Cellular/Molecular

Compensatory Activation of Cannabinoid CB2 Receptor Inhibition of GABA Release in the Rostral Ventromedial Medulla in Inflammatory Pain

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The rostral ventromedial medulla (RVM) is a relay in the descending pain modulatory system and an important site of endocannabinoid modulation of pain. Endocannabinoids inhibit GABA release in the RVM, but it is not known whether this effect persists in chronic pain states. In the present studies, persistent inflammation induced by complete Freund's adjuvant (CFA) increased GABAergic miniature IPSCs (mIPSCs). Endocannabinoid activation of cannabinoid (CB1) receptors known to inhibit presynaptic GABA release was significantly reduced in the RVM of CFA-treated rats compared with naive rats. The reduction in CFA-treated rats correlated with decreased CB1 receptor protein expression and function in the RVM. Paradoxically, the nonselective CB1/CB2 receptor agonist WIN55212 inhibited GABAergic mIPSCs in both naive and CFA-treated rats. However, WIN55212 inhibition was reversed by the CB1 receptor antagonist rimonabant in naive rats but not in CFA-treated rats. WIN55212-mediated inhibition in CFA-treated rats was blocked by the CB2 receptor-selective antagonist SR144528, indicating that CB2 receptor function in the RVM is increased during persistent inflammation. Consistent with these results, CB2 receptor agonists AM1241 and GW405833 inhibited GABAergic mIPSC frequency only in CFA-treated rats, and the inhibition was reversed with SR144528. When administered alone, SR144528 and another CB2 receptor-selective antagonist AM630 increased mIPSC frequency in the RVM of CFA-treated rats, indicating that CB2 receptors are tonically activated by endocannabinoids. Our data provide evidence that CB2 receptor function emerges in the RVM in persistent inflammation and that selective CB2 receptor agonists may be useful for treatment of persistent inflammatory pain.

Key words: endocannabinoids; GABAergic transmission; persistent inflammation; RVM; whole-cell patch clamp

Significance Statement

These studies demonstrate that endocannabinoid signaling to CB1 and CB2 receptors in adult rostral ventromedial medulla is altered in persistent inflammation. The emergence of CB2 receptor function in the rostral ventromedial medulla provides additional rationale for the development of CB2 receptor-selective agonists as useful therapeutics for chronic inflammatory pain.

Introduction

Chronic pain affects ~30% of the U.S. population (Johannes et al., 2010) and is a significant public health problem. The treatment of chronic pain has been challenging to date for myriad reasons, including ineffective drugs and/or serious side effects with prolonged use of prescribed analgesics (Trang et al., 2015). However, emerging data indicate that drugs that target the endocannabinoid system might produce analgesia with fewer side effects compared with opioids, drugs that are considered to be the gold standard in analgesic treatment (Woodhams et al., 2015).

The endocannabinoid system is comprised of the cannabinoid receptors (CB1 and CB2), endogenous cannabinoid ligands (endocannabinoids) and the enzymes that synthesize and metabolize endogenous ligands to control the bioavailability of endocannabinoids. Endocannabinoids are synthesized in neurons in response to activation of postsynaptic neurons, released and act as retrograde messengers to inhibit presynaptic neurotransmitter release (Kreitzer and Regehr, 2002; Wilson and Nicoll, 2002). CB1 and CB2 receptor inhibitors block endocannabinoid signaling in both central and peripheral sites, promoting and/or prolonging hyperalgesia (Richardson et al., 1997; Calignano et al.,

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1998; Meng et al., 1998), indicating that these receptors are potential targets for novel analgesic drugs.

Endocannabinoids are localized throughout the brain and are known to modulate synapses within the descending pain modulatory system that includes the periaqueductal gray (PAG) area and rostral ventromedial medulla (RVM) projections to the spinal cord. This circuit modulates nociceptive transmission pathways during acute injury, as well as induction and maintenance of chronic pain (Ren and Dubner, 2002; Heinricher and Ingram, 2008). Inactivation of the RVM in animal models of neuropathic and inflammatory pain reveals a potent modulation of nociceptive transmission (Burgess et al., 2002; De Felice et al., 2011; Cleary and Heinricher, 2013; Havelin et al., 2016), and persistent inflammation affects the function and activity of RVM neurons (Hurley and Hammond, 2000, 2001; Guan et al., 2002, 2003; Cleary and Heinricher, 2013). Cannabinoid receptor agonists modulate activity of RVM neurons (Meng et al., 1998; Meng and Johansen, 2004), and these effects are reversed with CB1-selective antagonists. CB1 receptor activation decreases GABA release from presynaptic terminals (Vaughan et al., 1999; Atwood et al., 2012). These actions are similar to the cellular actions of opioids in the RVM, except that, in contrast to opioids, cannabinoids do not have postsynaptic effects (Vaughan et al., 1999; Vaughan and Christie, 2005). This difference in cannabinoid and opioid signaling is interesting in that it suggests a differential mechanism of cannabinoid-mediated analgesia compared with opioid-induced modulation of the descending pain pathway. To date, there are few data regarding how the endocannabinoid system in the RVM is regulated in chronic pain states. In addition, the effects of CB2 receptor activation on the descending pain modulatory system are not known, largely due to the fact that CB2 receptors were thought to be expressed in peripheral tissues. More recent studies provide evidence that CB2 receptors are expressed in the CNS (for review, see Atwood and Mackie, 2010; Dhopeshwarkar and Mackie, 2014). In addition, analgesic effects of drugs that inhibit degradation of endocannabinoids, thus promoting endocannabinoid actions, are partially reversed with CB2 receptor antagonists (Jayamanne et al., 2006; Anderson et al., 2014). The goal of the present studies was to determine the effects of persistent inflammation on endocannabinoid levels and CB1 and CB2 receptor function in the RVM. Our data show that CB1 receptor function and protein levels are reduced during persistent inflammation without a significant change in endocannabinoid levels. Furthermore, CB2 receptors modulate GABA release in the RVM only in rats pretreated with complete Freud's adjuvant (CFA).

Materials and Methods

Animals. Male Sprague Dawley rats (Harlan Laboratories and bred in-house; postnatal day 30–90) were used. All procedures were performed in strict accordance with the *Guide for the care and use of laboratory animals* as adopted and promulgated by the National Institutes of Health and approved by the Institutional Animal Care and Use Committee of Oregon Health & Science University.

Persistent inflammation. CFA (heat-killed *Mycobacterium tuberculosis* in mineral oil, 1 mg/ml, 0.1 ml volume injected, Sigma-Aldrich) was injected subcutaneously into the plantar surface of the left hindpaw. The CFA injection produced an intense tissue inflammation of the hindpaw characterized by erythema, edema, and hyperalgesia (Iadarola et al., 1988). Electrophysiological recordings from RVM neurons were made 5–7 d following injections of CFA.

RVM ON-cell labeling. RVM neurons have been previously categorized into μ-opioid-sensitive (presumed ON cells) or μ-opioid insensitive (presumed OFF or NEUTRAL cells) subtypes (Heinricher et al., 2009). A fluorescent opioid compound dermorphin-AlexaFluor-594 (DERM-A594) was used to label μ-opioid-expressing RVM neurons (Arttamangkul et al., 2006; Phillips et al., 2012; Li et al., 2015). Microinjection of DERM-A594 into RVM was performed as described previously (Li et al., 2015). Briefly, rats were deeply anesthetized with ketamine (37.5 mg/kg)/xylazine (7.5 mg/kg) /acepramozine (1.5 mg/kg) mixture (i.p.) and a 23-gauge stainless steel guide cannula was lowered into the RVM (anteroposterior: −2.1; mediolateral: 0.0 mm; dorsoventral: −7.9 mm from lambda). A 31-gauge injection cannula that extended 2 mm beyond the tip of the guide cannula was inserted and DERM-A594 (150–300 ng/0.5 μl in 32% DMSO and saline) was administered over 100 s. The injection cannula was left in place for an additional 60 s after injection to minimize backflow up the cannula tract. The injection and guide cannula were removed, and the brain was immediately extracted for electrophysiological recording.

