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

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ABSTRACT

Sickle cell anemia is a manifestation of a single point mutation in hemoglobin, 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 its side effects and ability to activate mast cells. We, therefore, examined cannabinoid receptor-specific mechanisms to mitigate mast cell activation, neurogenic inflammation and hyperalgesia, using HbSS-BERK sickle and cannabinoid receptor-2-deleted sickle mice. We show that cannabinoids mitigate 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 'proof of principle' for the potential of cannabinoid/cannabinoid receptor-based therapeutics to treat several manifestations of sickle cell anemia.

Introduction

Sickle-cell anemia (SCA) is one of the most common inherited disorders and is associated with both unpredictable recurrent acute pain and chronic pain[1]. Morphine, an opioid, has been the drug of choice for the treatment of severe pain associated with SCA.[1,2] However, 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 mitigate chronic and hypoxia/reoxygenation (H/R)-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 investigated cannabinoid receptor-specific modulation of vascular function, inflammation and hyperalgesia.

Cannabinoid receptors, CB1R and CB2R, are expressed in both the central nervous system and non-central nervous system 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 in neuroinflammation and the CB2R agonist, JWH-133, mitigates stress-related neuroinflammation-dependent pathologies.[18,19] Selective activation of peripheral cannaboid receptors is appealing because it would avoid neuropsychiatric adverse effects associated with activation of CB1R in the central nervous system.

Sickle mice display neurogenic inflammation and hyperalgesia *via* a mast-cell-dependent mechanism.[3] Cannaboid receptors 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/reperfu-

sion injury and inflammation are hallmark features of SCA, we hypothesized that targeting specific cannaboid receptors may have beneficial effects on sickle pathobiology and pain. We used transgenic HbSS-BERK mice, hereafter referred to as sickle mice, which show features of pain and inflammation similar to patients with SCA,[4,5,20] and sickle mice with deletion of CB2R, to examine the contribution of each cannaboid receptor in mast cell activation, neurogenic inflammation, and pain.

Methods

The procedures are described in detail 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 hemoglobin (S), demonstrating severe sickle cell disease, or normal (A) hemoglobin.[4,5,21]

CB2R knockout (CB2R-/-) mice: CB2R-/- mice (Stock # 005786; Jackson Laboratory, Bar Harbor, ME, USA) were backcrossed with BERK mice 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 polymerase chain reaction with primers specific for the CB2R (Cnr2) gene (Jackson Laboratory). Sickle (HbSS) and control (HbAA) mice were bred and phenotyped for sickle and normal human hemoglobin by iso-electric focusing[4] and genotyping for the knockout and hemoglobin 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% dimethylsulfoxide (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 cannaboid receptors, 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 3 h followed by re-oxygenation in room air for 1 h.[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 the endpoint of the study, 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 enzyme-linked immunosorbent assays.[3] Degranulating mast cells in skin sections were quantified and cultured mast cells from skin were immunostained 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, USA).

Hematopathology of blood

Hematocrit, total hemoglobin, complete blood counts and red cell indices (% sickle red blood cells) were determined as previously described.[3]

Statistical analysis

All data were analyzed using Prism software (v 5.0a, GraphPad Prism Inc., San Diego, CA, USA). Repeated measures analysis of variance (ANOVA) with the Bonferroni 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 statistically significant. All data are presented as mean ± standard error of mean (SEM).

Results

Cannabinoids mitigate 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 H/R evoked acute hyperalgesia simulating the pain of a vaso-occlusive crisis.[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 relieved 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; we, therefore, examined whether chronic treatment with CP55,940 had 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 1A-F). The effect of CP55,940 was sustained over a period of 3 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 animals treated with CP55,940 or vehicle (Figure 1G).

Cannabinoids mitigate hyperalgesia via cannabinoid receptors

Using pharmacological and genetic approaches we analyzed whether cannabinoids relieved chronic and acute

