



## Research Paper

## Cortical adrenoceptor expression, function and adaptation under conditions of cannabinoid receptor deletion

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## ABSTRACT

A neurochemical target at which cannabinoids interact to have global effects on behavior is brain noradrenergic circuitry. Acute and repeated administration of a cannabinoid receptor synthetic agonist is capable of increasing multiple indices of noradrenergic activity. This includes cannabinoid-induced 1) increases in norepinephrine (NE) release in the medial prefrontal cortex (mPFC); 2) desensitization of cortical  $\alpha$ 2-adrenoceptor-mediated effects; 3) activation of c-Fos in brainstem locus coeruleus (LC) noradrenergic neurons; and 4) increases in anxiety-like behaviors. In the present study, we sought to examine adaptations in adrenoceptor expression and function under conditions of cannabinoid receptor type 1 (CB1r) deletion using knockout (KO) mice and compare these to wild type (WT) controls. Electrophysiological analysis of  $\alpha$ 2-adrenoceptor-mediated responses in mPFC slices in WT mice showed a clonidine-induced  $\alpha$ 2-adrenoceptor-mediated increase in mPFC cell excitability coupled with an increase in input resistance. In contrast, CB1r KO mice showed an  $\alpha$ 2-adrenoceptor-mediated decrease in mPFC cell excitability. We then examined protein expression levels of  $\alpha$ 2- and  $\beta$ 1-adrenoceptor subtypes in the mPFC as well as TH expression in the locus coeruleus (LC) of mice deficient in CB1r. Both  $\alpha$ 2- and  $\beta$ 1-adrenoceptors exhibited a significant decrease in expression levels in CB1r KO mice when compared to WT in the mPFC, while a significant increase in TH was observed in the LC. To better define whether the same cortical neurons express  $\alpha$ 2A-adrenoceptor and CB1r in mPFC, we utilized high-resolution immunoelectron microscopy. We localized  $\alpha$ 2A-adrenoceptors in a knock-in mouse that expressed a hemoagglutinin (HA) tag downstream of the  $\alpha$ 2A-adrenoceptor promoter. Although the  $\alpha$ 2A-adrenoceptor was often identified pre-synaptically, we observed co-localization of CB1r with  $\alpha$ 2-adrenoceptors post-synaptically in the same mPFC neurons. Finally, using receptor binding, we confirmed prior results showing that  $\alpha$ 2A-adrenoceptor is unchanged in mPFC following acute or chronic exposure to the synthetic cannabinoid receptor agonist, WIN 55,212-2, but is increased, following chronic treatment followed by a period of abstinence. Taken together, these data provide convergent lines of evidence indicating cannabinoid regulation of the cortical adrenergic system.

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## 1. Introduction

Norepinephrine (NE), a biogenic amine integral to the stress-response system and regulation of higher cognitive functions (Degenhardt et al., 2001; Arendt and Munk-Jorgensen, 2004; Pattij et al., 2008) is modulated by the endocannabinoid (eCB) system (Page et al., 2007; Page et al., 2008; Reyes et al., 2009; Carvalho et al., 2010b; Wyrofsky et al., 2015). Emerging lines of evidence indicate that the neurobehavioral consequences of exogenous cannabinoid exposure

involve, in part, cannabinoid-induced cellular and molecular changes in adrenergic neurons. For example, pre-treatment of human subjects with the beta-adrenergic antagonist, propranolol, has been shown to prevent the acute effects of cannabis-induced impairment of learning (Sulkowski and Vachon, 1977). Likewise, using an animal model, pre-test administration of the  $\alpha$ 1-adrenergic agonist, phenylephrine, reversed post-training cannabinoid-induced retrieval impairment (Moshfegh et al., 2011). The efficacy of nabilone, a cannabinoid receptor agonist, in the treatment of post-traumatic stress disorder (PTSD) symptoms is attributed to actions of cannabinoids on NE circuitry (Reyes et al., 2009; Villanueva et al., 2009; Carvalho et al., 2010a; Carvalho et al., 2010b). Finally, mice deficient in CB1 receptor exhibit anxiogenic-like responses in various behavioral paradigms including

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the elevated plus-maze, the open field test and the light-dark box (Haller et al., 2002; Maccarrone et al., 2002; Martin et al., 2002; Uriguén et al., 2004; Thiemann et al., 2009). These mice also exhibited depressive-like phenotypes (Viveros et al., 2005; Valverde and Torrens, 2012), cognitive impairments including memory and learning deficits (Martin et al., 2002; Varvel and Lichtman, 2002; Bilkei-Gorzo et al., 2005; Madronal et al., 2012) as well as impairment in the extinction of aversive memories (Marsicano et al., 2002) with no changes in locomotor function (Haller et al., 2002) suggesting an effect on biogenic amine circuitry.

Cannabinoid receptor agonists have been shown to both increase, and decrease, indices of brain noradrenergic activity. Activation of pre-synaptic CB1 receptor on terminals of sympathetic axons innervating blood vessels has been shown to reduce the release of NE (Ishac et al., 1996; Pfitzer et al., 2005). Incubation of synaptosomes with low concentrations of tetrahydrocannabinol (THC) results in reductions in NE release (Poddar and Dewey, 1980) while systemic administration of rimobant (a CB1 receptor antagonist) increases NE in the anterior hypothalamus and medial prefrontal cortex (mPFC) not the nucleus accumbens (Tzavara et al., 2001; Tzavara et al., 2003). In addition to inhibition of NE release, several lines of evidence support cannabinoid-induced increases in NE release (Rodríguez de Fonseca et al., 1991; Molderings et al., 2002; Page et al., 2007). Acute systemic (Oropeza et al., 2005) or local (Page et al., 2008) administration of the synthetic cannabinoid receptor agonist, WIN 55,212-2, or administration of a FAAH inhibitor (Gobbi et al., 2005), increases NE efflux in the rat frontal cortex. Acute WIN 55,212-2 exposure stimulates c-Fos expression in noradrenergic neurons of the locus coeruleus (LC) (Oropeza et al., 2005; Page et al., 2008), enhances *N*-methyl-D-aspartate-induced firing of LC neurons (Mendiguren and Pineda, 2004) and increases NE synthesis (Moranta et al., 2009) and release in terminal regions (Oropeza et al., 2005). CB1 receptor have been localized to noradrenergic axon terminals in the mPFC (Oropeza et al., 2007) supporting the hypothesis that NE and eCBs can regulate each other's function.