RVM slice preparation. RVM slice preparation was performed as described previously (Li et al., 2015). Rats were deeply anesthetized with isoflurane and the brains were rapidly removed and placed in *N*-methyl-D-glucamine-sucrose-based "cutting buffer" containing the following (in mM): 52 *N*-methyl-D-glucamine, 2.5 KCl, 0.5 $CaCl_2$, 10 $MgSO_4$, 1.2 NaH_2PO_4, 30 $NaHCO_3$, 25 D-dextrose, 75 sucrose, 5 sodium ascorbate, 2 thiourea, 3 sodium pyruvate, pH 7.4, adjusted with HCl and 300–310 mOsm (Zhao et al., 2011; Ting et al., 2014). Coronal slices (180–200 μm) were cut in 95% O_2- and 5% CO_2-oxygenated cutting buffer. Slices were incubated at 35°C in a submerged chamber containing aCSF equilibrated with 95% O_2- and 5% CO_2-oxygenated for at least 30 min and maintained at room temperature afterward until transfer to a recording chamber maintained at 30°C–31°C. The aCSF contained the following (in mM): 126 NaCl, 2.5 KCl, 2.4 $CaCl_2$, 1.2 $MgCl_2$, 1.2 NaH_2PO_4, 21.4 $NaHCO_3$, 11.1 D-dextrose, pH 7.4, and the osmolarity was adjusted to 300–310 mOsm.

Whole-cell patch-clamp recordings. RVM neurons were visualized and labeled with DERM-A594. Voltage-clamp recordings (holding potential −70 mV) were made from visually identified RVM ON cells (DERM-A594-labeled) or unlabeled cells in the whole-cell configuration using an Axopatch 200B amplifier (Molecular Devices). Patch-clamp electrodes were pulled from borosilicate glass (1.5 mm diameter; WPI) on a two-stage puller (PP83, Narishige). Pipettes had a resistance of 2–4 MΩ. IPSCs were recorded in an intracellular pipette solution containing the following (in mM): 140 CsCl, 10 HEPES, 4 MgATP, 3 NaGTP, 1 EGTA, 1 $MgCl_2$, 0.3 $CaCl_2$, pH adjusted to 7.3, with CsOH, 290 mOsm. Series resistance (<20 MΩ) was compensated by 70%–80% and continuously monitored during experiments. A junction potential of −5 mV was corrected during recording. GABAergic events were isolated in the presence of glutamate receptor antagonists, NBQX (10 μM) and APV (50 μM). Spontaneous miniature IPSCs (mIPSCs) were recorded in the presence of 500 nM TTX. Events were low-pass filtered at 2 kHz and sampled at 10–20 kHz for on-line and later off-line analysis (Axograph 1.4.3), and individual events were visually confirmed. Recordings in which access resistance or capacitance changed by >15% during the experiment were excluded from data analysis. One neuron was recorded per slice, and four or five slices were recorded per rat. Each set of experiments was repeated using at least 3 or 4 distinct rats with no more than 2 cells from a single rat included in a specific dataset.

Liquid chromatography-mass spectrometry analysis of endocannabinoids

Reagents and chemicals. Silanized glass 13 × 100 mm tubes, and siliconized 1.7 ml Eppendorf tubes were from Fisher Scientific, formic acid was from JT Baker, acetonitrile (ACN), methanol, and water were purchased from Burdick and Jackson, trifluoroacetic acid and ammonium acetate were from Sigma-Aldrich, 2-arachidonoylglycerol (2-AG), 1-arachidonoylglycerol (1-AG), anandamide (AEA), and 2-arachidonoylglycerol-d5, and anandamide-d4 were from Cayman Chemical.

The analysis of tissue endocannabinoids was developed adapting methods previously described (Richardson et al., 2007; Chen et al., 2009; Zhang et al., 2010). Briefly, RVM slices were weighed and placed into 13 × 100 mm screw-top silanized glass tubes, and 100 μl of 0.02% trifluoroacetic acid was added. The slices were sonicated for 30 s in an ice bath. ACN (4 ml) with 10 ng of 2-arachidonoylglycerol-d5, and 1 ng of

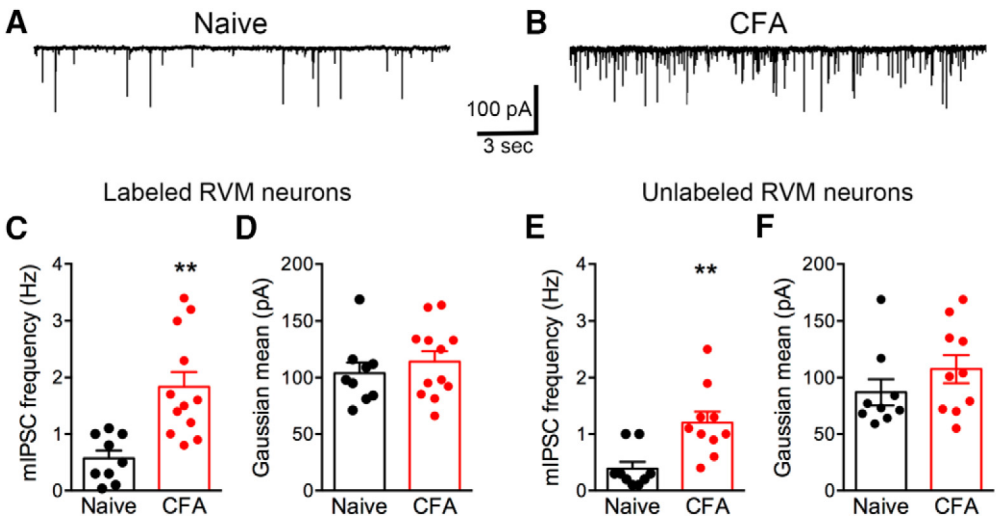

Figure 1. GABA release in the RVM is increased during persistent inflammation. ***A***, Representative traces of mIPSCs in RVM slices of naive rats. ***B***, Representative traces of mIPSCs in RVM slices of CFA-treated rats. ***C***, ***D***, Summary data of mIPSC frequency and amplitude recorded from DERM-A594-labeled neurons showing that mIPSC frequency is increased in CFA-treated compared with naive RVM (Mann–Whitney, $U = 8.50$) $^{**}p = 0.001$ without a change in mIPSC amplitude (unpaired t test, $t_{(19)} = 0.75$), $p = 0.46$. ***E***, ***F***, Summary data of mIPSC frequency and amplitude recorded from unlabeled RVM neurons showing that mIPSC frequency is increased in CFA-treated rats compared with naive rats (Mann–Whitney $U = 8.0$), $^{**}p = 0.001$ without a change in mIPSC amplitude (unpaired t test, $t_{(17)} = 1.20$). $p = 0.25$.