hyperalgesia *via* CB1R and/or CB2R. Sickle mice were treated with vehicle, CP55,940, the CB1R agonist ACEA, or the CB2R agonist JWH-133, for a week (normoxia), followed by 3 h of hypoxia and 1 h 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 periods. Under normoxic conditions 7 days of treatment with CP55,940 and the CB1R agonist ACEA significantly reduced deep tissue, mechanical and thermal (heat and cold) hyperalgesia as compared to the levels at baseline (*P*<0.05) or in 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
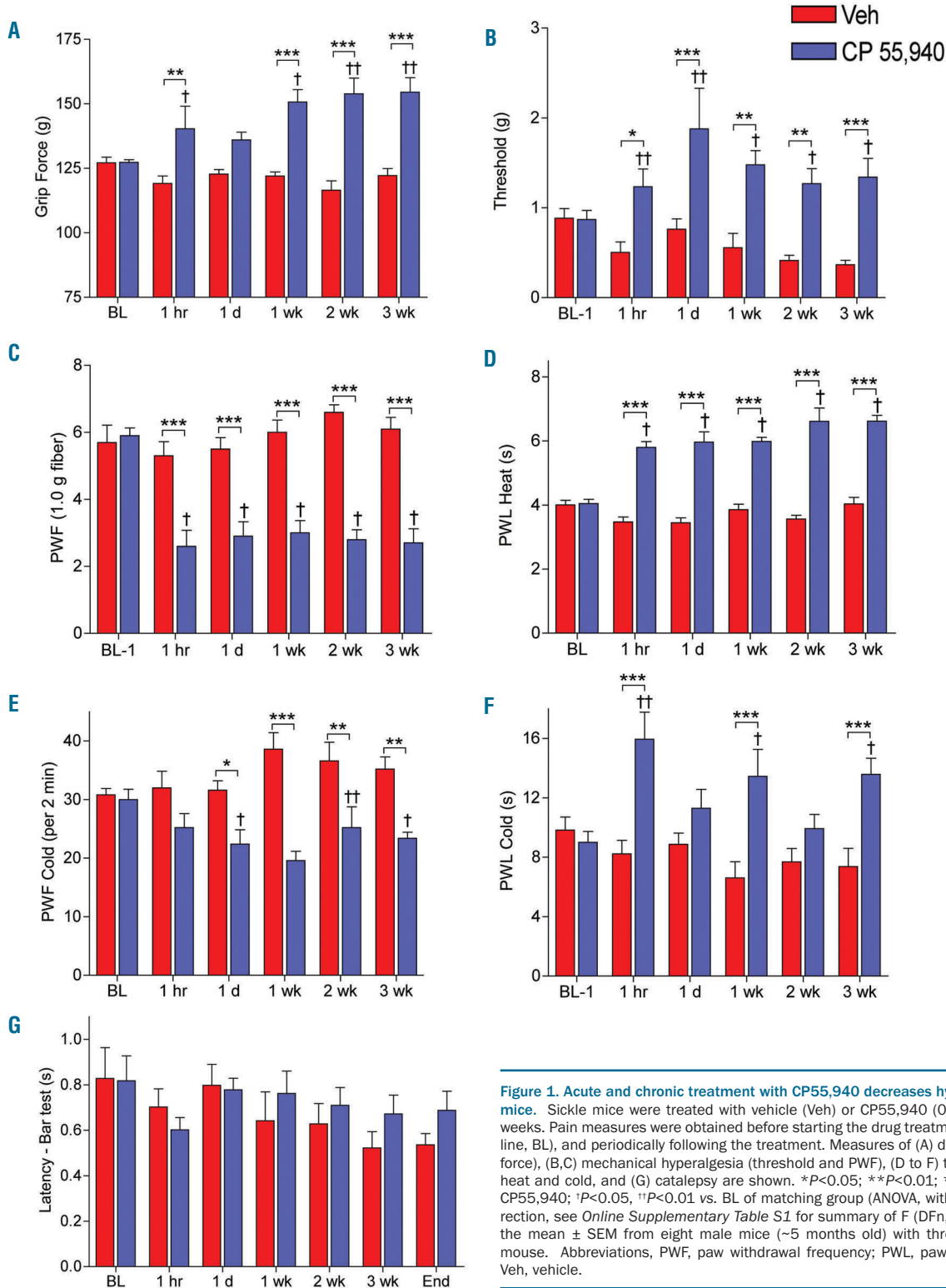

Figure 1. Acute and chronic treatment with CP55,940 decreases hyperalgesia in sickle mice. Sickle mice were treated with vehicle (Veh) or CP55,940 (0.3 mg/kg/day) for 3 weeks. Pain measures were obtained before starting the drug treatments on day 0 (baseline, BL), and periodically following the treatment. 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.* CP55,940; †P<0.05, ††P<0.01 *vs.* BL of matching group (ANOVA, with the Bonferroni correction, see *Online Supplementary Table S1* for summary of F (DFn, DFd). Each value is the mean ± SEM from eight male mice (~5 months old) with three observations per mouse. Abbreviations, PWF, paw withdrawal frequency; PWL, paw withdrawal latency; Veh, vehicle.

of treatment ($P<0.05$ *versus* baseline or vehicle; Figure 2A). The CB2R agonist did not show a significant effect on mechanical or thermal (heat and cold) hyperalgesia (Figure 2B-D). Thus, under normoxic conditions representative of chronic pain in SCA, the CB1R agonist as well as the non-selective cannaboid receptor 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, the CB2R agonist only mitigated 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 attenuated by a single injection of CP55,940.[4] Here we examined whether treatment with cananbinoids could prevent HR-evoked hyperalgesia. Pre-treatment of mice with CP55,940, and the CB1R agonist for 7 days decreased tonic hyperalgesia and also prevented H/R-evoked deep tissue, mechanical and thermal hyperalgesia (Figure 2A-D). However, treatment with the CB2R agonist decreased tonic as well as H/R-