We have also described functional interactions between CB1 receptor and adrenoceptor systems in the mPFC. Whole cell patch clamp recordings of layer V/VI cortical pyramidal neurons in rats revealed that clonidine-induced  $\alpha$ 2-adrenoceptor-mediated elevations in cortical pyramidal cell excitability are significantly decreased following pre-treatment with the synthetic CB1 receptor agonist, WIN 55,212-2, suggesting cannabinoid stimulation of NE release and desensitization of  $\alpha$ 2-adrenoceptors (Reyes et al., 2012). The receptor interaction was both action potential and GABA<sub>A</sub> receptor-independent as the desensitization occurred similarly in the presence or absence of tetrodotoxin or the GABA<sub>A</sub> receptor antagonist bicuculline indicating that CB1- $\alpha$ 2-AR interactions are likely direct rather than mediated by synaptic afferents. We also showed that  $\alpha$ 2A-adrenoceptors-immunoreactivity is distributed in axon terminals, somata and dendrites in the mPFC using immunoelectron microscopy (Cathel et al., 2014), consistent with other reports (Aoki et al., 1994; Venkatesan et al., 1996; Aoki et al., 1998; Aoki et al., 2000). Systemic administration of WIN 55,212-2, tetrahydrocannabinol ( $\Delta^9$  THC) and CP 55940 increases the spontaneous firing rate of LC neurons in a dose dependent manner (Mendiguren and Pineda, 2006; Muntoni et al., 2006). These effects were prevented by pretreatment with the cannabinoid receptor (CB1 receptor) antagonist, SR 141716 (Oropeza et al., 2005; Mendiguren and Pineda, 2004) supporting the involvement of CB1 receptors.

Adaptations occur following chronic exposure to WIN 55212-2 and withdrawal. For example, repeated administration of WIN 55212-2 increases TH protein expression in the LC and this is accompanied by potentiated NE efflux in response to an acute injection of WIN 55212-2 without a change in baseline NE efflux (Page et al., 2007). Chronic WIN 55,212-2 administration produces an anxiogenic-like response that reverts to pre-drug levels following a period of abstinence (Page et al., 2007). Chronic WIN 55212-2 treatment completely abolishes the ability of clonidine to induce an increase in excitability of

PFC neurons (Reyes et al., 2012) and reduces the binding site density of  $\beta$ 1-adrenoceptors in neocortex (Hillard and Bloom, 1982; Reyes et al., 2009), and both  $\alpha$ 2- and  $\beta$ 1-adrenoceptors in the accumbens (Carvalho et al., 2010b). Meanwhile withdrawal following chronic WIN 55212-2 exposure alters  $\beta$ 1-adrenoceptors in the mPFC (Reyes et al., 2009). Taken together, these data indicate that sustained CB1 receptor activation results in a sustained increase in NE release which induces down-regulation of adrenergic receptors.

However, several gaps in knowledge remain that we addressed in the present study using a multidisciplinary approach. First, we measured electrophysiological properties of  $\alpha$ 2-adrenoceptor responses under conditions of CB1 receptor deletion using CB1 receptor-knockout (KO) mice and compared these to wild type (WT) controls. Next, we examined expression levels of  $\alpha$ 2- and  $\beta$ 1-adrenoceptors in the mPFC in mice lacking the CB1 receptor as well as the catecholamine synthesizing enzyme, tyrosine hydroxylase (TH), in the LC, which provides the sole source of NE to the mPFC (Halliday, 2004; Aston-Jones and Cohen, 2005; Aston-Jones et al., 2007). To better define sites of cortical cannabinoid-adrenoceptor interactions, we examined the ultrastructural localization of CB1 receptors with respect to neurons expressing  $\alpha$ 2-adrenoceptors in a knock-in mouse that expressed a hemoagglutinin (HA)-tag downstream of the  $\alpha$ 2-adrenoceptor promoter (Lu et al., 2009). Given the results showing significant adaptations in the adrenergic system under conditions of CB1r deletion, we next investigated  $\alpha$ 2A-adrenoceptor binding in the mPFC following acute and chronic WIN 55,212-2 and following abstinence from chronic WIN 55,212-2 exposure in rats. These data are important considering the increasing prevalence of clinical studies examining exogenous cannabinoid administration for the treatment of a variety of pathophysiological conditions. Furthermore, because the noradrenergic and eCB systems are both dynamically regulated by stress, where stress decreases anandamide and CB1 receptor levels while increasing 2-arachidonoyl glycerol levels (Morena et al., 2016), understanding the mechanism underlying the cannabinoid-adrenoceptor interactions in the mPFC may help unravel the mechanism underlying cannabinoid-induced impairments in attention and cognition.

## 2. Methods

The procedures employed in the present study conformed to Drexel University Institutional Animal Care and Use Committee, *National Institute 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.

### 2.1. Specificity of CB1 receptor antibody

Three male CB1r KO mice and three WT controls mice (9–12 weeks old) were deeply anesthetized with sodium pentobarbital (40 mg/kg) and perfused transcardially through the ascending aorta with heparinized saline followed with 25 ml of 4% formaldehyde in 0.1 M phosphate buffer (PB; pH 7.4). Immediately following perfusion/fixation, the brains were removed and postfixed for 30 min. Brains were sectioned in the coronal plane at a setting of 40  $\mu$ m using a Vibratome (Technical Product International, St. Louis, MO, USA) through the forebrain and hippocampus, and collected into 0.1 M PB. Sections through the rostrocaudal extent of mPFC and hippocampus were processed for light microscopic detection of CB1 receptor in the mPFC. Tissue sections were incubated in rabbit anti-CB1 receptor at 1:1000 in 0.1% bovine serum albumin (BSA), 0.25% Triton X-100 and 0.1 M tris buffered saline (TBS; pH 7.6) for 15–18 h at room temperature. The following day, tissue sections were rinsed three times in 0.1 M TBS and incubated in biotinylated donkey anti-rabbit (1:400; Vector Laboratories, Burlingame,

CA, USA) for 30 min followed by rinses in 0.1 M TBS. Subsequently, a 30-min incubation of avidin-biotin complex (Vector Laboratories) was done. For all incubations and washes, sections were continuously agitated with a rotary shaker. CB1 receptor was visualized by a 4-minute reaction in 22 mg of 3,3'-diaminobenzidine (Sigma-Aldrich) and 10  $\mu$ l of 30% hydrogen peroxide in 100 ml of 0.1 M TBS. Sections were collected, dehydrated and coverslipped with Permount (Sigma-Aldrich) for light microscopic analysis of CB1 receptor immunoreactivity.

## 2.2. *In vitro* electrophysiology

For electrophysiology experiments, male wild-type (WT) and CB1 receptor KO mice (9–12 weeks old) were housed three per cage in a controlled environment (12-h light schedule, temperature at 20 °C). CB1 receptor 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 CB1 receptor KO mice and WT littermates. Food and water were provided ad libitum. The care and use of animals were approved by the Institutional Animal Care and Use Committee of Temple University and were conducted in accordance with the NIH guide for the care and use of laboratory animals.

