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Sex differences in the effect of cannabinoid type 1 receptor deletion on locus coeruleus-norepinephrine neurons and corticotropin releasing factor-mediated responses

DR. Ryan R. Wyrofsky^{1,*}, Beverly A. S. Reyes¹, Daohai Yu², Lynn G. Kirby³, and Elisabeth J. Van Bockstaele¹

¹Department of Pharmacology and Physiology, College of Medicine, Drexel University, Philadelphia, PA 19102

²Department of Clinical Sciences, Temple Clinical Research Institute, Lewis Katz School of Medicine at Temple University, Philadelphia, PA 19140

³Center for Substance Abuse Research, Department of Anatomy and Cell Biology, Lewis Katz School of Medicine at Temple University, Philadelphia, PA 19140

Abstract

Cannabinoids are capable of modulating mood, arousal, cognition, and behavior, in part via their effects on the noradrenergic nucleus locus coeruleus (LC). Dysregulation of LC signaling and norepinephrine (NE) efflux in the medial prefrontal cortex (mPFC) can lead to the development of psychiatric disorders, and CB1r deletion results in alterations of α 2- and β 1-adrenoceptors in the mPFC, suggestive of increased LC activity. To determine how CB1r deletion alters LC signaling, whole-cell patch-clamp electrophysiology was conducted in LC-NE neurons of male and female wild type (WT) and CB1r-knock out (KO) mice. CB1r deletion caused a significant increase in LC-NE excitability and input resistance in male but not female mice when compared to WT. CB1r deletion also caused adaptations in several indices of noradrenergic function. CB1r/CB2r-KO male mice had a significant increase in cortical NE levels and tyrosine hydroxylase and CRF levels in the LC compared to WT males. CB1r/CB2r-KO female mice showed a significant increase in LC α 2-AR levels compared to WT females. To further probe actions of the endocannabinoid system as an anti-stress neuromediator, the effect of CB1r deletion on CRF-induced responses in the LC was investigated. The increase in LC-NE excitability observed in male and female WT mice following CRF (300 nM) bath application was not observed in CB1r-KO mice. These results indicate that cellular adaptations following CB1r deletion cause a disruption in LC-NE signaling in males but not females, suggesting underlying sex differences in compensatory mechanisms in KO mice as well as basal endocannabinoid regulation of LC-NE activity.

*Corresponding Author: Ryan Wyrofsky, Department of Pharmacology and Physiology, College of Medicine, Drexel University, 245 S. 15th Street, Philadelphia, PA 19102, Voice: (215) 762-2386, rrw47@drexel.edu.

Author Contributions

RRW, LGK, and EJVB conceived and designed the study. RRW and BASR collected data. RRW and DY performed statistical analyses. RRW wrote the manuscript, with contributions from DY, LGK, and EJVB.

Conflict of Interest

The authors report no conflict of interest.

Data Accessibility

Data are available upon request from the corresponding author.

Graphical Abstract

Novel electrophysiological data in mice reveals that cannabinoid receptor (CB1r) deletion results in a significant increase in locus coeruleus-norepinephrine (LC-NE) excitability in male mice, but not in females. Basal differences in CB1r expression were also observed, with male wild type (WT) having higher CB1r levels than female WT mice. Taken together, these data suggest that females may have less tonic endocannabinoid signaling in the LC-NE system compared to males, potentially contributing to the increased vulnerability of females to stress-induced psychiatric disorders.

Keywords

stress; sex differences; arousal; psychiatric disorders

Introduction

The brainstem nucleus locus coeruleus (LC) plays an important role in regulating mood, arousal, and emotional states (Aston-Jones and Cohen, 2005; Valentino and Van Bockstaele, 2008), and is the sole provider of norepinephrine (NE) to the medial prefrontal cortex (mPFC) (Sara, 2009). Dysregulation of the LC-NE system resulting in subsequent alterations in mPFC NE levels has been shown to precipitate the development of psychiatric disorders (Mueller et al., 2008; Mueller and Cahill, 2010). One of the most widely used recreational drugs in the world, cannabis, often causes feelings of relaxation and euphoria (Velez et al., 1989; Green et al., 2003; Di Marzo et al., 2004); however, chronic usage can be anxiogenic and produce dysphoria (Reilly et al., 1998). Via activation of cannabinoid type 1 receptors (CB1r), cannabinoids are known to affect attention and anxiety (Witkin et al., 2005; Pattij et al., 2008; Hill and Gorzalka, 2009), in part via actions on noradrenergic

circuitry (Carvalho et al., 2010; Carvalho and Van Bockstaele, 2012; Wyrofsky et al., 2017). There are, however, some discrepancies within the cannabinoid field. While some studies find anxiolytic effects and therapeutic promise in cannabinoid treatments, others show that CB1r agonists can have a negative impact and promote anxiogenesis, and the exact dosage and experimental conditions can have a profound effect on whether targeting the endocannabinoid system could be beneficial (Di Marzo, 2008; Wyrofsky et al., 2015). Further investigation of how the eCB system targets neurotransmitter systems, like norepinephrine, can provide further insight into the differential effects of cannabinoid agonism.

Immunofluorescence and immunoelectron microscopy studies have provided direct anatomical evidence for CB1r co-localization to noradrenergic terminals in the mPFC and localization to TH-containing neurons in the LC (Carvalho et al., 2010; Wyrofsky et al., 2017). Several studies have also shown that CB1r activation increases NE release. CB1r agonists WIN 55,212–2, tetrahydrocannabinol (THC), and CP 55940 dose-dependently increase the spontaneous firing rate of LC neurons (Mendiguren and Pineda, 2006; Muntoni et al., 2006). Both systemic and local administration of CB1r agonist WIN 55,212–2 increases c-Fos expression in LC-NE neurons and NE efflux in the rat mPFC (Oropeza et al., 2005; Page et al., 2008), as does inhibition of eCB catabolism with a fatty acid amide hydrolase (FAAH) inhibitor (Gobbi et al., 2005). Additionally, administration of the CB1r antagonist SR141716A causes a decrease in LC activity, suggesting that during basal conditions (Muntoni et al., 2006), the LC is under tonic eCB regulation (Carvalho and Van Bockstaele, 2012; Wyrofsky et al., 2015). Conversely, systemic administration of a CB1r antagonist rimonabant causes an increase in mPFC and hypothalamic NE levels (Tzavara et al., 2001; Tzavara et al., 2003), and low levels of THC can reduce NE release from synaptosomes (Poddar and Dewey, 1980). These anatomical and functional studies provide evidence that the eCB system can modulate the LC-NE system.

The eCB system is considered an “anti-stress” neuromediator (Viveros et al., 2007; Cota, 2008), playing a role in the hypothalamic pituitary adrenal (HPA) axis negative feedback loop as well as initiating the stress response in the amygdala (Hill and McEwen, 2009; Hill et al., 2010a; Hill et al., 2010b). The LC-NE system is involved in the cognitive limb of the stress response, and is activated in parallel with the HPA axis via corticotropin releasing factor (CRF), the pro-stress neuropeptide (Valentino and Van Bockstaele, 2008). Following a stressor, CRF is released from limbic and autonomic afferent sources such as the paraventricular nucleus of the hypothalamus, central nucleus of the amygdala, and bed nucleus of the stria terminalis (Van Bockstaele et al., 1996; Van Bockstaele et al., 1999; Van Bockstaele et al., 2001; Valentino and Van Bockstaele, 2008), and can lead to increases in LC-NE firing and dysregulation of NE release in target regions, including the mPFC (Curtis et al., 1996). We have recently shown that CB1r are positioned both pre- and post-synaptically with respect to CRF-containing afferents within the LC, providing a neural substrate for eCB modulation of CRF in this noradrenergic nucleus (Wyrofsky et al., 2017).

Research examining male CB1r-knockout (KO) mice show that CB1r deletion increases anxiety and depressive-like behaviors in CB1r KO mice compared to wild type (WT) controls (Aso et al., 2008; Steiner et al., 2008; Parolaro et al., 2010; Wyrofsky et al., 2015).

Additionally, CB1r-KO mice have increased plasma levels of adrenocorticotropin and corticosterone (Uriguen et al., 2004), and increased CRF mRNA expression in the hypothalamus, suggesting heightened HPA activity (Cota, 2008). We have also demonstrated that CB1r-KO mice have reduced basal mPFC neuronal excitability due to desensitization of the normally excitatory mPFC α 2-adrenoceptors (ARs) (Reyes et al., 2017). These KO studies suggest that CB1r-KO mice have increased LC-NE release, which desensitized mPFC α 2-ARs, resulting in decreased mPFC output (Reyes et al., 2017).

