS BERK CP55,940
HbSS BERK CB1-R Ag
HbSS BERK CB2-R Ag

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

Lucile Vincent[1], Derek Vang[1], Julia Nguyen[1], Barbara Benson[1], Jianxun Lei[1], and Kalpna Gupta[1*]

[1]Vascular Biology Center, Division of Hematology, Oncology and Transplantation, Department of Medicine, University of Minnesota, Minneapolis, MN, USA.

Correspondence should be addressed to
Kalpna Gupta; E-mail: gupta014@umn.edu

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.

Supplementary References

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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.