### 2.2.1. Drug preparation and administration

A stock solution of clonidine (Sigma-Aldrich, St. Louis, MO) was prepared in de-ionized water at a stock concentration of 10 mM and diluted on the day of the experiment to a final concentration in the perfusion bath of 10  $\mu$ M.

### 2.2.2. Electrophysiology

Male CB1 receptor KO mice and WT controls (3–5 mo.) were euthanized, brains rapidly removed, 250  $\mu$ m slices were taken containing layer V/VI pyramidal neurons of the infralimbic region of mPFC, which is implicated in conditioning and extinction of fear (Giustino and Maren, 2015), drug seeking behavior (Kalivas, 2009; Moorman et al., 2015) and resilience to social stress (Cooper et al., 2015). Whole-cell recordings under current clamp ( $I = 0$  pA) conditions were conducted as described previously (Reyes et al., 2012; Cathel et al., 2014). At baseline, membrane potential was recorded and input resistance calculated using the current-voltage relationship. Neuronal excitability was assessed in each cell by recording voltage responses to a series of current pulses (0–160 pA). Membrane potential, input resistance and neuronal excitability were also measured following bath application of the  $\alpha$ 2-adrenoceptor agonist, clonidine (10  $\mu$ M). Recordings were analyzed and pyramidal cell morphology of recorded cells determined by immunohistochemistry as described previously (Reyes et al., 2012; Cathel et al., 2014).

### 2.2.3. Statistical analysis

Statistical analysis was performed using SPSS 16.0 software. For electrophysiology data, membrane voltage, input resistance and excitability responses to individual current pulses were compared between genotypes by unpaired Student's *t*-test and drug effects on these endpoints compared by paired Student's *t*-test. Significance was set at  $P < 0.05$ .

## 2.3. Protein extraction and Western blot analysis

For Western blotting experiments, WT and CB1 receptor KO littermates (9–15 weeks old) were housed three per cage in a controlled environment (12-h light schedule, temperature at 20 °C). The CB1 receptor KO was generated in CD1 mice as previously reported (Ledent et al., 1999). Brain tissue was rapidly removed from each animal on ice. Using a trephine, the mPFC brain region was microdissected from each animal. mPFC was homogenized with a pestle and extracted in radioimmunoprecipitation assay lysis buffer (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 41 °C. Supernatants or protein extracts were diluted with an equal volume of Novex 2<sup>®</sup> tris glycine sodium dodecyl sulfate sample buffer (Invitrogen, Carlsbad, CA, USA) containing dithiothreitol (Sigma-Aldrich Inc., St. Louis, MO, USA). Protein concentrations of the undiluted supernatants were quantified using the bicinchoninic acid protein assay reagent (Pierce, Rockford, IL, USA).

Cell lysates containing equal amounts of protein were separated on 4–12% tris-glycine polyacrylamide gels and then electrophoretically transferred to Immobilon-P polyvinylidene fluoride membranes (Millipore, Bedford, MA, USA). Membranes were incubated in rabbit anti-tyrosine hydroxylase (1:4000; Immunostar Inc., Hudson, WI), rabbit anti- $\alpha$ 2-adrenoceptor (1:500; Sigma-Aldrich Inc.) or rabbit anti- $\beta$ 1-adrenoceptor (1:1000) primary antibodies overnight at 4 °C and then in alkaline phosphatase-conjugated secondary antibodies for 30 min to probe for the presence of proteins using a Western blotting detection system (Western Breeze Chemiluminescent Kit; Invitrogen). Membranes containing proteins obtained from LC were incubated with mouse anti-TH while membranes containing proteins obtained from the mPFC were incubated with rabbit anti- $\alpha$ 2-adrenoceptor or rabbit anti- $\beta$ 1-adrenoceptor. Following incubation in a chemiluminescent substrate (Western Breeze Chemiluminescent Kit), blots were exposed to X-OMAT AR film (Kodak, Rochester, NY, USA) for different lengths of time to optimize exposures. CB1 receptor was readily detected by immunoblotting in rat mPFC extracts, and was visualized as a single band that migrates at approximately 60 kDa whereas  $\alpha$ 2-adrenoceptor, or  $\beta$ 1-adrenoceptor, migrate at approximately 45 kDa and 64 kDa. Blots were incubated in stripping buffer (Restore Stripping Buffer, Pierce) to disrupt previous antibody-antigen interactions and then re-probed with  $\beta$ -actin (1:5000, Sigma-Aldrich Inc.) with 1-hour incubation to ensure proper protein loading. The density of each band was quantified using Un-Scan-It blot analysis software (Silk Scientific Inc., Orem, UT, USA). CB1 receptor,  $\alpha$ 2-adrenoceptors, or  $\beta$ 1-adrenoceptors was normalized to  $\beta$ -actin immunoreactivity on each respective blot. Student's *t*-test was used to analyze the difference in CB1 receptor,  $\alpha$ 2-adrenoceptors, or  $\beta$ 1-adrenoceptors protein expression between WT and CB1 receptor KO mice. Results are presented as mean  $\pm$  SEM. Data on  $\alpha$ 2A-adrenoceptor binding and Western blot data were analyzed using Student's *t*-test.

## 2.4. Immunoelectron microscopy

Five male hemagglutinin (HA) epitope-tagged wild type  $\alpha$ 2A-adrenoceptors knock-in mice, six months of age were kindly provided by Dr. Qin Wang of the University of Alabama in Birmingham, Alabama. The HA- $\alpha$ 2A line is a gene targeting knock-in line not transgenic line. The HA tag was inserted right after the first ATG of the alpha2A coding sequence (this gene is intronless). Hence,  $\alpha$ 2A expression is at a level/localization/pattern identical to the endogenous one (same gene structure, under the same transcriptional control) (Lu et al., 2009). These mice were housed two or three to a cage (20 °C, 12-h light, 12-h dark cycle lights on 0700) and were quarantined prior to perfusion. These mice were used for examining the subcellular localization of HA epitope-tagged  $\alpha$ 2A-adrenoceptor and CB1 receptor. Food and water were freely available. On the day of perfusion these male HA- $\alpha$ 2A-adrenoceptor knock-in mice were deeply anesthetized with sodium pentobarbital (40 mg/kg) and transcardially through the ascending aorta with heparinized saline followed with 25 ml of 3.75% acrolein (Electron Microscopy Sciences) and formaldehyde in 0.1 M PB; pH 7.4, respectively. Brains were collected and sectioned as described earlier. Sections through the rostrocaudal extent of mPFC were processed for electron microscopic analysis of HA- $\alpha$ 2A-adrenoceptor and CB1 receptor. Tissue sections were incubated in mouse anti-HA (Covance, Emeryville, CA, USA) at 1:1000 and rabbit anti-CB1 receptor at 1:1000 in 0.1% bovine serum albumin (BSA) and 0.1 M TBS for 15–18 h at

room temperature. The following day, tissue sections were rinsed three times in 0.1 M TBS and incubated in biotinylated donkey anti-mouse (1:400; Vector Laboratories, Burlingame, CA, USA) for 30 min followed by rinses in 0.1 M TBS. Subsequently, a 30-minute incubation of avidin-biotin complex (Vector Laboratories) was done. For all incubations and washes, sections were continuously agitated with a rotary shaker. HA- $\alpha$ 2A-adrenoceptor was visualized by a 4-min reaction in 22 mg of 3,3'-diaminobenzidine (Sigma-Aldrich) and 10  $\mu$ l of 30% hydrogen peroxide in 100 ml of 0.1 M TBS.