In the present study, we used two strains of cannabinoid receptor KO mice to define electrophysiological properties of LC-NE neurons as well as effects on CRF-mediated responses, and cellular adaptations that occur in absence of the cannabinoid receptors: CB1r-KO mice for *in vitro* slice electrophysiology studies and dual CB1r/CB2r-KO mice for Western and ELISA experiments. First, whole-cell patch clamp electrophysiological recordings were conducted in WT and CB1r-KO mice to measure basal properties of LC-NE neurons and their excitability. Next, we used Western blot analysis to measure expression levels of the catecholamine synthesizing enzyme tyrosine hydroxylase (TH) in the LC and assessed cortical NE levels via ELISA in CB1r/CB2r-KO mice. We also investigated expression levels of CRF, α 2-AR, NET, and CB1r in the coeruleo-cortical pathway. Finally, the effect of CRF administration on LC-NE activity in CB1r-KO mice was assessed using whole-cell patch-clamp recordings. Considering previous reports showing female rodents are more sensitive to the reinforcing effects of cannabinoids and are more likely to self-administer WIN 55,212-2 than males (Barna et al., 2004; Fattore et al., 2007; Roberts et al., 2014) and sex differences in CRF signaling within the LC, with female rodents having increased sensitivity to CRF following a stressor compared to males (Bangasser et al., 2010; Valentino et al., 2013), we included both male and female mice in the study. These experiments help address the current gap in our understanding of how CB1r-KO differentially affects the LC-NE and stress systems across sexes.

Methods

Animals

For electrophysiology studies, male and female wild-type (WT) and CB1r KO mice (9–12 weeks old) were housed four per cage in a controlled environment (12-hour light schedule, temperature at 20°C). Electrophysiology data for input resistance and excitability was obtained from N=5 LC cells from 5 WT male mice, N=4 cells from 4 WT female mice, N=6 cells from 5 KO male mice, and N=7 cells from 4 KO female mice. Electrophysiology data for membrane characteristics was obtained from N=4 cells from 4 WT male mice, N=4 cells from 4 WT female mice, N=5 cells from KO male mice, and N=4 cells from KO female mice. For Western blot and ELISA analysis, male and female WT and CB1r/CB2r dual KO mice (9–18 weeks old) were used. For LC Western blot analysis of CRF, α 2-AR, and NET, data represents N=3 WT male mice, N=6 KO male mice, N=3 WT female mice, and N=6 KO female mice. For LC Western blot analysis of CB1r, data represents N=7 WT male mice and N=5 WT female mice. For mPFC Western blot and ELISA analyses, data represents N=3 WT male mice, N=5 KO male mice, N=3 WT female mice, and N=4 KO female mice.

Both CB1r and CB1r/CB2r KO mice were originally generated on a C57Bl/6 background by Zimmer et al. (Zimmer et al., 1999) at the National Institutes of Health. Heterozygous breeding pairs were generously donated by Dr. Carl Lupica at the National Institutes of Health and were bred and genotyped at Temple University to obtain CB1r and CB1r/CB2r KO mice and WT littermates. All WT mice used in comparison with CB1r or CB1r/CB2r KO mice were littermates and shared a common genetic background. Food and water were provided *ad libitum*.

The care and use of animals were approved by the Institutional Animal Care and Use Committee of both Drexel University and Temple University, and were conducted in accordance with the *National Institutes of Health's Guide for the Care and Use of Laboratory Animals* (1996), the Health Research Extension Act (1985), and the PHS Policy on Humane Care and Use of Laboratory Animals (1986). All efforts were made to utilize only the minimum number of animals necessary to produce reliable scientific data, and experiments were designed to minimize any animal distress.

Drug preparation and administration

Ovine CRF, generously provided by Dr. Jean Rivier (Clayton Foundation Laboratories for Peptide Biology, The Salk Institute, La Jolla, CA), was dissolved in water to make a 1 mg/mL solution and separated into 10 μ L aliquots, which were concentrated using a Savant Speed Vac concentrator. Aliquots were stored at -80°C until the day of the experiment, when they were then reconstituted in aCSF and added to the perfusion bath at a final concentration of 300 nM CRF. 300 nM CRF was established as the optimal concentration based on a concentration-response curve (100–400 nM) tested in brain slices from male WT mice (data not shown). Additionally, this dose matches with previous sources confirming that 300 nM CRF produces maximal increases in LC-NE excitability (Jedema and Grace, 2004).

Electrophysiology

All electrophysiology procedures were conducted as described previously (Reyes et al., 2012; Cathel et al., 2014). Male and female CB1r KO and WT mice were rapidly decapitated and brains rapidly extracted and placed in ice-cold artificial cerebrospinal fluid (aCSF), in which sucrose (248 mM) was substituted for NaCl. The brains were trimmed down to isolate the brainstem, and 250 μ m horizontal slices containing the LC were cut on a Vibratome 3000 Plus (Vibratome, St. Louis, MO, USA). Slices were then incubated for 1h in aCSF at 35°C , and bubbled with 95% O_2 /5% CO_2 . Slices were then maintained at aCSF at room temperature and bubbled with 95% O_2 /5% CO_2 . The aCSF was composed of the following: 124 mM NaCl, 2.5 mM KCl, 2 mM NaH_2PO_4 , 2.5 mM CaCl_2 , 2 mM MgSO_4 , 10 mM dextrose, and 26 mM NaHCO_3 .

Slices were transferred to a recording chamber (Warner Instruments, Hamden, CT, USA) and continuously perfused with aCSF at a rate of 1.5–2.0 mL/min at a temperature of 34°C , maintained by an inline solution heater (TC-324; Warner Instruments, Hamden, CT, USA). Data were obtained from one to two neurons per mouse; however, only one neuron was recorded per brain slice. Neurons in the LC were visualized using a Nikon E600 upright

microscope fitted with a 40x water immersion objective, differential interference contrast and infrared filter (Optical Apparatus, Ardmore, PA, USA). The microscope was connected to a CCD camera and computer monitor. LC-NE cells were tentatively identified by their morphology and electrophysiological characteristics (Williams et al., 1984), using the fourth ventricle as a marker for the location of the LC nucleus. Whole-cell recording pipettes were made with borosilicate glass capillary tubing (1.2 mm outer diameter, 0.69 mm inner diameter; Warner Instruments) on a P-97 micropipette puller (Sutter Instruments, Novato, CA, USA). Electrodes were pulled to a resistance of 4–8 M Ω when filled with an intracellular solution containing 120 mM K-gluconate, 10 mM KCl, 1 mM MgCl₂, 10 mM EGTA, 10 mM HEPES, 2 mM MgATP, 0.5 mM Na₂GTP, 10 mM Na phosphocreatinine, and 0.1% biocytin, pH 7.3. Cells were approached with the electrode until a G Ω seal was established, and the cell membrane ruptured to obtain a whole-cell recording using HEKA patch-clamp EPC-10 amplifier (HEKA Elektronik, Pfalz, Germany) under current clamp conditions ($I = 0$ pA). The series resistance was monitored throughout the experiment, and if it appeared unstable or exceeded four times the electrode resistance, the cell was discarded. Signals were stored on-line using Pulse software, filtered at 1 kHz and digitized at 10 kHz. The liquid junction potential was approximately 9 mV between the pipette solution and the aCSF, and was not subtracted from the data obtained.

At baseline, membrane potential was recorded and input resistance calculated by averaging the voltage change that occurred during a 300pA current pulse and using the current/voltage relationship. Neuronal excitability was assessed in each cell by injecting a series of current pulses (0–180 pA, 30 pA increments), and the number of action potential per pulse was determined. Additionally membrane characteristics were recorded, including action potential (AP) amplitude, AP duration, AP threshold, afterhyperpolarization potential (AHP) duration, and AHP amplitude. After baseline recordings were performed, 300 nM CRF was bath applied, and membrane potential, input resistance, neuronal excitability, and membrane characteristics were re-assessed 6 minutes after drug application.