evoked deep tissue hyperalgesia (Figure 2A) but did not reduce tonic or H/R-evoked mechanical or thermal (heat and cold) hyperalgesia (Figure 2B-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 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 or CB2R$^{+/+}$ mice (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$^{+/+}$ mice displayed similar
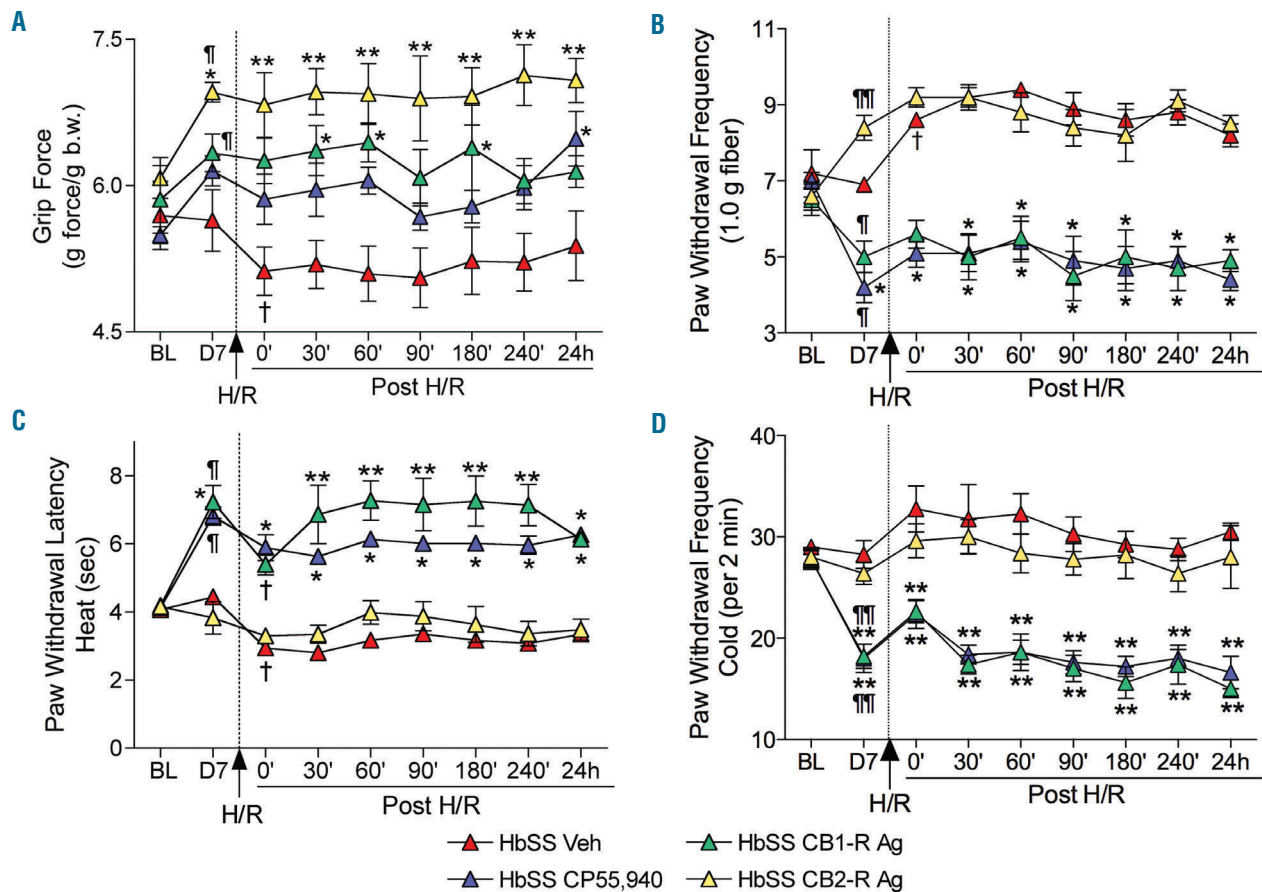

Figure 2. Cannabioids attenuate hypoxia/reoxygenation-evoked hyperalgesia in a receptor-specific manner. Sickle mice (HbSS) were treated with vehicle (Veh), CP55,940, CB1R agonist (ACEA) or CB2R agonist (JWH-133) for 7 days. All mice were then treated with 3 h of hypoxia and 1 h of reoxygenation (H/R). Pain measures were obtained before starting the drug treatments on day 0 (baseline, BL) and at the conclusion of drug treatments, day 7 (D7) prior to H/R, immediately after H/R and periodically up to 24 h after H/R. Measures of (A) deep pain, (B) mechanical hyperalgesia and (C-D) thermal sensitivity to heat and cold are shown. †$P<0.05$, ¶¶$P<0.01$ *vs.* 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 the Bonferroni correction, see *Online Supplementary Table S1* for the summary of F (DFn, DFd). Each value is the mean ± SEM from five male mice (4-5 months old) with three observations per mouse. Abbreviations, H/R, hypoxia/reoxygenation.

pain behaviors at baseline and a significant decrease in hyperalgesia following CP55,940 treatment (Figure 3A-D). However, significantly greater relief from heat (*P*<0.001) and deep tissue hyperalgesia (*P*<0.01) was observed in sickle CB2R$^{-/-}$ mice compared to sickle CB2R$^{+/+}$ mice following CP55,940 treatment. Thus, CB1R or CB2R may be used to variable extents to respond to cannabinoid therapy in cases of different pain phenotypes. Together, these data suggest that under conditions of both chronic and acute pain, activation of CB1R is critical to attenuate hyperalgesia, and CB2R may partly contribute to cananbinoid analgesia, perhaps by modulating inflammatory sickle pathobiology.