Following rinses in 0.1 M TBS, tissues sections were rinsed with 0.1 M PB and 0.01 M PBS, and were incubated in a 0.2% gelatin-PBS and 0.8% BSA buffer for 10 min. This was followed by incubation in goat anti-rabbit IgG conjugate in <1 nm gold particles (Amersham Bioscience Corp., Piscataway, NJ, USA) at room temperature for 2 h. Sections were then rinsed in buffer containing the same concentration of gelatin and BSA as above and subsequently rinsed with 0.01 M PBS. Sections were then incubated in 2% glutaraldehyde (Electron Microscopy Sciences) in 0.01 M PBS for 10 min followed by washes in 0.01 M PBS and 0.2 M sodium citrate buffer (pH 7.4). A silver enhancement kit (Amersham Bioscience Corp.) was used for silver intensification of the gold particles. The optimal times for silver enhancement time were determined by empirical observation for each experiment and ranged 7–8 min. Following intensification, tissue sections were rinsed in 0.2 M citrate buffer and 0.1 M PB, and incubated in 2% osmium tetroxide (Electron Microscopy Sciences) in 0.1 M PB for 1 h, washed in 0.1 M PB, dehydrated in an ascending series of ethanol followed by propylene oxide and flat embedded in Epon 812. Thin sections of approximately 50–100 nm in thickness were cut with a diamond knife (Diatome-US, Fort Washington, PA, USA) using a Leica Ultracut (Leica Microsystems, Wetzlar, Germany). Sections were collected on copper mesh grids and counterstained stained with 5% uranyl acetate followed by Reynold's lead citrate. Captured images of selected sections were compared with captured light microscopic images of the block face before sectioning. Sections were examined with a Morgagni transmission electron microscope (Fei Company, Hillsboro, OR, USA) and digital images were captured using the AMT advantage HR/HR-B CCD camera system (Advance Microscopy Techniques Corp., Danvers, MA, USA). Figures were assembled and adjusted for brightness and contrast in Adobe Photoshop.

#### 2.4.1. Identification of gold-silver labeling in profiles

Selective gold-silver labeled profiles were identified by the presence, in single thin sections, of at least two gold particles within a cellular compartment. As were previously reported (Reyes et al., 2006), spurious silver grains can contribute to false positive labeling and can be detected on blood vessels, myelin or nuclei. Considering that a minimal spurious labeling was identified, we have set the criteria that a process is immunolabeled if two immunogold-silver particles were present. Thus, a profile containing only one gold particle in adjacent thin sections was designated as lacking detectable immunoreactivity. Whenever possible, the more lightly labeled axonal labeling for HA- $\alpha$ 2A-adrenoceptors was confirmed by detection in at least two serial sections. As observed in low magnification electron micrographs, background labeling in the neuropil, deemed spurious, was not commonly encountered.

#### 2.5. Data analysis for electron microscopy

Tissue sections were taken from four male HA epitope-tagged wild type  $\alpha$ 2A-adrenoceptor knock-in mice with the best immunocytochemical labeling and preservation of ultrastructural morphology. These tissue sections were used in the analysis and the information is presented in Table 1. At least three Vibratome sections were examined per mouse. Vibratome sections were selected from non-adjacent 40- $\mu$ m-thick tissue sections. At least 8 grids containing four to eight ultrathin sections were collected from the surface of individual Vibratome

**Table 1**

Experiments conducted for the semi-quantitative analysis.

Mouse number	Number of Vibratome tissue sections analyzed	Number of grids analyzed per Vibratome tissue section
Mouse 1	Three	8
Mouse 2	Three	8
Mouse 6	Three	8
Mouse 7	Three	8

sections. For quantification of labeled profiles in 40  $\mu$ m-thick sections immunolabeled before embedding for electron microscopy, we have observed that the collection of sections only from the surface of the section minimizes artifacts that may be associated with incomplete penetration of antisera. The analysis of tissue sections collected at the plastic-tissue interface ensured that both markers were detectable in all sections used for analysis (Chan et al., 1990).

The classification of identified cellular elements was based on the method of Peters et al. (Peters et al., 1991). Neuronal perikarya were distinguished from proximal dendrites by the presence of a nucleus. Dendrites usually contained endoplasmic reticulum and were postsynaptic to axon terminals. They were defined as proximal if their size was larger than 0.7  $\mu$ m in diameter. Axon terminals contained synaptic vesicles and were at least 0.3  $\mu$ m in diameter. A varicosity was considered as synaptic when it showed a junctional complex, a restricted zone of apposed parallel membranes with slight enlargement of the intercellular space, and/or associated postsynaptic thickening. Asymmetric synapses were identified by thick postsynaptic densities (Gray's type I), whereas symmetric synapses had thin densities (Gray's type II). The terms 'contact' or 'apposition' were also used to denote close parallel membrane associations of axon terminals with perikarya and/or dendrites, which lacked recognizable specializations, but were otherwise not separated by glial processes.

#### 2.6. Drug preparation and administration

WIN 55,212-2, a synthetic cannabinoid agonist, (Sigma-Aldrich Inc., St. Louis, MO) was dissolved in 5% dimethyl sulfoxide (DMSO) in 0.9% sodium chloride solution at a concentration of 3 mg/ml. Experimental and control rats were injected intraperitoneally (i.p.) with 0.1 ml/100 g body weight of either WIN 55,212-2 or DMSO, respectively.

Rats were randomly divided into three groups at the beginning of the study. For the acute group, animals were administered WIN 55,212-2 at 3.0 mg/kg once. For the chronic group, animals were administered WIN 55,212-2 at 3.0 mg/kg once daily for seven days. Another group consisted of animals that were administered WIN 55,212-2 for seven days and were subsequently abstinent from WIN 55,212-2 for seven days. For each group examined, the control animals received a solution of DMSO solution i.p. at 0.1 ml/100 g body weight. Thirty minutes following the last WIN 55,212-2 injection, rats were briefly exposed to isoflurane (Abbott Laboratories, North Chicago, IL; 0.5–1.0%, in air) and euthanized by decapitation. Brains were removed for subsequent  $\alpha$ 2A-AR mRNA expression and binding analysis.