Following electrophysiological experiments, dual fluorescence immunohistochemistry techniques were used to confirm that recordings were performed in LC-NE cells. Slices that were used for recordings were post-fixed in 4% formaldehyde solution on 0.1 M phosphate buffer (PB; pH 7.4) for 72 hours. Biocytin-filled (recorded) neurons were visualized using an Alexa Fluor 488-conjugated streptavidin antibody (1:500, ThermoFisher Scientific, Waltham, MA, USA). Tyrosine hydroxylase (TH) was visualized using a primary TH antibody raised in mouse (1:1000, 48 h incubation, Immunostar, Hudson, WI) followed by Alexa Fluor 647-conjugated mouse secondary antibody (1:400, Jackson ImmunoResearch, West Grove, PA, USA). Images were examined using an Olympus IX81 inverted microscope (Olympus, Hatagaya, Shibuya-Ku, Tokyo, Japan) equipped with lasers (Helium Neon laser and Argon laser; models GLG 7000; GLS 5414A and GLG 3135, Showa Optonics Co., Tokyo, Japan) with the excitation wavelength of 488, 543, and 635. Data from recorded cells that were not co-stained with TH were excluded.

Data analysis for electrophysiology

Electrophysiological recordings were analyzed using Clampfit 9.2 (Axon Instruments, Foster City, CA, USA). The effect of genotype on input resistance and membrane characteristics across males and females was tested using two-way ANOVA (sex*genotype) followed by post-hoc Tukey's multiple comparison adjustments. The effect of CRF on input resistance and membrane characteristics across genotypes of males and females was tested using two-way ANOVA (sex*genotype) followed by post-hoc Tukey's multiple comparison adjustments. These statistics were performed using GraphPad Prism 7.03 (GraphPad Software, San Diego, CA, USA). The effect of sex and genotype on neuronal excitability was tested using a three-way repeated measure ANOVA/mixed-effects regression model (with sex, genotype, and injected current as covariates, along with a random effect for repeated measures from injected current), while the effect of CRF on neuronal excitability was tested by using a second mixed-effects regression model (with sex, genotype, injected current, and drug treatment as covariates, along with a random effect for repeated measures from injected current and drug treatment), both followed by post-hoc Tukey's multiple comparison adjustments when appropriate. Statistical analyses for neuronal excitability were performed using SAS version 9.4 (SAS Institute Inc., Cary, NC). Results are presented as mean $\pm$ SEM, based off of the number of cells per group. P-values less than 0.05 were considered statistically significant.

Protein extraction

Brain tissue from male and female WT and CB1r/CB2r KO mice was rapidly removed from each animal on ice. Using a trephine, the LC and mPFC brain regions were microdissected from each animal. Tissue punches were homogenized with a pestle, sonicated, and extracted in radioimmunoprecipitation assay lysis buffer with a protease inhibitor cocktail (Santa Cruz Biotechnology, Santa Cruz, CA, USA) on ice for 20 min. Lysates were cleared by centrifugation at 13,000 rpm for 12 min at 4°C, and supernatants were extracted. Protein concentrations were quantified using the bicinchoninic acid protein assay reagent (Pierce, Rockford, IL, USA).

Western blot analysis

Protein extracts were diluted with an equal volume of Novex 2× tris glycine sodium dodecyl sulfate sample buffer (Invitrogen, Carlsbad, CA, USA) containing dithiothreitol (Sigma-Aldrich Inc., St. Louis, MO, USA). Cell lysates containing equal amounts of protein (30 μ g per condition) were separated on 10% tris-glycine polyacrylamide gels and then electrophoretically transferred to Immobilon-P polyvinylidene fluoride membranes (Millipore, Bedford, MA, USA). Membranes were blocked with Odyssey buffer (1 h, diluted in 0.01M PBS 1:1) and incubated in the various combinations of the following primary antibodies overnight at room temperature (Table I): mouse anti-tyrosine hydroxylase (TH; 1:1000, Immunostar Inc., Hudson, WI), rabbit anti- α 2-adrenoceptor (α 2-AR; 1:500; Millipore Sigma, Billerica, MA, USA), guinea-pig anti-CRF (1:2000, Peninsula Laboratories, San Carlos, CA, USA), mouse anti-norepinephrine transporter (NET; 1:1000, Millipore Sigma, Billerica, MA, USA), guinea-pig anti-CB1r (1:500; gift from Dr. Mackie). Mouse anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH; 1:2000, ProteinTech

Group, Inc., Rosemont, IL, USA) was used as a loading control, to account for potential variability in amount of sample loaded. Membranes were then rinsed and incubated with infrared fluorescent secondary antibodies (Licor, Lincoln, NE, USA) for 1 h: donkey anti-rabbit IRDye 800CW (1:15,000), donkey anti-guinea-pig IRDye800 CW (1:15000), goat anti-mouse IRDye680LT (1:20,000). Membranes were scanned using the Odyssey Infrared Imaging System (Licor, Lincoln, NE, USA), and protein quantification was determined using Odyssey Infrared Imaging software. Protein quantification was normalized to the loading control, and all data is presented as a ratio of sample protein level to GAPDH level, to allow for comparison between groups.

Additionally, Chameleon Duo Pre-stained Protein Ladder (Licor, Lincoln, NE, USA) was used to determine the molecular weights of protein bands observed: GAPDH – ~37 kDa, CRF – ~25 kDa, α 2-AR - ~45 kDa, TH - ~60 kDa, NET - ~80 kDa, CB1r - ~53 kDa. Only proteins that did not fall around the same molecular weight were probed for at the same time. After imaging, membranes were stripped with NewBlot PVDF 5x Stripping Buffer (Licor, Lincoln, NE, USA) mixed with four parts DiH₂O for 20 min at room temperature. Blots were then rinsed and imaged to ensure removal of antibodies. Then, the blot could be incubated again with other primary antibodies to detect additional proteins.

ELISA

Sandwich ELISA was conducted in accordance with the instructions provided in the High Sensitivity NE Kit (Eagle, Nashua, NH). Tissue lysates containing equal amounts of protein were dispensed into an extraction plate and incubated for 60 min in 100 μ l extraction buffer at room temperature on an orbital shaker. The solution was then discarded and the extraction plates were washed before 200 μ l acylation buffer was dispensed into each well of the extraction plate and left rotating at room temperature for 20 min. Liquid was then decanted and washed 3 times prior to the dispense of 125 μ l 0.025 M hydrochloric acid into each well for an additional 20 min for elution. Next, 100 μ l eluent was transferred to the enzyme plate with 20 μ l of freshly prepared enzyme mix (2 ml Catechol-O-methyltransferase with 0.3 ml S-adenosyl-L-methionine in 0.7 ml enzyme buffer) into all wells of the enzyme plate and left at room temperature for 90 min. Finally, 100 μ l of the supernatant of each well was transferred to the NE ELISA with 20 μ l of rabbit NE-antiserum and left overnight at 4°C. The following day all wells were decanted and washed 3 times before incubation with 100 μ l anti-rabbit IgG-POD-conjugate for 60 min at room temperature on an orbital shaker. The wells were subsequently washed 4 times and incubated with 100 μ l of TMB solution for 40 min before 100 μ l of stop solution was dispensed into the wells and the plate was read at 450 nm within 15 min. A standard curve was run for each replicate and was used to estimate the concentration of NE in each sample.

Data analysis for Western blot and ELISA

Differences in CRF, α 2-AR, NET, TH, and NE levels were tested using two-way ANOVA/mixed-effects regression model (sex*genotype) followed by post-hoc Tukey's multiple comparison adjustments. Differences in CB1r expression across sexes were tested using a two-sample t-test. Statistics for the Western blot and ELISA experiments were performed using GraphPad Prism 7.03 (GraphPad Software, San Diego, CA, USA). Results are

presented as mean $\pm$ SEM. P-values less than 0.05 were considered statistically significant.