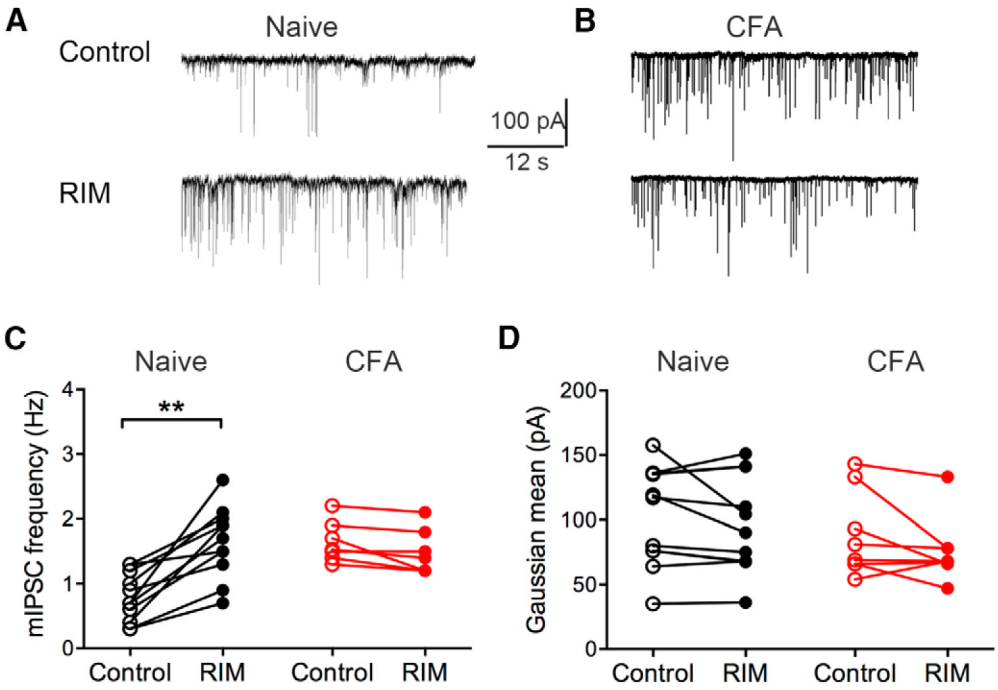

Figure 2. Reduced endocannabinoid inhibition of presynaptic GABA release in persistent inflammation. ***A***, Representative traces showing mIPSC frequency in the absence and presence of the CB1 antagonist rimonabant (RIM) in RVM neurons of naive rats. ***B***, Representative traces showing mIPSC frequency in the absence and presence of the CB1 antagonist RIM in RVM neurons of CFA-treated animals. ***C***, Summary data showing the increase in mIPSC frequency in the presence of rimonabant recorded from naive RVM neurons (two-way ANOVA, interaction, $F_{(1,17)} = 28.66$). $^{**}p < 0.001$. $p < 0.01$ (Sidak's multiple comparison). There was no significant change in mIPSC frequency in RVM neurons from CFA-treated rats. $p > 0.05$ (Sidak's multiple comparison). ***D***, Mean amplitudes determined from Gaussian fits to mIPSC amplitude histograms were not different in the presence of rimonabant between naive and CFA-treated RVM neurons (two-way ANOVA, interaction, $F_{(1,17)} = 0.071$), $p = 0.79$.

anandamide-d4 was added. Samples were then vortex mixed for 5 min at 2500 rpm in a multitube vortexer and then placed on ice for 15 min. Samples were then centrifuged for 15 min at 4°C at 2000 × *g* to pellet insoluble material. The supernatant was removed to new silanized 13 × 100 mm culture tube and evaporated to dryness in a speed vacuum evaporator at 35°C. Dried samples were dissolved in 100 μl of ACN, transferred to silanized inserts, and 5 μl was injected for analysis. Standards were prepared identically, except there was no tissue present.

Endocannabinoid content was analyzed using a 5500 Q-TRAP hybrid/triple quadrupole linear ion trap mass spectrometer (Applied Biosystems) with electrospray ionization in positive mode. The mass spectrometer was interfaced to a Shimadzu SIL-20AC XR auto-sampler followed by 2 LC-20AD XR LC pumps. The instrument was operated with the following settings: source voltage 5500 kV, GS1 30, GS2 60, CUR 30, TEM 650, and CAD gas medium. Compounds were quantified with multiple reaction monitoring and instrument parameters

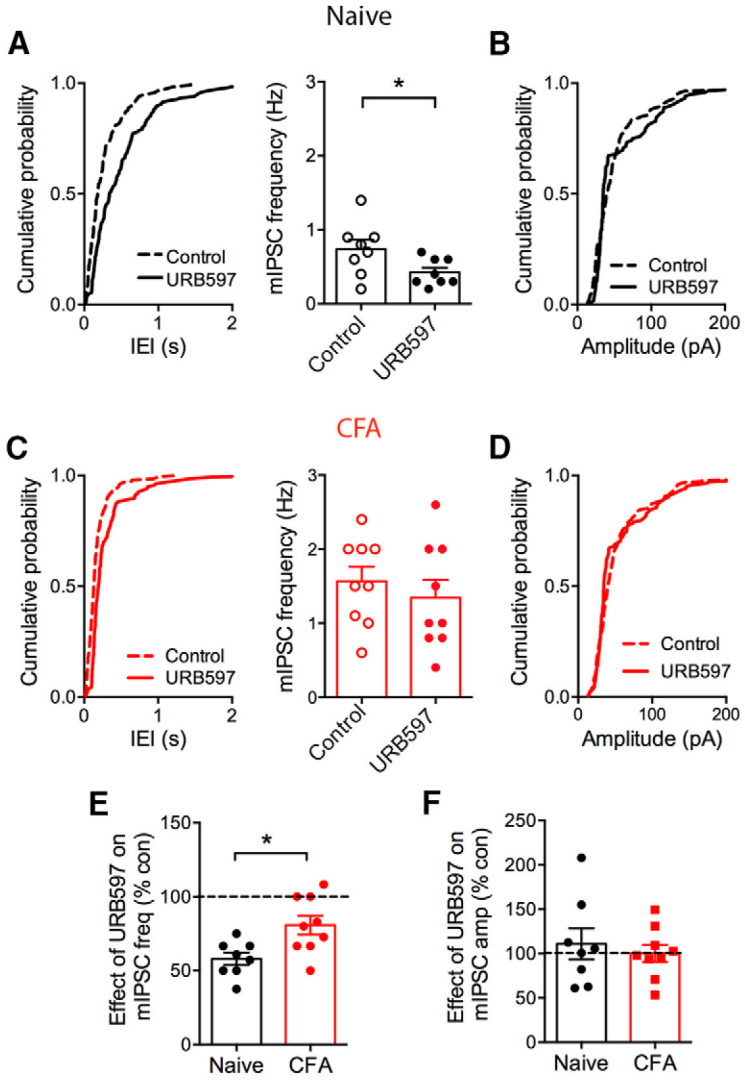

Figure 3. Inhibition of FAAH reduces mIPSC frequency in naive but not CFA-treated rats. ***A***, Cumulative frequency plot and summary data showing that the FAAH inhibitor URB597 increases interevent interval (IEI; left) and decreases mIPSC frequency in naive RVM neurons (right; Wilcoxon matched-pairs signed rank test, W = −28). *$p = 0.016$. $n = 8$. ***B***, There were no significant differences in mIPSC amplitude (paired *t* test, $t_{(7)} = 1.05$) $p = 0.33$. ***C***, Cumulative frequency plot and summary data showing that the FAAH inhibitor URB597 has a reduced effect on IEI (left) and mIPSC frequency in RVM neurons from CFA-treated rats (right; Wilcoxon matched-pairs signed rank test, W = −19). $p = 0.063$, $n = 9$. ***D***, There were no significant differences in the Gaussian mean of mIPSC amplitude distributions (paired *t* test, $t_{(8)} = 0.48$). $p = 0.65$. ***E***, Summary bar graph comparing the percentage inhibition by URB597 between RVM neurons from naive and CFA-treated rats (unpaired *t* test, $t_{(15)} = 2.91$). *$p = 0.011$. ***F***, mIPSC amplitude was not different in the presence of URB597 in naive compared with CFA-treated rats (unpaired *t* test, $t_{(15)} = 0.55$). $p = 0.59$.