##### 2.6.1. $\alpha$ 2-Adrenoceptor binding

$^3$ H-RX821002 (55.0 Ci/mmol, Perkin Elmer, Waltham, MA) was used to quantitate  $\alpha$ 2-adrenoceptor binding sites in mPFC.  $^3$ H-RX821002 binding was performed as previously described (Szot et al., 2006; Szot et al., 2010). Briefly, slides were thawed, and then 600  $\mu$ l/slide of incubation buffer (~2 nM  $^3$ H-RX 821002 in 50 mM NaPO<sub>4</sub> buffer, pH 7.4) was placed over the tissue. Non-specific binding was defined in the presence of 10  $\mu$ M rauwolscine. Slides were incubated for 45 min at room temperature and then washed for 2 min in ice-cold 50 mM NaPO<sub>4</sub> buffer, pH 7.4, dipped in ice-cold distilled water and dried rapidly under a stream of cool air. Slides were apposed to Biomax MR film (Eastman Kodak Co., Horsham, PA) for 2 months. Films were developed by

standard procedures (Szot et al., 1997; Szot et al., 2007). 3H-RX821002 binding sites were quantitated as optical density (OD) using MCID system (InterFocus Imaging Ltd., Cambridge, England).

### 3. Results

#### 3.1. Specificity of antibodies and control experiments

The characterization of all the primary antibodies used in the present study is presented in Table 2. Immunoperoxidase detection of CB1 receptor antibody was conducted in tissue sections obtained from mice with CB1 receptor deletion in parallel with the tissue sections obtained from the wild-type mice. CB1 receptor immunoperoxidase labeling was detected in multiple brain regions in WT mice including the cerebral cortex and hippocampus (not shown) consistent with previous studies (Katona et al., 2006; Scavone et al., 2010; Fitzgerald et al., 2012; Cathel et al., 2014; Reyes et al., 2015). Furthermore, the specificity of CB1 receptor was also demonstrated using immunoblotting where a protein of the expected molecular size was detected (Suarez et al., 2008). CB1 receptor specificity was also verified using preabsorption of the primary antisera with the blocking peptide corresponding to the last 15 amino acids of the CB1 receptor C-terminus. Tissue sections obtained from the rat forebrain incubated in this solution did not exhibit CB1 receptor immunoreactivity compared with tissue sections in which standard immunohistochemistry was performed. In addition, preabsorption experiment was also performed using Western blot analysis in which CB1 receptor immunoreactivity was also abolished (Scavone et al., 2010; Reyes et al., 2015). Tissue sections obtained from the rat forebrain incubated in this solution did not exhibit CB1 receptor immunoreactivity compared with tissue sections in which standard immunohistochemistry was performed. In addition, preabsorption experiment was also performed using Western blot analysis in which CB1 receptor immunoreactivity was also abolished (Scavone et al., 2010; Reyes et al., 2015). The monoclonal antibody against HA- $\alpha$ 2A-adrenoceptor was generated in mouse against the 12-amino-acid peptide CYPYDVPDYASL. The specificity of the TH antibody has been examined by preabsorption of the antibody with a high concentration of TH (Van Bockstaele and Pickel, 1993). The specificity of the  $\alpha$ 2-adrenoceptor and  $\beta$ 1-adrenoceptor was investigated using immunoperoxidase, immunofluorescence and Western blotting. We showed specific immunoreactivity of  $\beta$ 1-adrenoceptor in the amygdala using light, immunofluorescence and electron microscopy as well as Western blotting (Rudoy et al., 2009). We also showed specific immunoreactivity of  $\alpha$ 2-adrenoceptor in the mPFC using light and immunofluorescence (Reyes et al., 2009; Carvalho et al., 2010b). Preabsorption of  $\alpha$ 2-adrenoceptor and  $\beta$ 1-adrenoceptor with the antigenic peptide at 10  $\mu$ M blocked the  $\alpha$ 2-adrenoceptor and  $\beta$ 1-adrenoceptor immunoreactivities in the forebrain. Blots incubated with 10  $\mu$ g/ml of affinity-purified  $\alpha$ 2-adrenoceptor and  $\beta$ 1-adrenoceptor antisera preincubated with antigenic peptide blocked the  $\alpha$ 2-adrenoceptor and  $\beta$ 1-adrenoceptor expression in rat FC extracts (Reyes et al., 2009). Furthermore, some sections were processed in parallel with the rest of the immunohistochemical procedures identical except that one of the primary

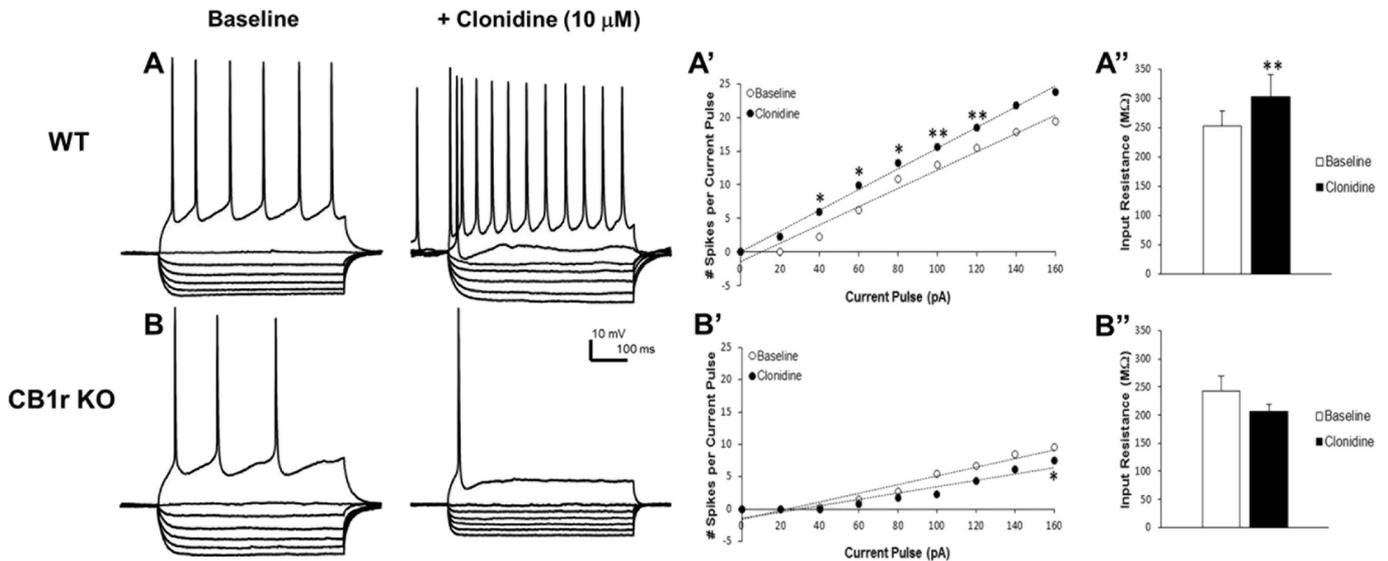
antisera was omitted. Sections processed with the omission of primary antibody (CB1 receptor,  $\alpha$ 2-adrenoceptor,  $\beta$ 1-adrenoceptor) abolished any detectable immunoreactivity (CB1 receptor,  $\alpha$ 2-adrenoceptor- or  $\beta$ 1-adrenoceptor-immunoreactivity). Furthermore, control of specificity for CB1 receptor, HA- $\alpha$ 2A-adrenoceptor, TH,  $\alpha$ 2-adrenoceptor and  $\beta$ 1-adrenoceptor was also carried out, including control of the secondary antibody where control sections were also processed without primary antibody/ies but with secondary antibody and control for primary antibody/ies where some sections were processed with primary antibody/ies but without secondary antibody/ies. In those control tissue sections, run in parallel, peroxidase immunoreactivity or immunogold-silver particles were not demonstrated in tissue sections from which primary or secondary antibody/ies was/were omitted. To evaluate the possible cross-immunoreactivity of secondary antibodies with the primary antibodies in the dual-labeling experiment, some sections were processed for dual immunolabeling with omission of one of the primary antibodies.