Cannabinoids attenuate mast cell activation in sickle cell anemia

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] We, therefore, analyzed whether 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 h after CP55,940 injection and the decrease persisted for the entire duration of treatment (*P*<0.01 *versus* vehicle 1 h after treatment and *P*<0.001 1 day, and 1, 2 and 3 weeks; Figure 4A). Treatment with
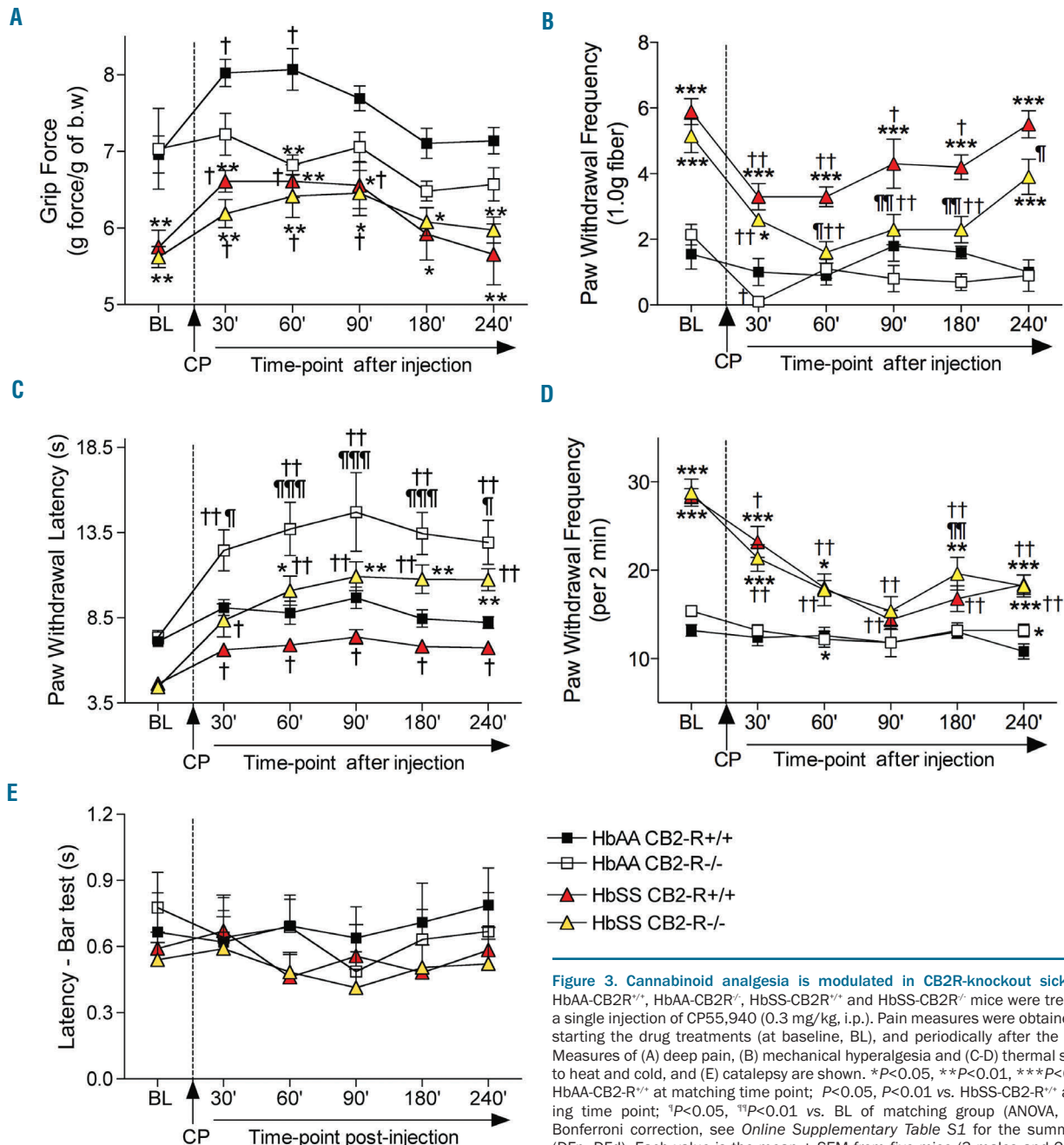

Figure 3. Cannabinoid analgesia is modulated in CB2R-knockout sickle mice. HbAA-CB2R$^{+/+}$, HbAA-CB2R$^{-/-}$, HbSS-CB2R$^{+/+}$ and HbSS-CB2R$^{-/-}$ mice were treated with a single injection of CP55,940 (0.3 mg/kg, i.p.). Pain measures were obtained before starting the drug treatments (at baseline,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

CP55,940 significantly decreased activation (degranulation) of mast cells in sickle mice compared to sickle mice treated with vehicle (Figure 4B-D). Sickle mice treated with CP55,940 showed about 40% less activated mast cells compared to those treated with the 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 and tryptase as compared to mast cells from vehicle-treated mice (P<0.05 for both; Figure 4F-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, compared to vehicle treatment, CP55,940 treatment of sickle mice for 3 weeks significantly decreased the cytokines released from skin biopsies (IL-1α, IL-6, TNF-α, MCP-1; P<0.01, Figure 2H). Consistent with decreased mast cell activation, treatment with CP55,940 lowered the levels of granulocte macrophage colony-stimulating factor (GM-CSF) and regulated on activation, normal T-cell expressed and secreted (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, which are often elevated in SCA.[26] We have previously reported leukocytosis in sickle mice and have shown that mast cells play a role in this process.[3] Treatment with CP55,940 significantly decreased white blood cell counts and sickle red blood cells, compared to the effect of vehicle, both under normoxia and following H/R incitement (Table 1). Thus CP55,940 treatment dampens the inflammatory response and sickling of red blood cells by decreasing the activation of mast cells.

Hypoxia/reoxygenation-induced mast cell activation is attenuated by cannabinoids in a receptor-specific manner

Next we determined cannaboid receptor-specific inhibition of mast cell activation in sickle mice under normoxia and H/R. Sickle mice showed a trend towards increased mast cell activation following H/R injury as compared to normoxia (Figure 5A,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 the respective conditions (P<0.05 for each condition; Figure 5B). Although the CB1R agonist ACEA caused appreciable inhibition of mast cell activation, the CB2R agonist JWH-133 produced a significant decrease in degranulating mast cells (P<0.05). Consistent with the inhibitory effect on mast cell activation, administration of CP55,940, compared to treatment with only the vehicle, significantly reduced plasma tryptase, β-hexosaminidase and serum amyloid protein after H/R injury in sickle mice (P<0.05; Figure 5C). The level of serum substance P was elevated after H/R injury compared to the level in normoxia in sickle mice (P<0.05; Figure 5D). CP55,940 treatment decreased the levels of substance P both under normoxia and following H/R injury (P<0.01; Figure 5D,E). Following H/R, the CB2R agonist significantly reduced substance P levels as compared to the levels in vehicle-treated mice (P<0.05; Figure 5E). The CB1R agonist tended to decrease serum substance P but the difference 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 described above, we examined the role of cannaboid receptors in relieving neurogenic inflammation. Evans blue leakage increased significantly in the skin of sickle mice following H/R incitement compared to the leakage in normoxia (P<0.05; Figure 6A). CP55,940 decreased Evans blue leakage in sickle mice under normoxia as well as following H/R (P<0.001; Figure 6A). Evoked leakage of Evans blue by intradermal injection of capsaicin or substance P is higher in sickle mice than in control mice under normoxia.[3] Treatment with CP55,940 or CB1R and CB2R agonists significantly reduced Evans blue leakage evoked by capsaicin (CP55,940, P<0.001; CB1R and CB2R P<0.05) and substance P (CP55,940, CB1R and CB2R P<0.001) as com-