Results

Heightened LC-NE activity in male CB1r-KO mice

As previously stated, exogenous cannabinoids are known to increase LC-NE activity (Patel and Hillard, 2003; Muntoni et al., 2006). Conversely, others studies have shown that the LC is under tonic eCB regulation and male CB1r-KO mice have decreased $\alpha 2$ - and $\beta 1$ -adrenoceptor levels in the mPFC, suggestive of compensatory responses to heightened LC-NE activity (Reyes et al., 2017). In order to elucidate the effect of CB1r-KO on LC-NE signaling across sexes, whole-cell patch-clamp recordings from LC-NE neurons in 250 μ m thick horizontal brain slices from CB1r-knock out (KO) mice and their wild type (WT) C57/Bl6 mice littermates were analyzed (Fig. 1). There was a significant effect of sex on input resistance ($F_{1,17}=4.485$, $p=0.049$) and genotype on input resistance ($F_{1,17}=13.24$, $p=0.002$), but no significant effect of interaction between sex and genotype on input resistance ($F_{1,17}=2.972$, $p=0.10$). Tukey's post-hoc analysis revealed that male CB1r-KO mice showed a significant increase in input resistance (376 ± 31 M Ω) when compared to male WT mice (224 ± 13 M Ω ; $p=0.006$), female WT mice (213 ± 39 M Ω ; $p=0.005$), and female KO mice (267 ± 25 M Ω ; $p=0.041$), while there was no significant change in input resistance between female WT and KO mice ($p=0.57$) (Fig. 1B). A three-way repeated measures ANOVA/mixed effects regression model (genotype effect by sex*current pulse (repeated measure); sex effect by genotype*current pulse (repeated measure)) showed a significant effect of the following in the collected electrophysiology data: genotype ($F_{1,18}=11.41$, $p=0.003$), current pulse ($F_{6,104}=75.78$, $p<0.0001$), interaction between sex and genotype ($F_{1,18}=7.23$, $p=0.015$), interaction sex*genotype*current pulse ($F_{5,104}=2.72$, $p=0.024$), indicating all three factors were statistically significant in the regression model. Baseline membrane voltage was not significantly different between CB1r KO and WT for male and female mice. Further statistical examination of the data via Tukey post-hoc analysis revealed that genotype had a significant effect across all current pulses in male mice ($p=0.016$), but not females ($p=0.24$). Male CB1r-KO mice exhibited an increase in LC-NE cell excitability compared to male WT mice [leftward shift of the stimulus-response curve; significantly increased excitability responses to 30 ($p=0.016$), 60 ($p=0.0005$), 90 ($p=0.0001$), 120 ($p<0.0001$), 150 ($p<0.0001$), and 180 pA ($p<0.0001$) current pulses] (Fig. 1C). However, female CB1r-KO mice did not show a significant increase in excitability compared to female WT mice (Fig. 1D). Additionally, a significant effect of sex on WT mice across all current pulses was also observed. When comparing sexes, female WT mice showed a significant increase in LC-NE excitability compared to male WT mice [significantly increased excitability responses to 30 ($p=0.028$), 60 ($p=0.020$), 90 ($p=0.004$), 120 ($p=0.0003$), 150 ($p=0.0008$), and 180 pA ($p=0.0006$). No significant effect of sex was observed between CB1r-KO mice at any of the current pulses. Representative traces from each group showing the number of spikes caused by 120pA current pulse: male WT – 11 spikes, male KO – 25, female WT – 25, female KO – 28 (Fig. 1A). Slices containing patched cells were processed for immunohistochemistry, to confirm that the data was collected from NE producing cells within the LC. Only cells that were labeled with both

tyrosine hydroxylase, a marker for NE production in the LC, and biocytin were included in the analyses (Fig. 2). These data provide evidence that genetic deletion of CB1r increases LC-NE neuron excitability in males, increasing the ability of these male CB1r-KO LC-NE cells to respond to excitatory synaptic inputs.

Another way to assess alterations in LC-NE activity is to look at changes in NE production (Fig. 3). Tyrosine hydroxylase (TH) is involved in the rate-limiting step in NE synthesis, and is used as a marker for NE production within the LC. There was a significant effect of genotype on TH expression levels ($F_{1,14}=11.00$, $p=0.005$), but no significant effect of sex on TH expression ($F_{1,14}=4.287$, $p=0.057$) or interaction sex*genotype on TH expression ($F_{1,14}=3.753$, $p=0.073$). Tukey's post-hoc analysis revealed that WT male mice have significantly lower normalized levels of TH (0.313 ± 0.049) compared to CB1r/CB2r-KO males (0.751 ± 0.064 ; $p=0.011$) and KO females (0.672 ± 0.061 ; $p=0.009$), while no significant increase is observed between WT (0.639 ± 0.141) and KO females (0.672 ± 0.061 ; $p=0.77$) (Fig. 3A). By using an ELISA, it is possible to more precisely determine changes in NE levels in the mPFC, representing a concrete endpoint for the effect of cannabinoid receptor deletion on LC-NE activity. Two-way ANOVA indicated a significant effect of genotype on NE levels ($F_{1,11}=9.719$, $p=0.01$), but no significant effect of sex on NE levels ($F_{1,11}=4.825$, $p=0.050$) or interaction sex*genotype on NE levels ($F_{1,11}=3.151$, $p=0.10$). Male CB1r/CB2r-KO mice have significantly increased levels of NE in the mPFC (0.151 ± 0.032 pg/mL; $p=0.021$) compared to all other groups (Fig. 3B): WT males (0.049 ± 0.008 pg/mL), WT females (0.039 ± 0.003 pg/mL; $p=0.012$), and KO females (0.071 ± 0.011 pg/mL; $p=0.041$). There was no significant difference in NE levels between WT and KO females ($p=0.79$). The Western and ELISA data confirms that deletion of the CB1r causes a significant increase in NE indices in male but not female mice.

Sex differences in cellular adaptations following cannabinoid receptor deletion

In order to better understand what might be causing the increase in LC-NE activity in male CB1r-KO mice but not females, Western blot analyses were performed to determine what other changes in protein levels might be occurring (Fig. 4). Within the LC, the $\alpha 2$ -AR functions to auto-inhibit LC-NE neurons. When examining changes in normalized $\alpha 2$ -AR protein levels following CB1r/CB2r deletion (Fig. 4A), a two-way ANOVA determined a significant effect of genotype on $\alpha 2$ -AR expression ($F_{1,14}=10.62$, $p=0.006$), but no significant effect of sex ($F_{1,14}=3.004$, $p=0.11$) or interaction of sex*genotype ($F_{1,14}=2.927$, $p=0.11$) on $\alpha 2$ -AR expression. Tukey's post-hoc analysis showed no significant change between WT males (0.560 ± 0.027) and KO males (0.721 ± 0.054 ; $p=0.70$); however, KO females (0.720 ± 0.059) showed a significant increase compared to WT females (0.386 ± 0.098 ; $p=0.016$). CB1r-KO mice have heightened HPA-activity and the LC is involved in the cognitive limb of the stress response; therefore, changes in CRF levels within the LC were also determined (Fig. 4B). Two-way ANOVA revealed a significant effect of genotype on CRF expression ($F_{1,14}=8.57$, $p=0.011$), but no significant effect of sex on CRF expression ($F_{1,14}=0.3233$, $p=0.58$) or interaction sex*genotype on CRF expression ($F_{1,14}=1.586$, $p=0.23$). Tukey's post-hoc analysis determined that CB1r/CB2r deletion resulted in a significant increase in normalized CRF levels in KO males (0.467 ± 0.088) compared to WT males (0.752 ± 0.062 ; $p=0.045$), but no significant change was observed between WT (0.581

± 0.139) and KO females (0.715 ± 0.059 ; $p=0.65$). Finally, changes in LC expression of the norepinephrine transporter (NET) were assessed (Fig. 4C). Two-way ANOVA revealed a significant effect of sex ($F_{1,14}=16.11$, $p=0.001$), genotype ($F_{1,14}=13.32$, $p=0.003$), and interaction sex* genotype ($F_{1,14}=12.07$, $p=0.004$) on LC NET expression. Tukey's post-hoc analysis showed significantly greater expression of NET in WT male mice (0.893 ± 0.088) compared to all other groups: KO males (0.541 ± 0.048 ; $p=0.001$), WT females (0.522 ± 0.071 ; $p=0.002$), and KO females (0.512 ± 0.041 ; $p=0.0005$). Within the mPFC, there was no significant effect of genotype ($F_{1,12}=0.5998$, $p=0.45$), sex ($F_{1,12}=0.1215$, $p=0.73$), or interaction sex*genotype ($F_{1,12}=3.674$, $p=0.08$) on NET expression (Fig. 4D): WT males (0.842 ± 0.047), KO males (0.693 ± 0.070), WT females (0.756 ± 0.013), and KO females (0.819 ± 0.036). The increase in CRF expression in male KO mice compared to WT and $\alpha 2$ -AR expression in female KO mice compared to WT might provide some insight into why CB1r-deletion causes an increase in LC-NE excitability in males but not females.