for each transition optimized by direct infusion of pure compounds. The 2-AG was monitored using the [M+H]+ (m/z 379→287) and [M+NH_4]+ parent ions (m/z 396→287). 1-AG was monitored as a coeluting peak with the same multiple reaction monitoring transitions as the 2-AG. Other multiple reaction monitoring transitions were as follows: 2-arachidonoylglycerol-d5, m/z 401→287; AEA, m/z 348→62; anandamide-d4, m/z 352→66. The gradient mobile phase was delivered at a flow rate of 0.3 ml/min and consisted of two solvents, A: 1 g/L of ammonium acetate, 0.1% formic acid in water; and B: 1 g/L of ammonium acetate, 0.1% formic acid in 75% methanol: 25% ACN. The initial concentration of solvent B was 45%, which was held for 1 min, followed by a linear increase to 98% by 11 min, held for 4 min, decreased back to starting 45% B over 0.1 min, and then held for 7 min, for a total of 22 min. Separation was achieved using a Kinetex 2.6 μ C8 100 Å 150 × 2.1 mm column (Phenomenex) kept at 45°C using a Shimadzu CTO-20AC column oven. Data were acquired using Analyst 1.6.2 and analyzed with Multiquant 3.0.1. Standard curves for the 2-AG were from 1 to 250 ng per slice; AEA was 10–2500 pg per slice. The relative SD was <15% for all concentrations.

Western blot. Rats were injected with CFA in a hindpaw or were left naive to treatment. Brains were collected 5–7 d later, and RVM tissue was microdissected and homogenized in Transmembrane Protein Extraction Reagent (FIVEphoton Biochemicals) with protease inhibitors. After incubating 30 min on ice, lysates were centrifuged at 13,000 RPM for 10 min and supernatant was collected. Protein concentration was determined by BCA protein assay (Thermo Scientific). Total protein was incubated at 60°C for 10 min and separated by electrophoresis on precast NuPAGE 4%–12% Bis-Tris gels (Invitrogen), then transferred to nitrocellulose membrane. Blots were blocked with 5% milk (CB1) or 3% BSA (CB2) in PBS-T buffer (10.6 mM NaH_2PO_4, 56.5 mM Na_2HPO_4, 70 mM NaCl, 0.1% Tween 20, pH 7.4) at room temperature for 1 h. Blots were then incubated with rabbit CB1-L15 antibody (1:500; generous gift from Dr. Ken Mackie, Indiana University) or rabbit CB2 antibody (ACR-002, 1:200, Alomone Labs) overnight at 4°C. Membranes were washed with PBS-T and incubated in goat anti-rabbit secondary antibody for 1 h at room temperature (Invitrogen; 1:2000). Immunoreactivity was detected using SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific) and captured using a FluroChem FC2 (AlphaInnotech). Analysis was performed using ImageJ software (National Institutes of Health). CB1 and CB2 receptor bands were analyzed relative to β-actin bands as a loading control.

Authentication of antibodies. G-protein-coupled receptor antibodies are notoriously nonspecific. The CB1-L15 antibody has been extensively tested using Western blot experiments and detects CB1 receptor bands in the expected range of 49–62 kDa in rat tissue (Grimsey et al., 2008). We analyzed the band at 62 kDa. The CB2 antibody recognizes a peptide sequence in the intracellular third loop, and these amino acids (228–242) are identical between rat CB2 and mouse CB2 receptors. Immunostaining was reduced in CB2 KO mice and with preabsorption with the specific immune peptide (Zhang et al., 2014). We confirmed that the band (40 kDa) that we analyzed was reduced with the specific immune peptide in our Western blot experiments. We are still concerned by the fact that we observed multiple bands with both antibodies in the size range expected for CB1 and CB2 receptors (shown in Fig. 4). We also recognize that testing in neither mouse KO models nor preabsorption controls is actually an appropriate control for specificity of antibodies in rat tissue. This is especially a concern for CB2 antibodies (Marchalant et al., 2014). Thus, the lack of change in CB2 receptor protein levels may indicate that total CB2 receptor protein is not changed, but there are more functional CB2 receptors on the plasma membrane of terminals in the RVM in CFA-treated rats or that the CB2 antibody recognizes additional protein(s) in a similar size range that are not altered by CFA treatment.

qPCR. Rats were injected with CFA in a hindpaw or were left naive to treatment. Brains were collected 5–7 d later, and RVM tissue was microdissected and flash frozen. RNA was isolated by the Oregon Health &

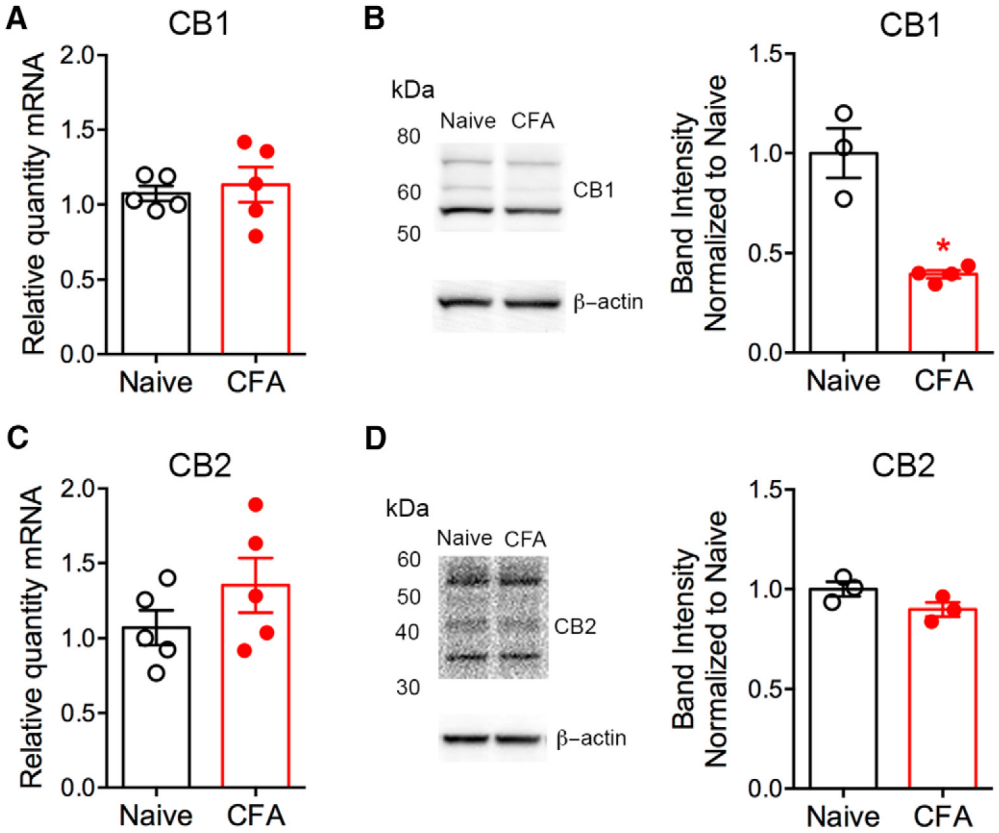

Figure 4. Persistent inflammation does not change CB1 and CB2 mRNA levels but decreases CB1 protein levels. ***A***, Relative quantities of mRNA for CB1 receptor expression in the RVM are not changed during persistent inflammation (unpaired *t* test, $t_{(8)} = 1.31$). $p = 0.6$. Expression levels were compared with mapk6 internal control. ***B***, Representative Western blot image and summary data show that the CB1 protein levels for the band running at 62 kDa are reduced in RVM from CFA compared with naive rats (unpaired *t* test, $t_{(5)} = 5.67$). $^*p = 0.002$. $n = 3$ or 4 rats/group. Other bands were not significantly changed ($p > 0.05$). Bands were analyzed relative to β-actin bands and then normalized to naive. ***C***, Relative quantities of mRNA for CB2 receptor expression in the RVM are not changed after 5 d of inflammation (unpaired *t* test, $t_{(8)} = 0.46$). $p = 0.66$. ***D***, Representative Western blot image and summary data show that the CB2 protein levels for the band running at 40 kDa were the same for RVM tissue from naive and CFA-treated rats (unpaired *t* test, $t_{(4)} = 2.0$). $p > 0.05$. $n = 3$ rats/group. Other bands were also not significantly changed ($p > 0.05$). Bands were analyzed relative to β-actin bands and then normalized to naive.