Table 1. The effect of CP55,940 on hematologic 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 (10^9/L)	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
Hematocrit (%)	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 (10^9/L)	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##°°
Neutrophils (10^9/L)	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#°
Lymphocytes (10^9/L)	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 (10^9/L)	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°
RBC indices								
Sickle RBC (% total)	n/a	n/a	28.9 ± 1.8	18.4 ± 1.2¶¶	n/a	n/a	37.9 ± 1.8¶¶	23.8 ± 1.5¶°°°

*Complete blood counts were measured in whole blood after 7 days of treatment with vehicle (Veh) or CP55,940. On day 7, mice were separated into two groups: the normoxia group (control condition) or the hypoxia/reoxygenation group in wich mice were treated with 3 h of hypoxia and 1 h of reoxygenation (H/R). Blood was collected on day 8, (24 h after the end of treatment or after H/R). RBC: red blood cells; Hb: hemoglobin; WBC: white blood cells; n/a: not applicable. *P<0.05, **P<0.01, ***P<0.001 vs. HbAA-BERK Veh Normoxia; ¶P<0.05, ¶¶P<0.01 vs. HbSS-BERK Veh Normoxia; #P<0.05, ##P<0.01, ###P<0.001 vs. HbAA-BERK Veh H/R; °P<0.05, °°P<0.01, °°°P<0.001 vs. HbSS-BERK Veh H/R. n = five male mice in each group. Data are mean ± SEM (ANOVA, with the Bonferroni correction).*

pared to vehicle (Figure 6B,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 indicated above, while CB1R mediates the response on 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 a "crisis" in addition to chronic pain further adds to the complexity and heterogeneity of SCA pain as compared to severe pain in other conditions. It is not, therefore, surprising that current pain management strategie, requiring identification of therapeutic modalities acting on multiple targets peripherally and in the central nervous system are not always effective. Cannabinoid receptors are unique targets because of their peripheral and central activity at a multicellular level.

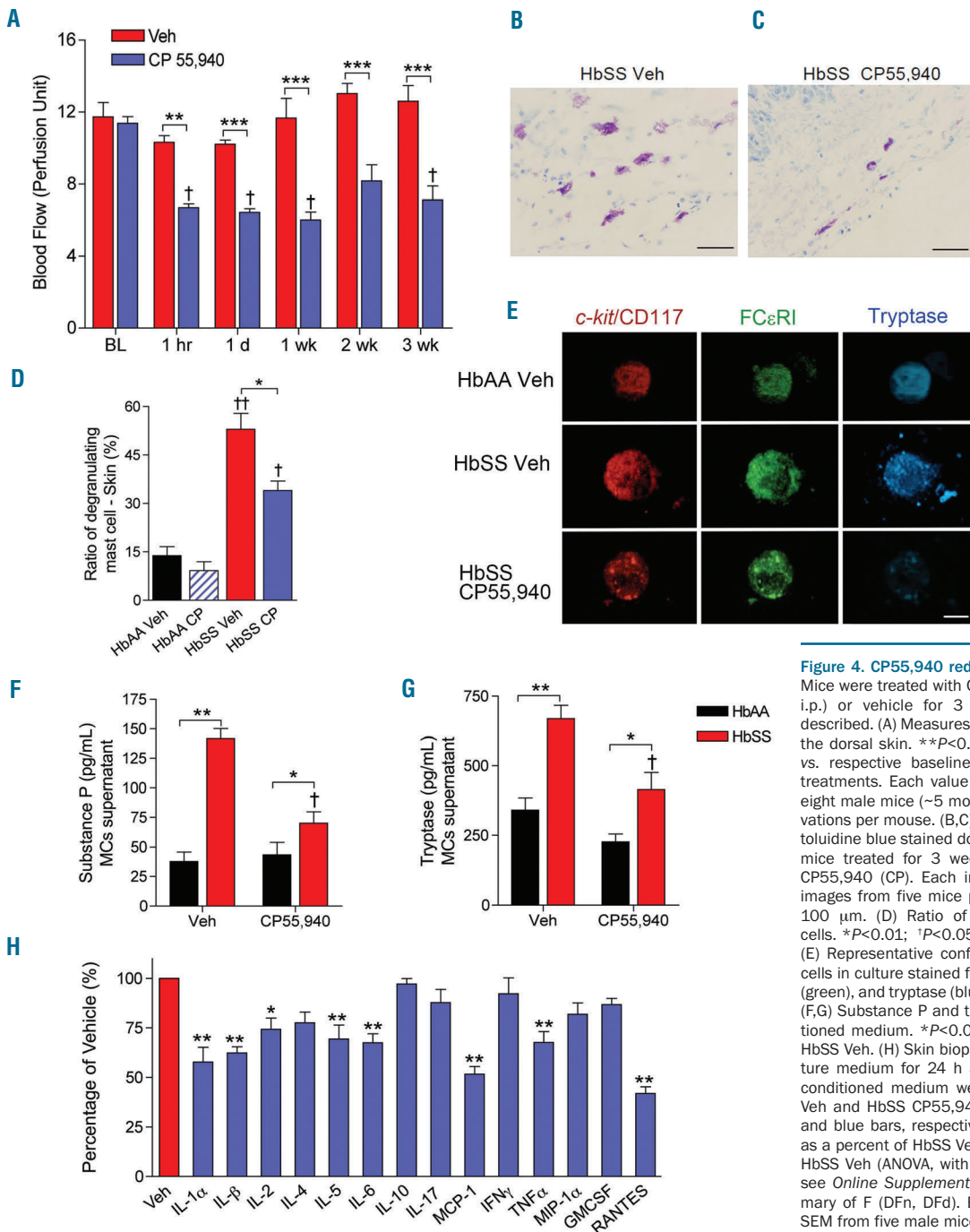Given the psychotropic effects of CB1R, attention is