#### 3.2. Indices of adrenergic activity in CB1 receptor KO mice

To define the adrenergic activity in CB1 receptor KO mice, we compared  $\alpha$ 2-adrenoceptor-mediated responses of pyramidal neurons in mPFC slices from WT and CB1 receptor KO mice (Fig. 1). Here, we used clonidine, an  $\alpha$ 2-adrenoceptor agonist that increased the excitability and input resistance of rat mPFC pyramidal neurons in vitro (Cathel et al., 2014). In the presence of WIN 55,212-2 the effects of clonidine on neuronal excitability and input resistance were blocked (Cathel et al., 2014). Fig. 1 depicts data that represent  $N = 14$  cells from seven WT mice and  $N = 7$  cells from three CB1 receptor KO mice. Baseline membrane voltage was not different between CB1 receptor KO and WT mice (WT:  $-58.7 \pm 2.8$  mV; CB1r KO:  $-56.0 \pm 3.0$  mV) and neither group showed a significant clonidine-induced change in membrane voltage. Similar to results that we and others reported for rats (Carr et al., 2007; Reyes et al., 2012; Cathel et al., 2014), WT mice exhibited a clonidine-induced  $\alpha$ 2-adrenoceptor-mediated increase in mPFC cell excitability [leftward shift of the stimulus-response curve; significantly increased excitability responses to 40, 60, 80 ( $P < 0.05$ ), 100 and 120 pA ( $P < 0.01$ ) current pulses] coupled with a significantly increased input resistance ( $252 \pm 27$  to  $303 \pm 37$  M $\Omega$ ,  $P < 0.01$ ). In contrast, CB1 receptor KO mice showed reduced basal cortical excitability compared to WT mice and an  $\alpha$ 2-adrenoceptor-mediated decrease in mPFC cell excitability (rightward shift of the stimulus-response curve; significantly decreased excitability response to 160 pA current pulse ( $P < 0.05$ ) coupled with a slight decrease in input resistance ( $243 \pm 27$  to  $206 \pm 12$  M $\Omega$ , n.s.). Clonidine effects on excitability and input resistance both reflect  $\alpha$ 2-adrenoceptor-mediated modulation of a hyperpolarization/cyclic nucleotide-gated (HCN) current in mPFC pyramidal dendrites that influences synaptic integration in these neurons (Carr et al., 2007). These data provide evidence that genetic deletion of CB1 receptor reduces basal mPFC pyramidal neuron excitability and desensitizes cortical  $\alpha$ 2-adrenoceptors, decreasing the ability of cortical neurons to respond to excitatory synaptic inputs.

**Table 2**  
Characterization of the primary antibodies.

Antigen	Immunogen	Manufacturer	Host, monoclonal/polyclonal	References
CB1r	Directed against the last 15 amino acids of the C-terminus of the rat CB1r	Generated in the laboratory of Dr. Ken Mackie.	Rabbit polyclonal	Scavone et al., 2010; Katona et al., 2006; Fitzgerald et al., 2012; Reyes et al., 2015; Cathel et al., 2014
HA	Directed against the 12-amino-acid peptide CYPYDVPDYASL	Covance	Mouse monoclonal	Lu et al., 2009; Wang et al., 2007
TH	Raised against denatured TH from rat pheochromocytoma	Immunostar	Mouse monoclonal	Van Bockstaele and Pickel, 1993
$\alpha$ 2A-AR	Synthetic peptide, amino acids 218–235 of human, rat and pig	Sigma-Aldrich	Rabbit polyclonal	Reyes et al., 2009; Carvalho et al., 2010a, 2010b
$\beta$ 1-AR	Synthetic peptide, amino acid 392–408	Sigma-Aldrich	Rabbit polyclonal	Reyes et al., 2009; Carvalho et al., 2010a, 2010b



**Fig. 1.**  $\alpha$ 2-Adrenoceptor responses in mPFC pyramidal neurons in WT and CB1 receptor KO mice. Panels A and B indicate voltage responses to 700 ms current steps (pA;  $-100$ ,  $-80$ ,  $-60$ ,  $-40$ ,  $-20$ ,  $0$ ,  $+80$ ) from a representative WT (A) and CB1 receptor KO mouse (B) both before and after bath application of clonidine ( $10 \mu\text{M}$ ). Panels A' and B' summarize mean excitability data as stimulus-response curves to a range of current steps ( $0$ – $160$  pA) in mice from each genotype. Panels A'' and B'' show clonidine effects on membrane input resistance in mice from each genotype. Acute stimulation of the  $\alpha$ 2-adrenoceptor with clonidine increases excitability of mPFC neurons (A and rightward shift in A') and increases input resistance (A'') in WT mice. In contrast, clonidine decreases excitability of mPFC neurons (B and leftward shift B') and slightly decreases input resistance (B'') in CB1 receptor KO mice. Data represent mean  $\pm$  SEM. Asterisks indicate a significant difference between baseline and clonidine by paired Student's *t*-test (\* $P < 0.05$ ; \*\* $P < 0.01$ ).