Science University GPSR Core from tissue using the RNeasy Micro kit (QIAGEN). Following RNA isolation, RNA quality assessment was performed using the Agilent 2100 Bioanalyzer with a Eukaryote total RNA Pico chip. Reverse transcription (RT) was performed using the SuperScript VILO cDNA synthesis kit (Invitrogen) with 750 ng of input RNA per 30 μl reaction for mRNA. Following reverse transcription, 2 μl of cDNA was used in the PCR using the TaqMan Universal mastermix (Invitrogen). The qPCR assays were performed on the QuantStudio RealTime PCR System (Invitrogen) using TaqMan probes for CB1 (CnR1-Rn02758689_s1) and CB2 (CnR2-Rn00571953_m1) receptors, β-actin (actb-Rn00667869_m1), and mitogen-activated protein kinase 6 (mapk6-Rn00581152_m1). Data were collected using QuantStudio 12K Flex Software version 1.0 (Applied Biosystems). All analysis settings were set to default. All samples generated C_t values within an acceptable linear range (between 15 and 30 cycles). Both actb and mapk6 were highly stable between all 10 samples with variance within 1 C_t. The SD between replicates was very low with the highest being 0.1.

Chemicals. NBQX, DL-AP5, TTX, and CFA were purchased from Sigma. DERM-A594 was a generous gift from Dr. Seksiri Arttamangkul and Dr. John Williams (Vollum Institute, Oregon Health & Science University). WIN55212, rimonabant (SR141716), *N*-cyclohexylcarbamic acid 3′-(aminocarbonyl)[1,1′-biphenyl]-3-yl ester (URB597), AM1241, GW405833, AM630, and SR144528 were purchased from Abcam. All chemicals were dissolved according to the manufacturer's instructions and made in stock solution kept in −20°C, then diluted to final concentration into aCSF on the day of the experiment. Stock solutions of cannabinoids were prepared in DMSO and diluted using aCSF to a final concentration of 0.03%–0.1% DMSO and 0.05% BSA to decrease adsorption to the perfusion system. We tested several concentrations of each cannabinoid agonist and antagonist to determine the concentration range for *in vitro* slice experiments. The lipophilicity of the drugs necessitates using higher concentrations than determined in cell culture experiments (Vaughan et al., 1999).

Data analyses. All data are reported as mean ± SEM. Unpaired or paired Student's *t* test or Mann–Whitney *U* test was used in two-group comparisons where appropriate. One- or two-way ANOVA followed by Dunnett's or Sidak multiple-comparisons test was used for comparisons with more than two groups where appropriate to determine statistical significance. The criterion for significance was set at $^*p < 0.05$ or $^{**}p < 0.001$ (GraphPad Prism 6).

Results

GABA release is enhanced in RVM neurons following persistent inflammation

Whole-cell patch-clamp recordings were performed in DERM-A594-labeled and unlabeled RVM neurons from naive and CFA-treated rats 5–7 d after CFA injections. Spontaneous mIPSC frequency was increased when recording from RVM neurons from CFA-treated compared with naive rats (Fig. 1). The mIPSC frequency was increased in both DERM-A594-labeled and unlabeled RVM neurons to similar extents (two-way ANOVA, cell type, $F_{(1,34)} = 2.28$, $p = 0.14$), and there were no changes in mean mIPSC amplitude distributions. In addition, no significant differences were noted in mean rise time and decay time of the

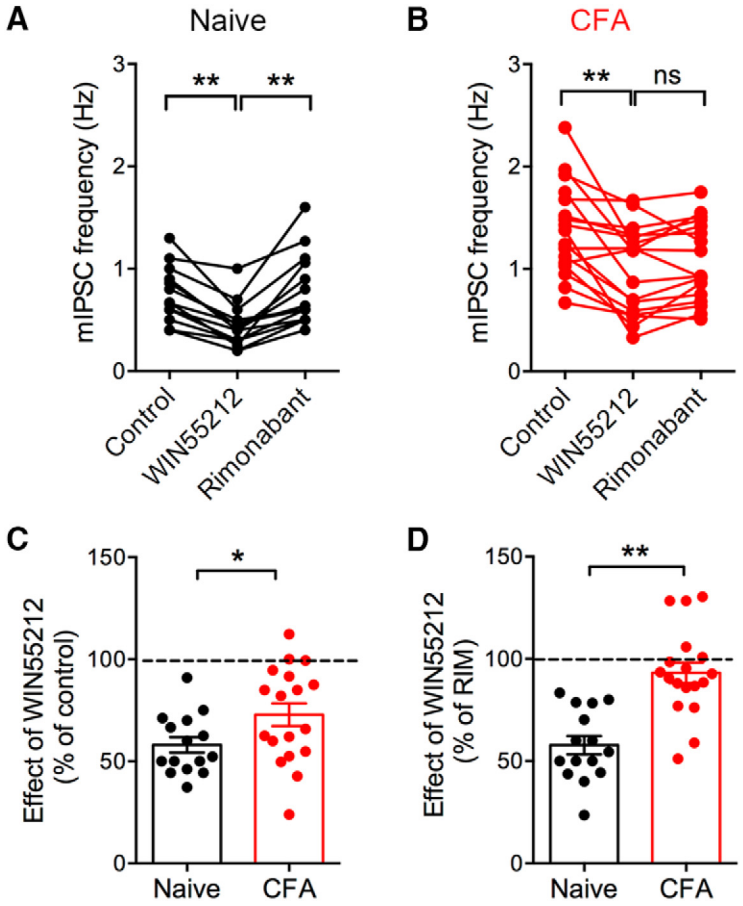

Figure 5. CB1 receptor function is reduced in RVM neurons from CFA-treated rats. ***A***, ***B***, Summary data showing the change in mIPSC frequency in the presence of WIN55212 and after addition of the CB1 receptor antagonist rimonabant in RVM neurons from naive and CFA-treated rats (two-way repeated-measures ANOVA, interaction, $F_{(4,60)} = 5.27$, $p = 0.001$; Sidak's multiple comparisons test). $^{**}p < 0.01$. Rimonabant reversed WIN55212 inhibition of mIPSC frequency in RVM neurons from naive but not in CFA-treated rats. ***C***, The effect of WIN55212 was greater in slices from naive rats (unpaired *t* test, $t_{(31)} = 2.12$, normalized to control mIPSC frequencies). $^{*}p = 0.04$. ***D***, The effect of WIN55212 was also greater in slices from naive rats (unpaired *t* test, $t_{(31)} = 5.15$, normalized to mIPSC frequencies in the presence of rimonabant). $^{**}p < 0.0001$.