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 (BL) before starting the treatments. Each value is the mean ± SEM from eight male mice (~5 months old) with three 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 is representative of images from five 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 h and cytokines released in conditioned medium were analyzed. HbSS BERK Veh and HbSS CP55,940 are represented by 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 the Bonferroni correction, see *Online Supplementary Table S1* for the summary of F (DFn, DFd). Each value is the mean ± SEM from five male mice (~5 months old).

being focused on the possibility of targeting CB2R, which does not have psychotropic effects.[27,28] CB2R agonists and/or knockout mice provide compelling evidence that CB2R activation mitigates 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 attenuat-

ing leukocyte adhesion to the endothelium, transendothelial migration, and interrelated oxidative-nitrosative damage,[31,32] all of which are consistent with the pathobiology of SCA. We show here that targeting the cannabinoid receptors is effective in reducing inflammation, mast cell activation and neurogenic inflammation, which orchestrate pain.
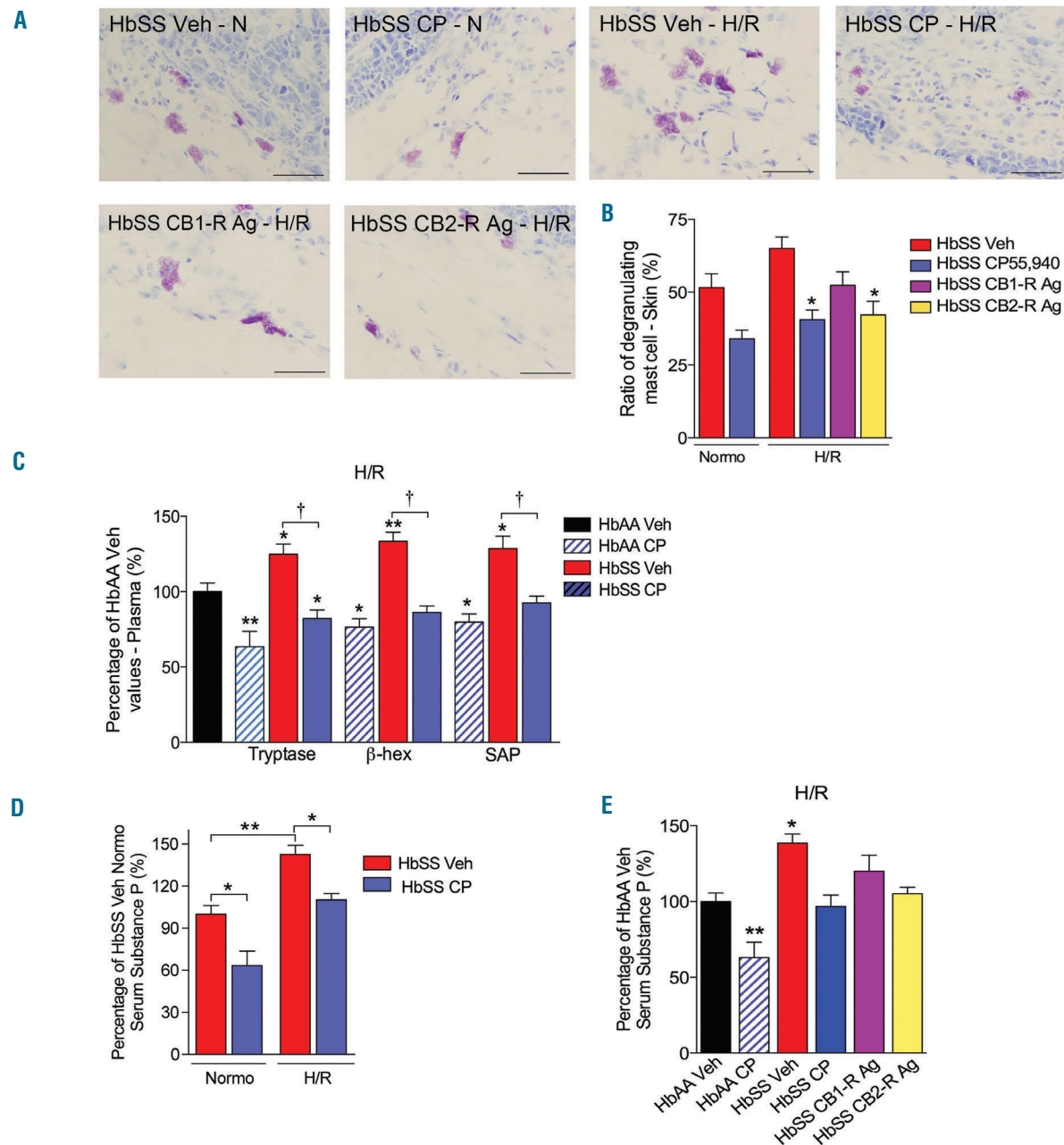