Additionally, basal differences in CB1r expression across sexes could account for the observed electrophysiological differences following CB1r deletion. To investigate this, Western blot analysis on male and female WT mice was performed (Fig. 4E). Two-sample t-test analysis revealed a significant difference in CB1r expression between males (0.379 ± 0.056) and females (0.162 ± 0.068 ; $t_{10}=2.469$, $p=0.033$). Less CB1r expression in females could be contributing to the increased basal LC-NE excitability in WT females compared to males, as well as the lack of increase in LC-NE excitability in CB1r-KO females compared to WT females.

Loss of CRF-induced increases in LC-NE excitability in CB1r-KO mice

We determined how CRF affects LC-NE excitability under conditions of CB1r deletion. After baseline measurements were recorded from male and female WT neurons, CRF (300 nM) was bath applied and the effect of drug treatment was then recorded. This dose of CRF did not cause a significant increase in the input resistance of male (Fig. 5B) or female (Fig. 5B') WT and CB1r-KO mice when compared to baseline: male WT CRF (274 ± 29 M Ω), male KO CRF (417 ± 52 M Ω), female WT CRF (259 ± 43 M Ω), and female KO CRF (282 ± 48 M Ω). Data for neuronal excitability was analyzed using a four-way repeated measure ANOVA/mixed-effects regression model (sex effect by genotype*current pulse, genotype effect by sex*current pulse, genotype effect by drug treatment*current pulse, drug effect by genotype*current pulse) and the following effects were found significant: drug treatment ($F_{1,20}=6.92$, $p=0.016$), current pulse ($F_{6,108}=159.27$, $p<0.0001$), interaction sex*genotype ($F_{1,18}=10.55$, $p=0.005$), interaction genotype*current pulse ($F_{6,108}=4.85$, $p=0.0002$), interaction sex*genotype*current pulse ($F_{6,108}=4.80$, $p=0.0002$), and interaction genotype*drug treatment ($F_{1,20}=5.48$, $p=0.03$), indicating all four factors were important predictors of neuronal excitability. CRF (300 nM) treatment caused a significant increase in LC-NE cell excitability in both male WT (Fig. 5C) and female WT (Fig. 5C') mice, as expected. Tukey post-hoc analyses showed a significant drug treatment effect across WT male and female mice ($p=0.0002$ for 30 pA current pulses and $p<0.0001$ for 60, 90, 120, 150, and 180 pA current pulses). Interestingly, CRF did not cause a significant increase in LC-NE cell excitability in male (Fig. 5D) and female (Fig. 5D') CB1r-KO mice, as the post-hoc analyses showed no significant effect of drug treatment across KO male and female

mice ($p = 0.63$). Representative traces from each group showing the number of spikes caused by 120pA current pulse are provided in figure 5: male WT baseline – 11 spikes, male WT CRF – 16 spikes, male KO baseline – 25, male KO CRF – 28 (Fig. 5A), female WT baseline – 25 baseline, female WT CRF – 33, female KO baseline – 28, female KO CRF – 30 (Fig. 5A). These electrophysiological results suggest that while 300 nM CRF causes a significant increase in LC-NE cell excitability in WT male and female mice, it is not capable of producing the same effect in CB1r-KO mice.

When examining single action potentials, CRF administration, sex, and genotype did not affect many of the analyzed characteristics (Table II). Resting membrane potential remained constant among all groups, as did action potential threshold, duration, amplitude, and after-hyperpolarization amplitude. CRF administration did cause a significant decrease in after-hyperpolarization duration in WT males ($p=0.004$), KO males ($p=0.047$), and WT females ($p=0.002$), but not in KO females ($p=0.069$) (Fig. 6). CB1r deletion also caused a significant reduction in after- hyperpolarization duration in males ($p=0.013$), but not in females ($p=0.29$).

Discussion

The present study highlights interesting sex differences in LC neurons following cannabinoid receptor deletion. Electrophysiological studies conducted using an in vitro slice preparation show that CB1r deletion results in a significant increase in LC-NE excitability in male mice, but not in females. Additionally, male CB1r-KO mice have a significant increase in TH expression in the LC and NE levels in the mPFC compared to WT males, which is not observed in females. Via Western blot analysis, changes in protein expression across genotype and sex were observed. Male CB1r/CB2r-KO mice exhibited an increase in CRF expression and a decrease in NET expression in the LC compared to male WT mice, and female CB1/CB2r-KO mice had an increase in $\alpha 2$ -AR levels in the LC compared to female WT mice. Basal differences in CB1r expression were also observed, with male WT mice having higher CB1r levels than female WT mice. Finally, CB1r deletion affected CRF-induced increases in LC-NE activity. Bath application of CRF caused an increase in LC-NE excitability in male and female WT mice; however, the effect of CRF was lost in CB1r-KO mice. When examining individual membrane characteristics, LC-NE cells from male CB1r-KO mice showed a decrease in after-hyperpolarization duration. Additionally, CRF treatment caused a further decrease in after-hyperpolarization duration in LC-NE neurons from WT males and CB1r-KO males, and also a decrease in WT females. These data further highlight the importance of the endocannabinoid system in maintaining normal brain adrenergic function, especially in male mice, where CB1r deletion had the most profound effect.

Methodological considerations

While electrophysiology experiments were carried out in CB1r-KO mice, Western blot and ELISA experiments were carried out in CB1r/CB2r-KO mice due to tissue availability. Many immunohistochemical studies have identified CB1r as an abundant protein in the LC, both directly on the noradrenergic neurons as well as on presynaptic afferents synapsing

onto LC-NE dendrites and cell bodies (Scavone et al., 2010; Wyrofsky et al., 2017). While CB2r is traditionally thought to play a role in the periphery and immune responses (Castillo et al., 2012), growing accumulating evidence suggests that it does in fact play a role in neuronal signaling in some select brain regions (Van Sickle et al., 2005; Gong et al., 2006). However, direct evidence of CB2r on LC-NE neurons has not been shown. Additionally, mixed CB1r/CB2r agonists such as THC, WIN 55,212-2, and CP 55,940 all dose-dependently increase LC-NE firing, and their effects are completely blocked by CB1r-selective antagonist SR141716A (Mendiguren & Pineda, 2006; Muntoni et al., 2006). These studies suggest that there is a significantly greater influence of CB1r than CB2r on LC-NE neurons, providing indirect evidence for a minimal role of CB2r signaling in the LC. Therefore, the effects of CB2r deletion in the LC should be less substantial than that of CB1r deletion, and the differences between CB1r-KO mice and CB1r/CB2r-KO mice should be minimal. However, the caveat exists that findings reported here are due to deletion of both receptor phenotypes.

Several studies have reported fluctuations in CB1r mRNA expression and eCB levels across the estrus cycle in several brain regions, including the anterior pituitary (Gonzales et al., 2000) and hypothalamus (Rodriguez de Fonseca et al., 1994), however the effect of ovarian hormones on CB1r and eCB levels in the LC has not been identified. While estrogen might alter the endocannabinoid system, no effect of the estrus cycle was observed in preliminary studies examining the anti-depressant like behavioral effects of CB1r antagonism (Steiner et al., 2008). Additionally, a study specifically examining the LC region controlled for the estrus cycle and found no notable effect of phase on female LC-NE excitability, firing rate, or CRF_{r1} compartmentalization in both WT and CRF-overexpressing mice (Bangasser et al., 2013). Since no direct effect of estrus cycle has been found on baseline WT LC-NE excitability, and since the effect of circulating sex hormones on the eCB system is less relevant when CB1r are genetically deleted, we did not note the estrus cycle prior to our experiments, though the caveat exists that controlling for the estrus cycle could alter our results.

Some experimental caveats exist with Western blot and ELISA analysis, including the accuracy of tissue punches and equal protein quantities across various animals and groups. In order to ensure that regions of interest were sampled accurately, one investigator preformed the micropunches for each animal. Additionally, Western blots were probed with GAPDH as an internal standard to ensure equal protein loading for each sample. Results were normalized to GAPDH expression, which was comparable across each animal group examined. Finally, while Western blot analysis allows us to examine changes in protein expression levels, subsequent studies examining changes in mRNA levels could be conducted to further elucidate the effects of CB1r deletion on noradrenergic indices in the LC.