In addition, to define adaptations in adrenergic systems under conditions of CB1 receptor deletion, we examined expression levels of the catecholamine synthesizing enzyme, TH (Fig. 2A) in the LC, as well as two adrenergic receptor subtypes,  $\beta$ 1-adrenoceptor (Fig. 2B) and  $\alpha$ 2-adrenoceptor (Fig. 2C) in the mPFC of mice lacking the CB1 receptor. This data is important considering that the release of NE is related to arousal states that have profound effects on cognitive and behavioral processes involving mPFC functions (Arnsten, 2007; Lapiz and Morilak, 2006). These modulatory systems engage multiple receptor subtypes, including  $\alpha$ 1-adrenoceptor,  $\alpha$ 2-adrenoceptor and  $\beta$ 1-adrenoceptor (Ramos and Arnsten, 2007; Lapiz and Morilak, 2006; Arnsten, 2007). In mice deficient in CB1 receptor (Fig. 2), a significant increase in TH was observed in the LC ( $P < 0.05$ ) while both  $\alpha$ 2-AR ( $P < 0.05$ ) and  $\beta$ 1-AR ( $P < 0.01$ ) receptors in the mPFC exhibited a significant decrease in expression levels when compared to WT.

### 3.3. Ultrastructural localization of CB1 and HA- $\alpha$ 2A-AR in frontal cortex

The region analyzed for ultrastructural analysis included the infralimbic portion of the mPFC as illustrated in the rat brain atlas (Paxinos and Watson, 1997) at the antero-posterior level of bregma  $2.20$ – $3.20$  mm. The infralimbic region of the mPFC is bounded anteriorly by the medial orbital cortex, dorsally by the prelimbic cortex, laterally by the forceps minor of corpus callosum, as well as ventrally and caudally by the dorsal peduncular cortex and lateral septal nucleus. Control tissue sections for immunoelectron microscopy were processed to determine the specificity of the primary antibodies used in the present study. Control tissue sections from mice with CB1 receptor deletion incubated with CB1 receptor antibody did not show any immunoreactivity at ultrastructural level (Fig. 3A). In addition, tissue section obtained from HA- $\alpha$ 2A-adrenoceptor-tagged mouse showed absence of HA- $\alpha$ 2A-adrenoceptor immunoreactivity when tissue sections were incubated without HA- $\alpha$ 2A-adrenoceptor primary antibody (Fig. 3B). HA- $\alpha$ 2A-adrenoceptor was also not present when tissue sections from non-HA- $\alpha$ 2A-adrenoceptor-tagged mouse were incubated with HA- $\alpha$ 2A-adrenoceptor primary antibody (Fig. 3C).

Using dual immunolabeling, HA- $\alpha$ 2A-adrenoceptor was detected with immunoperoxidase and CB1 receptor was detected with immunogold-silver particles (Fig. 4A). Our present study confirms previous reports demonstrating that CB1 receptor is abundant in axon terminals in the infralimbic portion of the mPFC (Kawamura et al., 2006; Monory et al., 2006; Oropeza et al., 2007; Kano et al., 2009). Likewise, our results show that HA- $\alpha$ 2A-adrenoceptor is prominently localized in axon terminals confirming results from our previous analysis (Cathel et al., 2014). There were several interactions observed between HA- $\alpha$ 2A-adrenoceptor and CB1r (Figs. 4–5). First, it was observed that HA- $\alpha$ 2A-adrenoceptor-immunoreactive axon terminals also express CB1 receptor (Fig. 4B–C). Semi-quantitative analysis conducted showed that out of 276 HA- $\alpha$ 2A-adrenoceptor-labeled axon terminals, 26% ( $72/276$ ) also exhibited CB1 receptor immunoreactivity. The dual-labeled axon terminals were directly contacting unlabeled dendrites. When synaptic specializations were observed, 64% ( $46/72$ ) were of the symmetric type (Fig. 4C) and 15% ( $11/72$ ) formed asymmetric synapses with unlabeled dendrites (Fig. 4B). The remaining 21% ( $15/72$ ) of synaptic contacts did not form detectable synaptic specializations. This type of interaction showing both HA- $\alpha$ 2A-adrenoceptor and CB1 receptor in axon terminals indicates that CB1 receptor could modulate NE release in the mPFC. It was also observed that appositions between HA- $\alpha$ 2A-adrenoceptor-labeled axon terminals and CB1 receptor-labeled dendrite were present (10%;  $27/276$ ). When synaptic specializations were distinguishable, 56% ( $15/27$ ) were of the symmetric type (Fig. 4F) and 18% ( $5/27$ ) formed asymmetric type synapses (Fig. 4F). The remaining 26% ( $7/27$ ) did not form detectable synaptic specializations. In some instances, HA- $\alpha$ 2A-adrenoceptor-labeled dendrites also exhibited CB1 receptor immunoreactivity (15%;  $40/276$ ). Of 40 HA- $\alpha$ 2A-adrenoceptor and CB1 receptor co-labeled dendrites, 60% ( $24/40$ ) received symmetric type synapses (Fig. 5B–D), 23% ( $9/40$ ) received asymmetric type synapses (Fig. 5B) and 17% ( $7/40$ ) did not form detectable synaptic specializations. In very few cases, 3% ( $7/276$ ) of HA- $\alpha$ 2A-adrenoceptor-labeled dendrites received a synaptic contact from a CB1 receptor-labeled axon terminal (Fig. 5D–E). Singly labeled HA- $\alpha$ 2A-adrenoceptor and CB1 receptor axon terminals converging onto unlabeled dendrites was infrequently observed (1%;  $2/276$ ). These data add to our growing