Figure 5. CP55,940 reduces hypoxia/reoxygenation-evoked mast cell activation. Mice were treated with vehicle (Veh), CP55,940 (CP), CB1R agonist (CB1-R Ag, ACEA) or CB2R agonist (CB2-R Ag, JWH-133) for 1 week followed by normoxia (N) or hypoxia/reoxygenation (H/R) and analyzed as described. (A) Representative images of toluidine blue stained dorsal skin sections of HbSS mice. Each image is representative of images from five 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) and 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 the Bonferroni correction, see *Online Supplementary Table S1* for the 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 in (B-E) is the mean ± SEM from five male mice, ~5 months old.
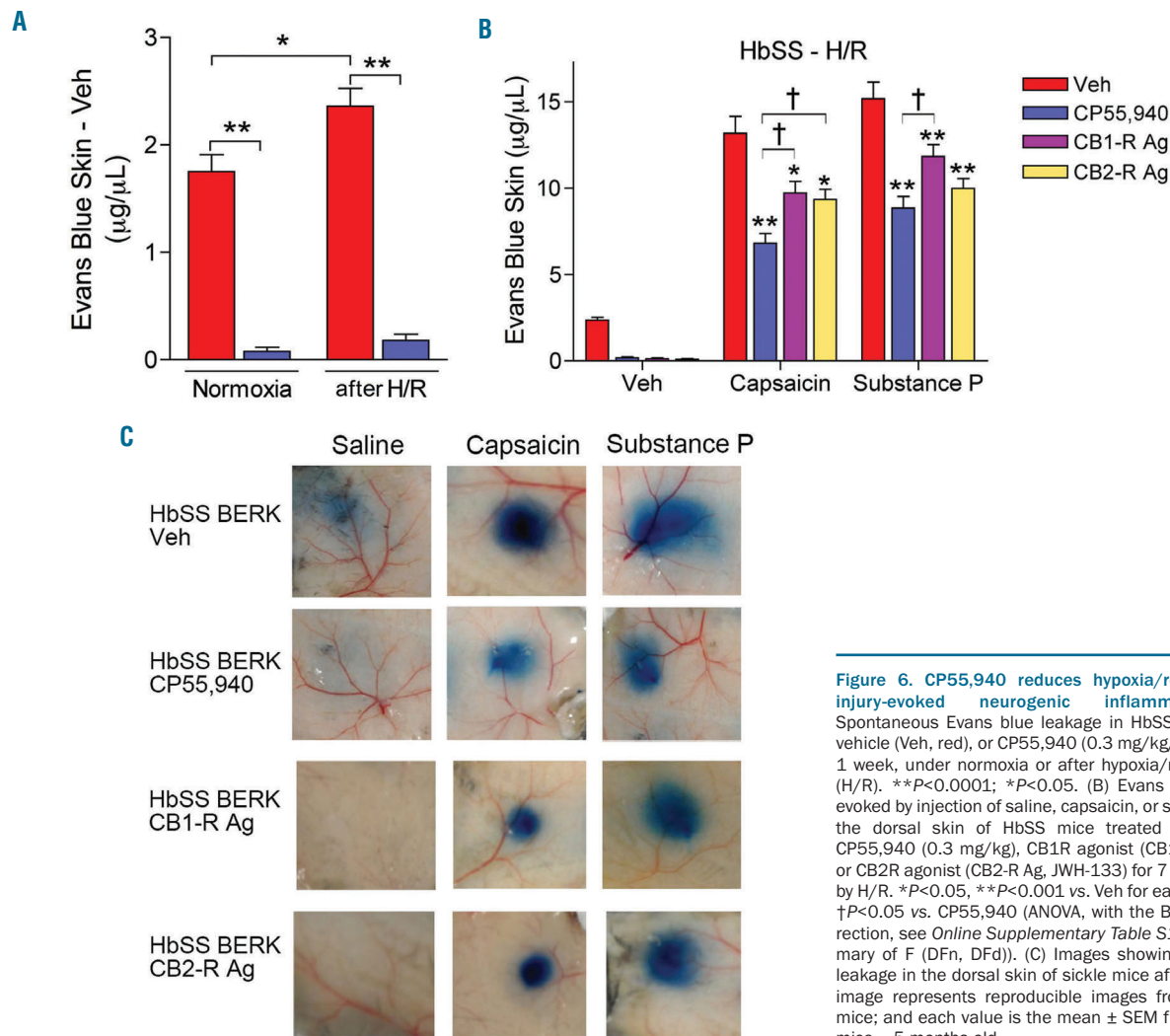
Figure 6. **CP55,940 reduces hypoxia/reoxygenation injury-evoked neurogenic inflammation.** (A) Spontaneous Evans blue leakage in HbSS treated with vehicle (Veh, red), or CP55,940 (0.3 mg/kg/day, blue) for 1 week, under normoxia or after hypoxia/reoxygenation (H/R). **P<0.0001; *P<0.05. (B) Evans blue 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 the Bonferroni correction, see *Online Supplementary Table S1* for the 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 five male mice; and each value is the mean ± SEM from five male mice, ~5 months old.

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 mechanical hyperalgesia induced by complete Freud's adjuvant (CFA) in sickle mice.[4,5] Our present observations that sickle mice exhibit sustained analgesia over 3 weeks of chronic treatment with CP55,940 suggest that tolerance to cannabinoid analgesia does not develop.