Basal Differences in LC-NE excitability across sexes

Whole-cell patch-clamp electrophysiology revealed that female WT mice have increased LC-NE excitability compared to male WT mice. This implies that female WT LC-NE cells have an increased sensitivity to depolarizing stimuli than male WT cells. It is important to

note that while WT females have heightened excitability, this does not correlate to a heightened basal firing rate. Previous studies by Bangasser et al. similarly found that female WT mice have increased LC-NE excitability compared to males, but the basal firing rate of these neurons is not significantly different across sexes (Bangasser et al., 2013). In corroboration with Bangasser's findings that male and female LC-NE neurons have similar firing rates, our study shows that there is no significant difference in NE levels in the mPFC across sexes – another endpoint for LC-NE firing. While we observe an increase in LC-NE excitability in male CB1r-KO mice, we did not examine the basal firing rates between male and female WT and CB1r-KO mice. It would be tempting to postulate that CB1r-KO males would also have an increase in LC-NE firing rate compared to the other groups, as they are the only group with increased mPFC NE levels. Subsequent electrophysiology studies could further elucidate this hypothesis.

CB1r-KO increases LC-NE excitability in males but not females

One of the most interesting and surprising findings from this study is that CB1r deletion affected LC-NE excitability selectively in male subjects. The changes observed in input resistance and after-hyperpolarization (AHP) duration across sex and genotype are consistent with the changes observed in LC-NE excitability. All groups that had heightened LC-NE excitability also showed a decrease in AHP duration: CB1r-KO males and WT females compared to WT males, CRF treated WT males and females compared to baseline. Additionally, CB1r-KO male and CRF treated WT males and females all had an increase in input resistance, suggesting a decrease in ion channel conductance. Neuronal increases in 3', 5'-cyclic adenosine monophosphate (cAMP) cause increased Ca^{2+} efflux. Ca^{2+} -mediated increases in K^{+} conductance occur in LC-NE cells, leading to more rapid repolarization (Adams et al., 1982; Osmanovic and Shefner, 1993; Nestler et al., 1999). An increased repolarization rate corresponds with a shorter AHP duration, and suggests a general decrease in overall K^{+} ion conductance (Adams et al., 1982). Results from the Western blot analysis provide further insight into why only certain groups had increased excitability.

In males, a potential mechanism underlying increased LC-NE excitability involves differences in CRF expression. Interestingly, only male CB1r/CB2r-KO mice showed an increase in CRF expression in the LC when compared to WT and female subjects. This was surprising initially, since many studies have shown that CB1-KO mice have heightened HPA axis activity and increased vulnerability to chronic stress (Martin et al., 2002; Uriguen et al., 2004), and female rodents are known to have increased susceptibility to stressors compared to males (Bangasser and Valentino, 2012; Handa and Weiser, 2014). However, more recent studies examining mice of both sexes report that females do not show HPA axis hyperactivity to the same degree as males following CB1r deletion or antagonism (Atkinson et al., 2010; Roberts et al., 2014). Thus, since the LC-NE cognitive limb of the stress response is engaged in parallel with the HPA axis (Van Bockstaele et al., 1998, Valentino and Van Bockstaele, 2008), it is tempting to speculate that female CB1r-KO mice might show less of an increase in CRF release in the LC compared to males. CRF binds to its receptor, CRFR1, on LC-NE neurons and increases LC-NE firing rate (Curtis et al., 1996). Both acute and chronic stress up-regulate the cAMP pathway (Nestler and Aghajanian, 1997), which, through downstream effects, increases neuronal re-polarization and decreases

LC-NE AHP duration (Adams et al., 1982; Nestler et al., 1999). Therefore, increased CRF expression represents one mechanism by which CB1r-KO males might have increased LC-NE excitability while female CB1r-KO mice do not (Fig. 7B).

Another potential mechanism underlying the observed sex differences in this study involves alterations in $\alpha 2$ -AR expression between female CB1r/CB2r-KO and WT mice. NE released from recurrent collaterals of LC neurons can bind to postsynaptic $\alpha 2$ -AR on the LC-NE dendrites resulting in auto-inhibition (Lee et al., 1998a, b). In response, an increase in $\alpha 2$ -AR would result in a decrease in cAMP and inhibition of LC-NE neurons, which is characterized by an increase in AHP and decrease in input resistance (Korf et al., 1973). While CB1r/CB2r deletion does not affect $\alpha 2$ -AR levels in males, it does cause a significant increase in $\alpha 2$ -AR expression in females. This could represent a compensatory mechanism in females, where another inhibitory receptor, $\alpha 2$ -AR, is up-regulated to compensate for the loss of another inhibitory receptor, CB1r (Fig. 7B'). This results in comparable excitability levels across LC-NE neurons, input resistance, and AHP duration in CB1r-KO and WT females.

Lastly, the observed sex difference in basal CB1r expression is likely a key contributor to the electrophysiological variances across sexes. While there is conflicting data surrounding the effects of CB1r antagonists on LC-NE activity, studies performed on male rodents show that they do alter LC-NE firing, suggesting there is tonic eCB activity in males (Carvalho & Van Bockstaele, 2012; Wyrofsky et al., 2015). One study found that a low dose of a CB1r antagonist resulted in an increase in NE release in the mPFC (Tzavara et al., 2003). This mimics what was observed in the male CB1r-KO mice, where abolishing CB1r signaling receptor deletion caused an increase in NE release. One final reason why female CB1r-KO mice might not have shown a significant increase in LC-NE excitability and NE efflux could be because they have less CB1r expression, so their deletion would have less profound of an effect on LC cells compared to males. Future studies examining the effect of CB1r antagonists in females would provide additional support for this hypothesis. Additionally, while less CB1r expression could indicate less tonic eCB signaling in the female LC, it is important to note that other eCB proteins play a role as well. Basal differences in FAAH and other eCB metabolic proteins might also exist between males and females, further implicating a difference in tonic eCB regulation of the LC. Subsequent Western blot analyses investigating their expression should be performed to determine their contribution to changes in LC-NE excitability across sexes and genotype.

Loss of CRF-mediated responses in CB1r-KO mice

Although CRF is known to modulate LC-NE neurons and increase their firing rate (Curtis et al., 1996; Valentino et al., 1997; Jedema and Grace, 2004), and this study confirmed that 300 nM CRF bath application is capable of doing so in LC neurons from WT males and females, this effect was not observed in CB1r-KO mice. This loss of CRF-mediated increases in LC-NE excitability in male CB1r-KO mice could be due to the increased endogenous CRF levels saturating CRF₁, rendering the exogenous application of oCRF ineffective (Fig. 7C). In female CB1r-KO mice, the lack of CRF-induced responses might be attributed to the

increased $\alpha 2$ -AR expression, causing local auto-inhibition and attenuation of LC-NE activity following oCRF administration (Fig. 7C').

Additionally, chronic stress has been shown to alter CRF₁ trafficking in a sex dependent manner, with males exhibiting increased stress-induced internalization while females exhibit stress-induced recruitment to the plasma membrane (Bangasser et al., 2010; Valentino et al., 2013). It is possible that increased HPA hyperactivity (Uriguen et al., 2004) and a chronic increase in CRF levels in the LC of male CB1r-KO mice could lead to sustained desensitization of CRF receptors. Immunoelectron microscopy experiments examining CRF receptor trafficking in LC-NE neurons of male and female CB1r-KO mice could provide further insight into changes in the stress circuitry following CB1r deletion.

Implications for pharmacotherapies

Dysregulation of NE is a key component in the development of anxiety and other stress-induced psychiatric disorders (Carvalho and Van Bockstaele, 2012). Furthermore, reduction in NET expression in the LC has been identified in individuals suffering from major depression and PTSD (Klimek et al., 1997; Pietrzak et al., 2013). While acute stress does not affect NET levels, chronic and repeated stress exposure can lead to downregulation (Zafar et al., 1997). Increased turnover and NE depletion, which can occur following a stressor (Korf et al., 1973), also leads to the downregulation of NET and upregulation of TH, and increased TH levels in the LC have also been correlated with the development of depression (Klimek et al., 1997). Male CB1r/CB2r-KO mice exhibit a decrease in NET and an increase in TH expression, while no change was observed between female CB1r/CB2r-KO and WT mice. These findings suggest that CB1r deletion or chronic antagonism may be more detrimental to males than females, creating a microenvironment in the LC of male KO mice that mimics conditions of chronic stress.