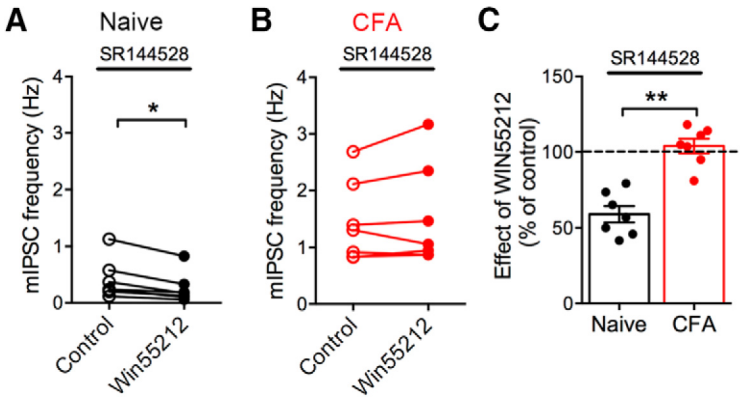

Figure 6. The selective CB2 antagonist SR144528 blocks WIN55212 effects in CFA-treated but not naive rats. ***A***, WIN55212 reduces mIPSC frequency in naive RVM neurons in the presence of the CB2 antagonist SR144528 (3 μM) (Wilcoxon matched-pairs signed rank test, $W = -28$). $^{*}p = 0.02$. ***B***, WIN55212 did not inhibit mIPSC frequency in CFA-treated rats in the presence of SR144528 (Wilcoxon matched-pairs signed rank test, $W = 12$). $p = 0.38$. ***C***, The effect of WIN55212 in the presence of CB2 antagonist SR144528 was greater in RVM neurons from naive compared with CFA-treated rats (unpaired *t* test, $t_{(12)} = 6.26$). $^{**}p < 0.0001$. Data are normalized to mIPSC frequency in the presence of SR144528.

mIPSCs (data not shown). The change in frequency without a significant change in the mIPSC amplitude or kinetics is consistent with a presynaptic change in GABA release in RVM caused by persistent inflammation.

Endocannabinoid activation of CB1 receptors in RVM neurons is reduced following persistent inflammation

There is evidence that endocannabinoids tonically inhibit GABA release in adult RVM neurons (Li et al., 2015). We tested the hypothesis that the enhanced GABA release in CFA-treated RVM slices is due to a reduction in endocannabinoid modulation of GABA release by CB1 receptors during persistent inflammation. The selective CB1 receptor antagonist rimonabant (also called SR141716, 5 μM) was used to detect the degree of tonic inhibition in the RVM. We previously observed that rimonabant inhibited GABA release similarly onto DERM-A594-labeled and unlabeled RVM neurons under control conditions (Li et al., 2015), and there were no differences in mIPSC frequency between the two cell types in CFA-treated animals (Fig. 1), so the data were combined for the remainder of the experiments. Rimonabant increased mIPSC frequency in RVM slices from naive animals (Fig. 2*A*,*C*). However, this effect was absent in CFA-treated animals (Fig. 2*B*,*C*). We did not observe differences in the effects of rimonabant on mIPSC amplitudes (Fig. 2*D*). These data indicate that tonic activation of CB1 receptors does not inhibit GABA release in the RVM during persistent inflammation.

To determine whether bioavailability of endocannabinoids is altered by persistent inflammation, a mass spectrophotometry lipidomics analysis was used to examine the levels of 2-AG and AEA using quantitative LC-MS/MS. There were no significant differences in 2-AG levels in RVM tissue between naive (2.9 ± 0.4 ng/mg wet weight, tissue from 11 rats) and CFA-treated animals (2.6 ± 0.3 ng/mg wet weight, tissue from 8 rats; $t_{(17)} = 0.46$, $p = 0.65$). Similarly, AEA levels did not differ in the RVM between naive (1.2 ± 0.2 pg/mg wet weight, $n = 11$ rats) and CFA-treated animals (1.1 ± 0.1 pg/mg wet weight, $n = 8$ rats; $t_{(17)} = 0.41$, $p = 0.69$).

The fatty-acid amide hydrolase (FAAH) inhibitor URB597 (McKinney and Cravatt, 2005) was used to examine whether the hydrolysis of endocannabinoids is enhanced in persistent inflammation. In RVM slices from naive animals,

URB597 (1 μM) promoted inhibition of GABAergic mIPSCs (Fig. 3*A*) but did not alter mIPSC amplitude (Fig. 3*B*). The degree of URB597 inhibition of mIPSC frequency was reduced in CFA-treated animals (Fig. 3*C*), again without affecting mIPSC amplitude (Fig. 3*D*). The inhibition in mIPSC frequency by URB597 was significantly different in naive slices compared with CFA-treated slices (Fig. 3*E*) without affecting mIPSC amplitude distributions (Fig. 3*F*). There were also no differences in mean rise time and decay time constant from naive and CFA rat RVM (data not shown). Thus, endocannabinoids hydrolyzed by FAAH are active in the RVM of naive rats but have substantially reduced effects in the RVM of CFA-treated rats. These data further indicate that increased FAAH activity does not underlie the changes in endocannabinoid modulation of GABA release in persistent inflammatory pain.

CB1 receptor function is reduced, but CB2-mediated inhibition of GABA release is increased in the RVM in persistent inflammation

We next determined whether levels of CB1 or CB2 receptor mRNA or protein in the RVM were altered as a consequence of inflammation. Both CB1 and CB2 receptor mRNA was detected by qPCR, but there was no difference in mRNA expression of these receptors in the RVM of CFA-treated compared with naive rats (Fig. 4*A*, *C*). Western blot analysis showed a significant decrease in the level of CB1 receptor protein in the RVM of CFA-treated compared with naive rats (Fig. 4*B*). These data, together with the lack of significant changes in endocannabinoid levels, imply that inflammatory pain decreases CB1 receptor expression. We did not observe changes in CB2 protein levels in the predicted size range (Fig. 4*D*).

To test whether reduced CB1 receptor levels in the RVM of CFA-treated rats are associated with changes in CB1 receptor function, the effects of a mixed CB1/CB2 receptor agonist WIN55212–2 (WIN55212) were examined in RVM slices from naive and CFA-treated rats. Superfusion of WIN55212 (5 μM) reduced mIPSC frequency in both naive and CFA-treated slices (Fig. 5*A*, *B*). However, the WIN55212-mediated reduction in mIPSC frequency was reversed by the selective CB1 receptor antagonist rimonabant (5 μM) only in naive animals. This is consistent with effects of rimonabant alone (Fig. 2). The inhibition of mIPSC frequency was greater in naive compared with CFA-treated rats regardless of whether WIN55212 inhibition was measured from control or after superfusion of rimonabant (Fig. 5*C*,*D*). WIN55212 and rimonabant had no effects on mIPSC amplitude (data not shown). Together, these data indicate that the function of CB1 receptors is reduced in RVM neurons during persistent inflammatory pain.

Because WIN55212 is a mixed CB1/CB2 receptor agonist, the lack of reversal of WIN55212 inhibition by rimonabant in CFA-treated rats suggested that WIN55212 activates CB2 receptors in the CFA-treated rats. To test this hypothesis, the CB2 antagonist SR144528 (3 μM) was applied before WIN55212 superfusion. WIN55212 significantly inhibited mIPSC frequency in the presence of SR144528 in recordings from RVM slices of naive rats (Fig. 6*A*), indicating that the effects of WIN55212 were not mediated by CB2 receptors. However, WIN55212 had no effect in the presence of SR144528 in RVM slices from CFA-treated animals (Fig. 6*B*). WIN55212-mediated inhibition of mIPSC frequency was also significantly greater in the presence of SR144528 in RVM neurons from naive compared with CFA-treated rats (Fig. 6*C*).