Cannabinoids have been found to be protective against ischemia/reperfusion 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 the CB1R agonist was able to reduce mechanical and thermal (heat and cold) hyperalgesia following H/R. Complementary to these observations, CP55,940 treatment had an antihyperalgesic effect in HbSS CB2R^{-/-} mice on mechanical and thermal

(heat and cold) hyperalgesia but not on deep tissue hyperalgesia under normoxia. Cannabinoid analgesia is, therefore, mediated through both CB1R and CB2R, which is specific to the sickle pain phenotype. Thus, cannaboid receptors 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] the present findings highlight the analgesic potential of cannabinoids to relieve different pain phenotypes under normoxia (representing chronic pain) and under H/R (representing the pain of a vaso-occlusive crisis). Importantly, the present data support the use of both CB1R and CB2R agonists for overall analgesia but, depending on the characteristics of the pain, one or the other agonist may potentially be more useful.

Mast cell activation contributes to sickle pathophysiology by mediating inflammation and pain.[3] Inflammatory mediators, proteases including tryptase and pro-inflammatory cytokines are released from mast cells upon activa-

tion 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, the sickle microenvironment favors persistent mast cell activation, consecutively causing nociceptor sensitization, which in turn aggravates hyperalgesia. Indeed our recent studies showed nociceptor sensitization and activation of the p38MAPK pathway in the spinal cords of sickle mice, suggestive of central sensitization.[36] The 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 the 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, substance P 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 the nitric oxide synthase inhibitor, N^G-monomethyl-L-arginine, but sickle males were not, suggesting that gender-based nitric oxide-dependent and -independent mechanisms are involved. Since, CP55,940 inhibited blood flow in male mice in our study, it may be acting *via* nitric oxide-independent mechanisms, but may also inhibit nitric oxide-dependent blood flow in females, an aspect that 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 white blood cell counts are elevated in SCA patients[26] and in sickle mice, and are both further increased by H/R incitement.[3] Our finding that CP55,940 decreased GM-CSF levels, leukocyte counts and also sickle red blood cells has important implications for improving vaso-occlusive crises and the accompanying pain. A direct effect of CP55,940 on reducing sickling of red blood cells is an exciting possibility, but the reduction could also be due to an indirect effect, which warrants further investigation. Importantly, the observed inhibitory effect of both CB1R and CB2R agonists on neurogenic inflammation and 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 been shown to be effective in treating symptoms of multiple sclerosis, including spasticity and neuropathic pain.[44,45] Sativex is also being tested in two phase 3 trials for cancer pain and neuropathic pain.[46] Furthermore, Abrams *et al.*[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 of addiction limit the use of cannabinoids for therapy. However, targeting the CB1R and CB2R receptors simultaneously in the periphery would minimize the side effects and concurrently help in managing pain. A recent report by Khasabova *et al.*[48] described that the activation of peripheral CB1R and CB2R synergistically reduced tumor-evoked hyperalgesia. A questionnaire-based study evaluating the use of marijuana 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] The CB1R agonist was able to improve hyperalgesia significantly in sickle mice, and the CB2R agonist significantly attenuated mast cell activation and neurogenic inflammation, which may improve the condition of the systemic disease, consequently reducing pain. Since, deep hyperalgesia was mitigated by the CB2R agonist (JWH-133), targeting both CB1R and CB2R simultaneously may be of advantage in treating the complex, mixed type of pain that typically occurs in SCA. CP55,940, *via* the CB2R, was demonstrated to stimulate serotonin 2A receptor activity in the pre-frontal cortex of rats, suggesting of an influence on cognitive and mood disorders.[51] The effect of cannabinoids on neuropsychiatric conditions in SCA does, therefore, require consideration.

Interestingly we did not see an increase in hyperalgesia with the deletion of CB2R in either control or sickle mice. Earlier studies in CB2R-deleted C57BL/6 mice, compared to wild-type C57BL/6 animals, did not show an effect on baseline hyperalgesia in paw withdrawal latency in response to heat or mechanical allodynia induced using von Frey filaments or in a 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 paw withdrawal latency 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 to alleviate deep hyperalgesia. Similar to our observations of no effect of CP55,940 on mechanical hyperalgesia but an increase in heat-provoked paw withdrawal latency in control CB2R$^{-/-}$ mice, in a previous study WIN 55,212-2 (a potent cannaboid receptor agonist) did not influence mechanical hyperalgesia but led to an increase in heat-provoked paw withdrawal latency in CB2R$^{-/-}$ C57BL/6 mice in a model of neuropathic pain.[28] These data suggest a role of CB2R in the anti-allodynic 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 involving inflammation and neuropathy, making both CB1R and CB2R agonists necessary to achieve analgesia.

CB1R-mediated psychotropic effects and utilization of smoked cannabis are major deterrants to the use of cannabis as a medicine.[53] However, the recent discovery of cannaboid receptor-specific agonists and delivery following

vaporization provide advantages to the use of 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 about cannabis-derived medications, their dosage and side effects needs to be acquired in disease-specific, pre-clinical and clinical investigations, as emphasized recently.[53,54] It is noteworthy that in the states of the USA in which cannabis has been legalized for medical use, the 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 in SCA is associated with a poor quality of life and increased morbidity and opioids, with all their side effects, remain the mainstay of therapy.[1] Our observations in a pre-clinical setting of SCA provide a compelling rationale to examine the potential of cannaboid receptor-specific agonists and cannabinoids to treat pain and ameliorate the associated pathobiology in SCA.

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