In addition to cellular adaptations that occur following cannabinoid receptor deletion, the lack of increase in LC-NE excitability in female CB1r-KO mice could be due to less basal eCB signaling in females. If the female LC is under less tonic regulation by eCBs, then removal of CB1r might have less profound of an effect. In the amygdala, basal differences in the eCB system have been observed across sex, with males having greater levels of 2-AG and AEA, while females have increased expression of the enzymes responsible for their degradation (Krebs-Kraft et al., 2010; Craft et al., 2013). A similar phenomenon might exist in the LC, and future Western blot and ELISA analyses of 2-AG, AEA, and their metabolic and synthesizing enzymes would provide further insight into potential basal sex differences.

We have demonstrated in previous studies that CB1r are localized to CRF-containing afferents from the amygdala, and their presynaptic distribution in the peri-LC suggests that they might be capable of attenuating CRF release via activation by endogenous or exogenous cannabinoids. A working model (Fig. 7A) is that CRF released from amygdalar afferents binds to postsynaptic CRF₁ on LC-NE neurons, causing membrane depolarization and increase in LC-NE excitability. However, the influx of Ca^{2+} then stimulates the synthesis and release of eCBs, which in turn retrogradely traverse the synapse. They then bind to presynaptic CB1r on CRF-containing amygdalar axon terminals, aiding in the suppression of subsequent CRF release and helping return LC-NE activity back to baseline.

CB1r antagonists and inverse agonists have been investigated for a variety of disorders ranging from obesity to schizophrenia (Wyrofsky et al., 2015). Rimonabant, a CB1r antagonist originally used for the treatment of obesity, was discontinued due to adverse psychological side effects (Nissen et al., 2008). Based on the results obtained in this study, CB1r antagonism might not cause an increase in LC-NE excitability to the same degree in females that it would in males, and the effects from CB1r antagonist treatment might be less significant in females, highlighting the importance of using both genders when performing pre-clinical trials. Indeed, this would be in line with analyses performed on the adverse effects of rimonabant, which suggest that the odds ratio for developing depression after taking this CB1r antagonist was greatest in males aged 35–38 (Pi-Sunyer et al., 2006; Nissen et al., 2008). This study adds to the growing literature that dysregulation of the cannabinoid system can lead to the dysregulation of noradrenergic signaling, especially in males, helping to advance our understanding of how these systems could be targeted for more effective treatment of psychological disorders.

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Abbreviations

2-AG	2-arachidonylglycerol
aCSF	artificial cerebrospinal fluid
AEA	N-arachidonylethanolamide/anandamide
AHP	afterhyperpolarization potential
ANOVA	analyses of variance
AP	action potential
AR	adrenoceptor
cAMP	cyclic adenosine monophosphate
CB1r	cannabinoid type 1 receptor
CB2r	cannabinoid type 2 receptor
CRF	corticotropin releasing factor
CRFr1	corticotropin releasing factor receptor type 1
eCB	endocannabinoid
ELISA	enzyme-linked immunosorbent assay
FAAH	fatty acid amide hydrolase
GAPDH	glyceraldehyde 3-phosphate dehydrogenase

HPA	hypothalamic-pituitary adrenal (axis)
KO	knockout
LC	locus coeruleus
mPFC	medial prefrontal cortex
mRNA	messenger ribonucleic acid
NE	norepinephrine
NET	norepinephrine transporter
oCRF	ovine corticotropin releasing factor
PTSD	posttraumatic stress disorder
PVDF	polyvinylidene difluoride
SEM	standard error of mean
TH	tyrosine hydroxylase
THC	–9 tetrahydrocannabinol
WT	wild type

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Figure 1: CB1r deletion causes an increase in LC-NE excitability in male mice.

a. Voltage responses to a 120 pA current pulse from representative WT and CB1r-KO male and female mice. **b.** LC-NE neurons from male CB1r-KO mice have a significantly larger input resistance compared to WT males ($p=0.006$) and WT females ($p=0.005$). CB1r deletion does not cause a change in input resistance in female LC-NE cells. Panels **c**, **d**, and **e** summarize excitability data in the form of stimulus-response curves to a range of current pulses (0–150 pA in 30 pA increments) in male and female WT and CB1r-KO mice. CB1r deletion results in a significant increase in excitability of LC-NE cells in male mice

(leftward shift in **c**), which it does not affect LC-NE excitability in female mice (no shift in **d**). **e**. Female WT mice have a significant increase in LC-NE excitability compared to male WT mice. N=5 cells from 5 WT males, N=4 cells from 4 WT females, N=6 cells from 5 KO males, N=6 cells from 4 KO females. Data represent mean $\pm$ SEM. Astrisks indicate a significant difference between groups as determined by two-way and three-way repeated measure ANOVAs/mixed-effects regression model (* $p < 0.05$; ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$).

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Figure 2: Confirming electrophysiological data is obtained from NE producing cells within the LC.

Confocal fluorescence micrographs showing TH (red), biocytin (green), and co-localization (yellow) in the LC. **a.** TH was detected using an Alexa Fluor 647-conjugated secondary antibody. **b.** Biocytin was detected using an Alexa Fluor 488-conjugated streptavidin antibody. **c.** *Arrow* depicts co-localization between biocytin and TH, confirming that the patched cell was an LC-NE neuron. *Single arrowheads* indicate TH labeled cell bodies in the LC that were not patched. Scale bar = 25 μ m.

Figure 3: Western blot and ELISA analyses of NE indices in the LC and mPFC of male and female WT and CB1r/CB2r-KO mice.

Bands shown are representative of one sample obtained from one animal per group. **a.** Western blot analysis for TH in protein extracts from the LC of male and female WT and CB1r/CB2r-KO mice. TH expression is significantly higher in male CB1r/CB2r-KO mice compared to male WT mice ($p=0.005$); however, there is no significant change in TH expression between female WT and CB1r/CB2r-KO ($p=0.77$). $N=3$ WT males, $N=6$ KO males, $N=3$ WT females, and $N=6$ KO females. **b.** ELISA for NE in protein extracts from

the mPFC of male and female WT and CB1r/CB2r-KO mice. NE levels are significantly increased in male CB1r-KO mice compared male WT ($p=0.021$), female WT ($p=0.012$), and female CB1r/CB2r-KO mice ($p=0.041$). CB1r deletion does not cause an increase in mPFC NE levels in female mice ($p=0.79$). $N=3$ WT males, $N=5$ KO males, $N=3$ WT females, and $N=4$ KO females. **c.** Standard curve used to determine the NE concentration in LC protein extracts from each sample animal. Standards were run on the same plate as samples to avoid variability, and the R^2 value for the curve equals 0.9904. Data represent mean $\pm$ SEM. Asterisks indicate a significant difference between groups as determined by two-way ANOVAs/mixed-effects regression model (* $p < 0.05$).

Figure 4: Western blot analysis of α 2-adrenoreceptor (α 2-AR), corticotropin-releasing factor (CRF), and norepinephrine transporter (NET) expression in the LC and mPFC in male and female WT and CB1r/CB2r-KO mice.

Bands shown are representative of one sample obtained from one animal per group. LC blots were stripped and reincubated, and multiple proteins were probed on the same blots. **a.** Western blot analysis for α 2-AR expression in protein extracts from the LC of male and female WT and CB1r/CB2r-KO mice. CB1r/CB2r deletion causes a significant increase in α 2-AR expression compared to WT mice in females ($p=0.016$), but no change in males ($p=0.70$). $N=3$ WT males, $N=6$ KO males, $N=3$ WT females, and $N=6$ KO females. **b.**

Western blot analysis for CRF expression in protein extracts from the LC of male and female WT and CB1r/CB2r-KO mice. There is a significant increase in CRF levels in the LC of male CB1r/CB2r-KO mice compared to male WT mice ($p=0.045$), while no change occurs in female mice ($p=0.65$). N=3 WT males, N=6 KO males, N=3 WT females, and N=6 KO females. **c.** Western blot analysis for NET expression in protein extracts from the LC of male and female WT and CB1r/CB2r-KO mice. Male WT mice have significantly greater NET levels in the LC compared to male CB1r/CB2r-KO ($p=0.001$), female WT ($p=0.002$), and female CB1r/CB2r-KO mice ($p=0.005$). N=3 WT males, N=6 KO males, N=3 WT females, and N=6 KO females. **d.** Western blot analysis for NET expression in protein extracts from the mPFC of male and female WT and CB1r/CB2r-KO mice. No significant change in NET expression is observed across sexes or genotype. N=3 WT males, N=5 KO males, N=3 WT females, and N=4 KO females. **e.** Western blot analysis for CB1r expression in protein extracts from the LC of male and female WT mice. Male WT mice have significantly greater CB1r expression in the LC compared to female WT mice ($p=0.033$). N=7 WT male mice and N=5 WT female. Data represent mean $\pm$ SEM. Asterisks indicate a significant difference between groups as determined by two-way ANOVAs/mixed-effects regression model (* $p < 0.05$).