We tested the ability of a selective CB2 agonist AM1241 (Malan et al., 2001) to inhibit mIPSC frequency in the RVM. AM1241 (3 μM) had no effect on RVM neurons from naive animals (Fig. 7*A*). However, AM1241 significantly reduced mIPSC frequency in RVM neurons from CFA-treated animals. The selective CB2 antagonist SR144528 reversed this effect. Inhibition by AM1241 was significantly increased in CFA-treated compared with naive animals (Fig. 7*B*). AM1241 did not change mIPSC amplitude in RVM neurons from naive (paired *t* test, $t_{(8)} = 1.25$, $p = 0.25$) or CFA-treated rats (paired *t* test, $t_{(7)} = 0.066$, $p = 0.95$). The kinetics of mIPSCs (rise time and decay constant) were

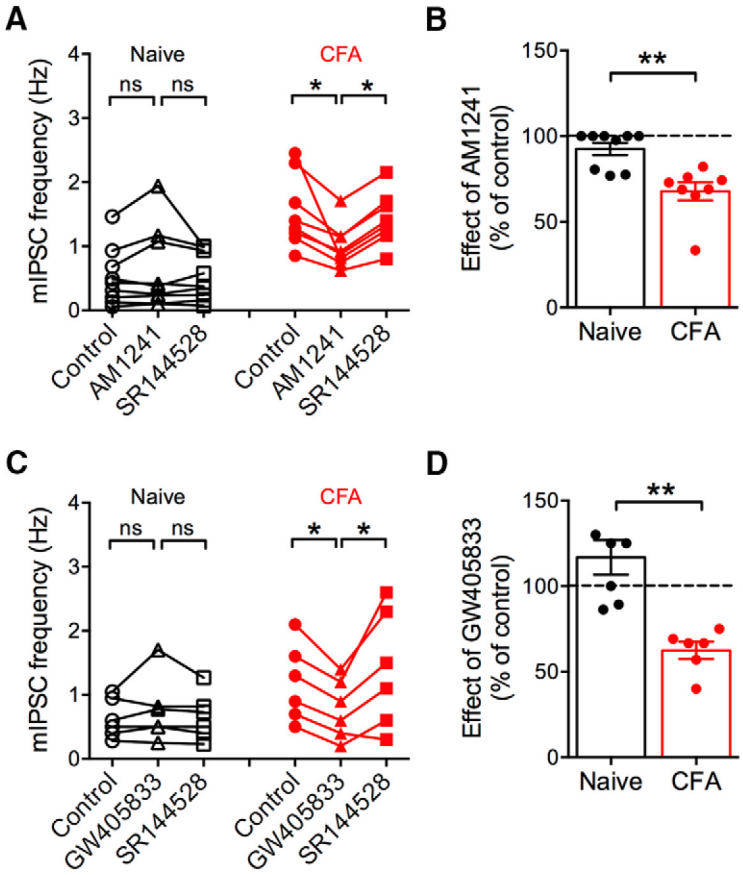

Figure 7. The selective CB2 agonists AM1241 and GW405833 inhibit GABA release in RVM neurons from CFA-treated rats. ***A***, The CB2 agonist AM1241 (3 μM) significantly inhibits mIPSC frequency in neurons from CFA-treated but not in naive rats (two-way repeated-measures ANOVA, interaction, $F_{(2,30)} = 9.90$, $p = 0.0005$; Dunnett's multiple comparisons test). *$p < 0.05$. ***B***, The percentage inhibition of mIPSC frequency by AM1241 was greater in RVM slices from CFA-treated compared with naive rats (unpaired *t* test, $t_{(15)} = 3.99$). **$p = 0.0012$. Data are normalized to control mIPSC frequency before AM1241 superfusion. ***C***, The CB2 agonist GW405833 (1 μM) significantly inhibits mIPSC frequency in neurons from CFA-treated but not in naive rats (two-way repeated-measures ANOVA, interaction, $F_{(2,22)} = 14.23$, $p = 0.0001$; Dunnett's multiple comparisons test). *$p < 0.05$. ***D***, The percentage inhibition of mIPSC frequency by GW405833 was greater in RVM slices from CFA-treated compared with naive rats (unpaired *t* test, $t_{(11)} = 4.54$). **$p = 0.001$. Data are normalized to control mIPSC frequency before GW405833 superfusion.

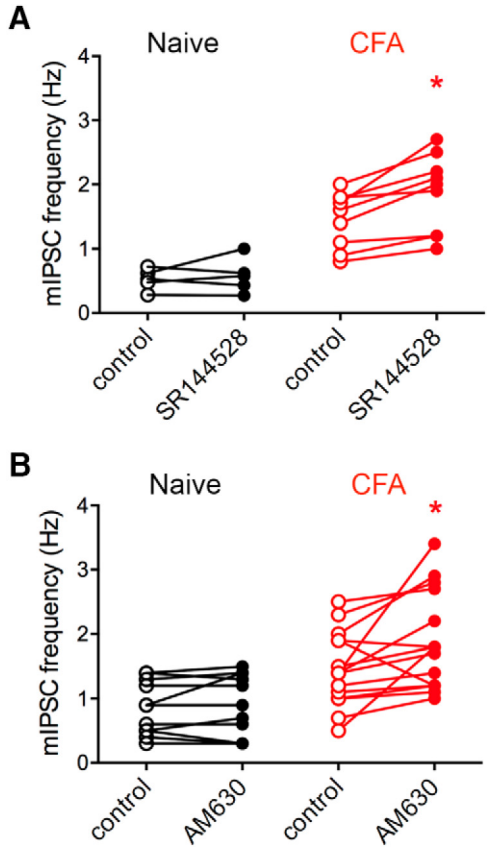

Figure 8. Endocannabinoid tone is present in the RVM of CFA-treated rats. ***A***, The CB2-selective antagonist SR144528 increases mIPSC frequency in CFA-treated rats but not naive rats (two-way repeated measure ANOVA, interaction, $F_{(1,13)} = 5.84$, $p = 0.03$, Sidak's multiple comparison). *$p < 0.05$. ***B***, The CB2-selective antagonist AM630 also increases mIPSC frequency in CFA-treated rats without an effect in naive rats (two-way repeated measure ANOVA, interaction, $F_{(1,26)} = 4.28$, $p = 0.049$, Sidak's multiple comparison). *$p < 0.05$.

not altered by AM1241 in either group (data not shown). We observed similar results using another CB2 receptor-selective agonist GW405833 (1 μM; Fig. 7*C*,*D*).

To further identify whether tonic activity of CB2 receptors modulate GABA release in CFA-treated RVM slices, we tested whether the CB2-selective antagonists SR144528 and AM630 had effects on presynaptic GABA release alone. In RVM slices from naive rats, SR144528 (3 μM) did not alter mIPSC frequency. In contrast, SR144528 significantly increased mIPSC frequency recorded from RVM neurons in CFA-treated rats (Fig. 8*A*). Similar results were obtained with another selective CB2 antagonist AM630 where mIPSC frequencies were increased only in RVM neurons from CFA-treated rats (Fig. 8*B*). These data indicate there is tonic activation of CB2 receptors by endocannabinoids in CFA-treated rats.

Discussion

The RVM is an integral brainstem relay for the descending modulation of pain (Heinricher and Ingram, 2008). *In vivo* single-unit recordings have shown that RVM neurons are dynamically modulated in adult rats in the transition from acute to chronic inflammation (Cleary and Heinricher, 2013). The present studies addressed whether $GABA_A$-mediated synaptic currents and endocannabinoid inhibition of GABAergic synaptic transmission are modulated in the RVM of adult rats during persistent inflammation. We observed increased GABA release and reduced tonic inhibition by endocannabinoid activation of CB1 receptors in the RVM of CFA-treated rats. We also show evidence for an emergence of CB2 receptor actions in the RVM after persistent inflammation.