Figure 5: CB1r deletion disrupts CRF-induced increases in LC-NE excitability in male and female mice.

Voltage responses to a 120 pA current pulse are shown from representative WT and CB1r-KO male (**a**) and female (**a'**) mice both at baseline and following CRF (300 nM) treatment. **b**, Bath application of CRF (300 nM) does not cause an increase in WT or CB1r-KO neuronal excitability in male or female (**b'**) mice. Panels **c**, **c'**, **d**, and **d'** summarize excitability data in the form of stimulus-response curves to a range of current pulses (0–150 pA in 30 pA increments). Bath application of CRF (300 nM) results in a significant increase

in excitability of LC-NE cells in male WT mice (leftward shift in **c**) and female WT mice (leftward shift in **c'**); however, 300 nM CRF does not alter the excitability of LC-NE cells in male CB1r-KO mice (no shift in **d**) and female CB1r-KO mice (no shift in **d'**). N=5 cells from 5 WT males, N=4 cells from 4 WT females, N=6 cells from 5 KO males, N=6 cells from 4 KO females. Data represent mean $\pm$ SEM. Asterisks indicate a significant difference between groups as determined by two-way ANOVA (**c** and **c'**) and four-way repeated measure ANOVAs/mixed-effects regression model (**d**, **d'**, **e**, and **e'**; * $p < 0.05$; *** $p < 0.001$).

Figure 6: Sex, genotype, and CRF treatment affect the after-hyperpolarization (AHP) duration of LC-NE neurons.

a. LC-NE cells from female WT mice have a significantly shorter AHP duration compared to cells from WT males ($p=0.001$). Additionally, there is a sex difference in the effect of CB1r deletion, with neurons from male CB1r-KO mice having a shorter AHP duration compared to neurons male WT mice ($p=0.002$), but no difference between female CB1r-KO and WT cells ($p=0.38$). **b** and **b'** show the effect of 300 nM CRF bath application on the AHP duration of LC-NE cells, separated by sex. **b.** CRF causes a significant reduction in AHP duration in both male WT ($p=0.004$) and male CB1r-KO neurons ($p=0.013$). **b'.** CRF causes a significant reduction in AHP duration in female WT ($p=0.002$) but not female CB1r-KO ($p=0.069$) neurons. $N=4$ cells from 4 WT males, $N=4$ cells from 4 WT females, $N=5$ cells from 5 KO males, $N=4$ cells from 4 KO females. Data represent mean $\pm$ SEM. Asterisks indicate a significant difference between groups as determined by two-way ANOVAs/mixed-effects regression model (* $p < 0.05$).

Figure 7: Schematic depicting the effects of CB1r deletion on LC-NE indices.

This schematic contains a combination of results from electrophysiology studies performed in CB1r-KO mice with Western blot and ELISA analysis performed on CB1r/CB2r-KO tissue. **a.** Our working model depicting how the endocannabinoid (eCB) system might modulate CRF-mediated responses within the LC. (1) Stressors cause CRF to be released from excitatory amygdalar afferents into the LC. (2) CRF binds to its postsynaptic CRF type 1 receptor (CRF1), and subsequent post-synaptic depolarization and Ca²⁺ influx leads to increased LC-NE excitability and NE efflux in the mPFC. (3) Increased intracellular

Ca^{2+} levels stimulate eCB production, which then cross the synapse in a retrograde fashion to bind to their cannabinoid type 1 receptors (CB1r). (4) CB1r activation leads to inhibition of pre-synaptic CRF and glutamate release, attenuating CRF-induced increases in LC-NE excitability and NE efflux in the mPFC. **b.** Male CB1r/CB2r-KO mice have increased CRF expression within the LC, which could be responsible for the heightened LC-NE excitability and subsequent NE efflux in the mPFC. **b'.** Female CB1r/CB2r-KO mice do not have a significant increase in CRF levels, which corresponds with the lack of change in LC-NE excitability and NE efflux in the mPFC of CB1r-KO mice compared to wild type mice. However, there is a significant increase in $\alpha 2$ -adrenoceptor ($\alpha 2$ -AR) expression in female CB1r/CB2r-KO mice compared to WT, while there is no significant change in $\alpha 2$ -AR expression in males. **c.** Bath application of ovine CRF (oCRF) causes a significant increase in male and female WT mice, but not in CB1r-KO mice. In males, this might be due to saturation of CRFr1 by the increased endogenous CRF levels. **c'.** In females, the lack of oCRF-induced increases in LC-NE excitability could be attributed to the significant increase in $\alpha 2$ -AR expression. oCRF could induce local NE release and subsequent auto-inhibition, preventing oCRF-induced increases in LC-NE activity.

Table 1:

Characterization of the primary antibodies used for immunofluorescence microscopy (IF) and Western blotting (WB)

Antigen	Immunogen	Host	Source	Catalog #	Dilution	References
Biocytin	A biotin-binding protein (streptavidin) covalently attached to a fluorescent label (Alexafluor 488)	n/a	ThermoFisher Scientific	S32354	1:500 (IF)	Li & Kirby (2016)
TH	TH purified from rat PC12 cells	Mouse	Immunostar Inc	22941	1:1000 (IF & WB)	Van Bockstaele & Pickel (1993), Oropeza et al. (2005)
α 2-AR	Synthetic peptide, amino acids 218–235 of human, rat, and pig	Rabbit	Millipore Sigma	SAB4500548	1:500 (WB)	Reyes et al. (2009), Reyes et al. (2017)
CRF	Synthetic CRF peptide	Guinea-pig	Peninsula Laboratories	T-5007	1:2000 (WB)	Rudoy et al. (2009)
NET	Sodium-dependent NET recombinant protein epitope signature tag (PrEST)	Mouse	Millipore Sigma	MAB5620	1:1000 (WB)	Reyes et al. (2009)
CB1r	Directed towards last 72 amino acid residues (401–473) of rat CB1r	Guinea-pig	Gift from Dr. Mackie	n/a	1:500 (WB)	Reyes et al. (2015)
GAPDH	GAPDH fusion protein Ag0766	Mouse	ProteinTech	60004-I-Ig	1:2000 (WB)	Ross et al. (2017)

Table II:

Action potential and after-hyperpolarization characteristics of LC-NE neurons before and after 300 nM CRF bath application in wild type (WT) and CB1r-knock out (KO) male and female mice.

	Male			Female		
	WT		CB1r KO	WT		CB1r KO
	Baseline	CRF	Baseline	Baseline	CRF	CRF
Resting Membrane Potential (mV)	-51.50 ± 4.99	-50.25 ± 5.17	-55.40 ± 2.32	-50.67 ± 0.67	-54.00 ± 2.08	-51.25 ± 6.88
Action Potential Threshold (mV)	-25.72 ± 5.20	-24.67 ± 5.62	-32.76 ± 2.60	-31.99 ± 2.72	-34.98 ± 4.98	-33.52 ± 5.83
Action Potential Amplitude (mV)	68.08 ± 2.65	61.48 ± 3.37	63.87 ± 4.78	68.82 ± 4.93	67.58 ± 5.86	25.58 ± 18.84
Action Potential Duration (ms)	0.88 ± 0.13	0.88 ± 9.17	0.77 ± 0.05	0.67 ± 0.09	0.74 ± 0.10	0.94 ± 0.32
Afterhyperpolarization Amplitude (mV)	-18.68 ± 2.63	-18.11 ± 2.93	-16.06 ± 1.14	-18.91 ± 1.38	-17.15 ± 2.41	-11.97 ± 2.45
Action Potential Duration (ms)	117.4 ± 8.7	80.9 ± 10.9 [*]	85.1 ± 5.2 [^]	73.3 ± 9.4 ⁺	45.6 ± 4.6 [*]	48.7 ± 2.5

N=4 cells from 4 WT males, N=4 cells from 4 WT females, N=5 cells from 5 KO males, N=4 cells from 4 KO females. Data represent mean ± SEM. Asterisks indicate a significant difference (p<0.05) between groups as determined by two-way ANOVAs/mixed-effects regression models

^{*} significant difference between CRF treatment and baseline

⁺ significant difference between sex

[^] significant difference between genotype.