Modulation of GABA release in the RVM during persistent inflammation

GABA signaling is integral to the coordination and activation of the descending pain circuit. Opioid-induced inhibition of GABA release disinhibits PAG and RVM output neurons (Heinricher and Ingram, 2008; Lau and Vaughan, 2014) and microinjection of $GABA_A$ receptor antagonists in either area results in antinociception (Moreau and Fields, 1986; Heinricher and Tortorici, 1994; Bobeck et al., 2009). Altered GABA release in the PAG and RVM is associated with chronic inflammatory or neuropathic pain models, although both increases and decreases in GABAergic mIPSCs have been reported (Hahm et al., 2011; Zhang et al., 2011; Zhang et al., 2013) indicating the complexity of the changes in the descending pain circuit. One key variable in these studies may be age of animals that are used for recording as we have previously reported a marked decrease in GABA release probability between neonatal and adult RVM neurons that is the result of increased endocannabinoid tone in the adult rat RVM (Li et al., 2015). Other laboratories have also observed important differences in the function of the RVM between neonatal and adult rats (Hathway et al., 2009, 2012). We focused our studies on adult rats to understand modulation of endocannabinoid signaling in the RVM during persistent inflammation.

Cannabinoid modulation of the descending pain pathway

Cannabinoid-mediated antinociception involves brainstem circuitry similar to that mediating opioid analgesia (Meng et al., 1998; Meng and Johansen, 2004). Indeed, cannabinoids inhibit GABA release in the PAG and RVM, as do opioids (Vaughan et al., 1999, 2000). Antinociception mediated by systemic administration of the nonselective CB1 and CB2 agonist WIN55212 is blocked by inactivation of the RVM with the $GABA_A$ receptor agonist muscimol, indicating that the RVM contributes to the analgesic effects of cannabinoids (Meng et al., 1998). Further, WIN55212 modulates RVM ON and OFF cells similarly to opioids (Meng and Johansen, 2004), supporting the idea that cannabinoid activation of the descending pain pathway occurs via a mechanism similar to opioids. The present studies show that the CB1-selective antagonist rimonabant increased GABAergic mIPSCs onto both putative ON (DERM-A594-labeled) and OFF (unlabeled) RVM neurons providing evidence for a tonic cannabinoid-mediated inhibition of GABA terminals in adult RVM. This is consistent with a previous report that both RVM ON- and OFF-cell firing activity were modulated following systemic administration of a CB1 receptor antagonist during *in vivo* microelectrode recordings (Meng et al., 1998). However, inhibition of GABA release would be expected to increase firing of both cell types, but ON cells exhibit reduced activity in the presence of cannabinoid agonists (Meng et al., 1998; Meng and Johansen, 2004). Cannabinoid agonists produce their effects via presynaptic actions (Vaughan et al., 1999; Atwood et al., 2012), so a direct postsynaptic effect of cannabinoid agonists on RVM ON cells is not likely. A more plausible explanation is that there are CB1 receptors on glutamatergic afferents to RVM ON cells or changes in other neurons within the descending pain circuit. Glutamate receptor activation is required for the RVM ON-cell burst in response to a nociceptive stimulus (Heinricher and Roychowdhury, 1997; Heinricher and McGaraughty, 1998), and cannabi-

noid agonists also inhibit glutamate release in the PAG (Vaughan and Christie, 2000). Future experiments will test the role of cannabinoid modulation of glutamatergic inputs in the adult RVM.

Loss of CB1 receptor function during persistent inflammation

The present studies provide evidence that CB1 receptors are downregulated during persistent inflammation. In naive rats, superfusion of the selective CB1 antagonist rimonabant significantly increased GABAergic mIPSC frequency. However, in CFA-treated rats, rimonabant had a reduced effect on mIPSC frequency. In addition, lower CB1 receptor levels were detected by Western blot analysis, suggesting that CB1 receptors are downregulated in the adult rat RVM during persistent inflammation. Our data are consistent with the loss of CB1 receptor function and protein levels in the PAG following chronic constriction injury (CCI), a neuropathic pain model (Palazzo et al., 2012). Other groups have reported desensitized and/or reduced CB1 levels following sustained or repeated cannabinoid administration (Sim et al., 1996; Breivogel et al., 1999; Dudok et al., 2015). Given that it has been reported that both AEA and 2-AG levels are significantly increased in the RVM 3–7 d after CCI (Petrosino et al., 2007), we hypothesized that reduced CB1 receptor function was the result of increased bioavailability of endocannabinoids and desensitization or downregulation of CB1 receptors. However, we did not observe changes in AEA or 2-AG levels in the RVM of CFA-treated animals compared with naive rats. Therefore, the mechanism underlying the loss of CB1 receptor levels and function following persistent inflammation remains to be elucidated. The difference in results from this study using a model of persistent inflammation and the previous study using CCI (Petrosino et al., 2007) indicates that endocannabinoid regulation in the RVM may be different depending on the chronic pain model. Nonetheless, CB1 receptor function is compromised in both CCI and persistent inflammation.

Emergence of CB2 receptor actions in RVM during persistent inflammation

Early reports characterizing the CB receptor system indicated that CB1 receptors are localized to the CNS and that CB2 receptors are localized to peripheral tissues, including the immune system and bone. More recently, numerous functional and anatomical evidence suggests that CB2 receptors are expressed in the nervous system (for review, see Atwood and Mackie, 2010). Both receptors are implicated in analgesic actions of cannabinoids (Malan et al., 2001; Jayamanne et al., 2006; Anderson et al., 2014), and CB2 receptors are dramatically upregulated in the spinal cord and periphery in inflammatory (Beltramo et al., 2006; Burston et al., 2013) and neuropathic (Ibrahim et al., 2003; Zhang et al., 2003; Sagar et al., 2005; Guindon and Hohmann, 2008) pain models. CB2 receptors also play an important role in inhibiting chemotherapy-induced neuropathic pain (Rahn et al., 2008; Deng et al., 2015a). We observed functional effects of selective CB2 agonists and antagonists in the RVM of CFA-treated rats that were not present in RVM recordings from naive rats. These data suggest that CB2 receptors are upregulated or may be trafficked to the plasma membrane of RVM neurons in persistent inflammation. We did not find an increase in CB2 mRNA in the RVM in CFA-treated rats (although CB2 receptor mRNA was detected in the RVM) or an increase in CB2 protein levels using Western blot analysis. However, both mRNA and protein levels were low in RVM due to the low expression levels of CB2 receptors and poor specificity of detection methods (Marchalant et al., 2014; Li and Kim, 2015). The lack of changes in mRNA expression of both CB1 and CB2 receptors is consistent with the functional data showing effects of both receptors on presynaptic GABA release. Presynaptic receptors are probably expressed in neurons outside of the RVM and trafficked to terminals projecting into the RVM.

An alternative hypothesis is that the emergence of CB2 receptor function may be a result of CB2 receptors expressed on microglia and other cells in the immune system (Stella, 2010). CB2 modulation of microglial activity has been observed in the periphery (Romero-Sandoval and Eisenach, 2007; Romero-Sandoval et al., 2008), as well as in the CNS (Racz et al., 2008; Luongo et al., 2010). Future studies will test the role of microglial activation in the CB2-mediated actions in adult RVM to determine whether inhibition is mediated by direct effects of CB2 receptors on presynaptic release or indirect modulation of release via neural-immune interactions.

Implications for pain modulation

The psychoactive ingredients of *Cannabis* and synthetic cannabinoid receptor agonists have analgesic activity in animal models of acute and chronic pain (Mackie, 2006), as well as in humans (Rukwied et al., 2003; Holdcroft et al., 2006; Ashton and Milligan, 2008). Altered descending control from the RVM has been implicated in chronic pain states (De Felice et al., 2011; Cleary and Heinricher, 2013). Importantly, our results showing increased function of CB2 receptor modulation of GABA signaling in CFA-treated RVM neurons suggest that selective activation of CB2 receptors may have therapeutic potential for treating persistent inflammatory pain. An additional benefit is that CB2 receptor agonists have a lower propensity than CB1 receptor agonists to induce tolerance and withdrawal effects (Deng et al., 2015b) and psychotropic side effects (Mackie, 2006; Atwood and Mackie, 2010; Parsons and Hurd, 2015).

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