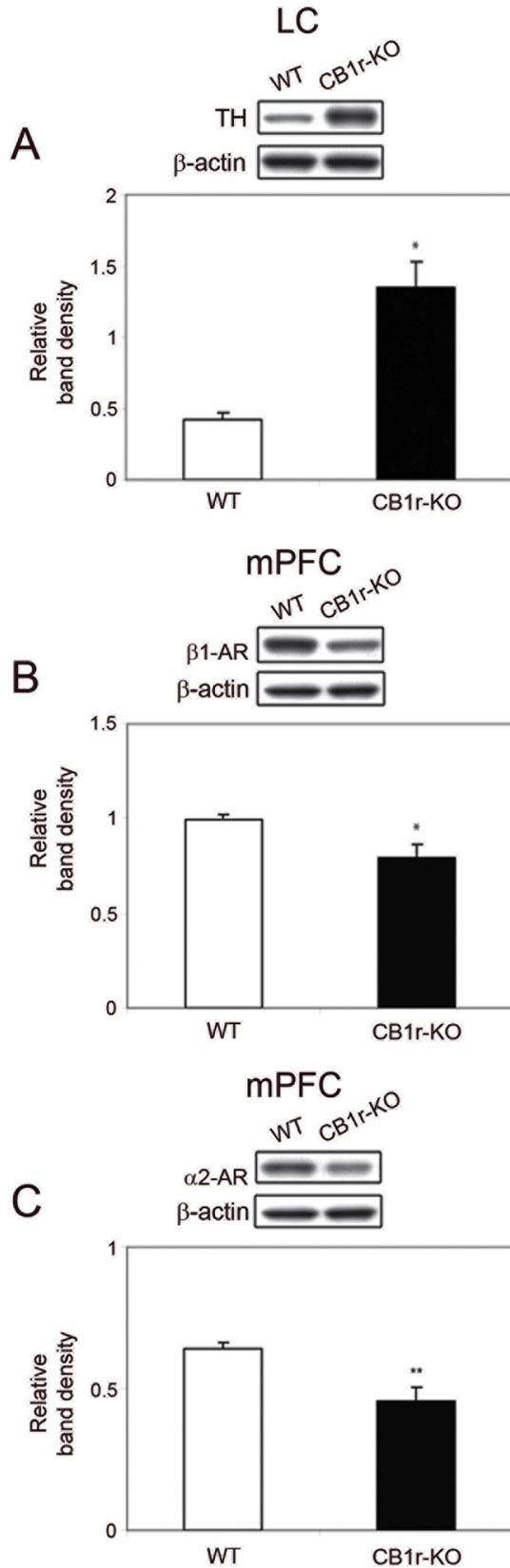


Figure 2

knowledge of cannabinoid modulation of noradrenergic circuitry and indicate that CB1 receptor-containing afferents are positioned to impact cortical activity via multiple synaptic configurations.

3.4. WIN 55,212-2 effects on α2A-adrenoceptor binding in the mPFC

Here, we examined the influence of WIN 55,212-2 on cortical adrenoceptor binding of α2A-adrenoceptor in the mPFC. We previously published protein analysis data (Reyes et al., 2009) showing that acute or chronic treatment with WIN 55,212-2 did not produce any change in α2A-adrenoceptor expression in the mPFC. However, this prior study did show a trend for an increase in α2A-adrenoceptor expression following abstinence from chronic exposure to WIN 55,212-2. Using receptor binding, we confirm that acute or chronic treatment with WIN 55,212-2 does not affect α2A-adrenoceptor binding in the mPFC; however, α2A-adrenoceptor binding was increased following abstinence from chronic WIN 55,212-2 exposure. Fig. 6 shows a representative autoradiogram of α2A-adrenoceptor binding in the mPFC following acute or chronic WIN 55,212-2 exposure and following abstinence from repeated WIN 55,212-2 treatment. The effect of abstinence on the α2-adrenoceptor binding in the mPFC was measured 7 days following withdrawal from 7-day WIN 55,212-2 administration. Acute or chronic WIN 55,212-2 exposure did not affect α2A-adrenoceptor binding in the mPFC; however, α2-adrenoceptor binding in the mPFC was significantly increased ( $P < 0.05$ ) following abstinence (Fig. 6).

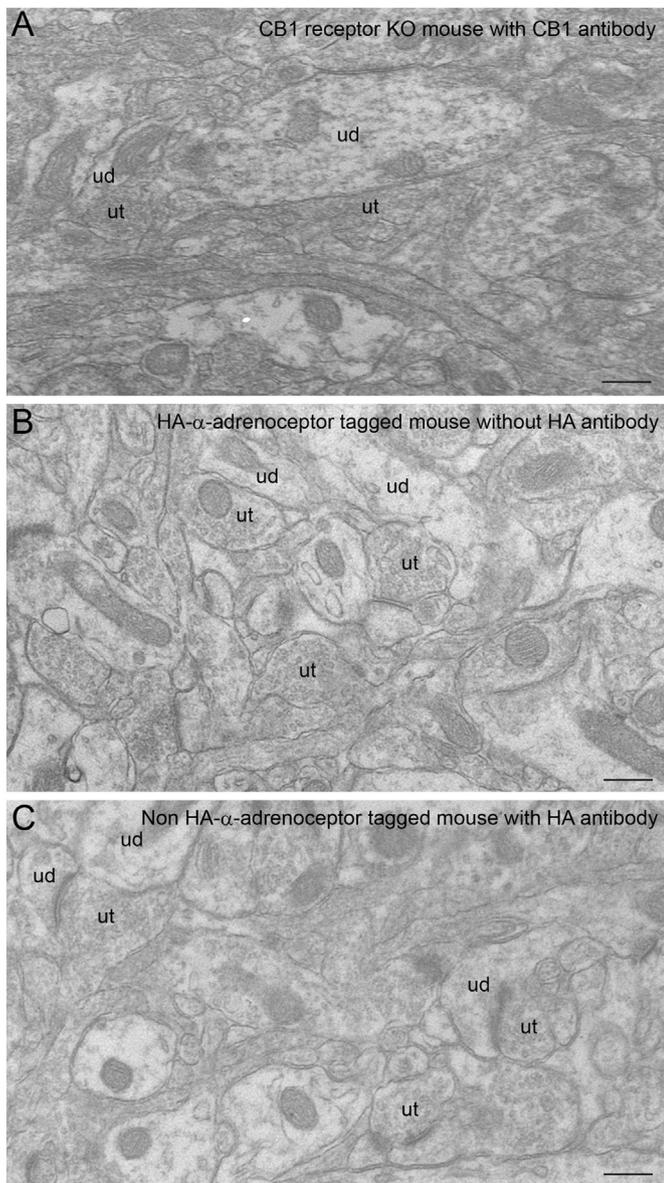
4. Discussion

Electrophysiological studies in a slice preparation reveal that acute stimulation of α2-adrenoceptor with clonidine generated a differential response in CB1 receptor-KO mice compared to WT mice. Acute stimulation of the α2-adrenoceptor with clonidine significantly increased excitability of mPFC neurons in the WT mice while decreasing excitability of mPFC neurons in the CB1 receptor-KO mice. Our results also indicate significant decreases in two adrenoceptor subtypes, α2- and β1-adrenoceptors, in the mPFC of CB1 receptor-KO mice when compared to WT mice. Interestingly, the catecholamine synthesizing enzyme, TH, is significantly increased in the LC of CB1 receptor-KO mice compared with WT mice. Ultrastructural analysis revealed co-localization of the α2-adrenoceptor with CB1 receptor in mPFC neurons in addition to other synaptic configurations. Finally, a significant increase in α2-adrenoceptor mRNA expression was only observed in the mPFC following abstinence from exposure to a chronic cannabinoid receptor agonist in rats suggesting a potential alteration of NE at the synapse. These data add to the accumulating evidence supporting a significant impact of cannabinoids on brain adrenergic function.

4.1. Methodological considerations

Experiments were carried out in both rats and mice. In order to examine adaptations in the noradrenergic system under conditions of

Fig. 2. Western blot analysis of tyrosine hydroxylase (TH) in the locus coeruleus (LC), β1-adrenoceptor (β1-AR) and α2-adrenoceptor expression in the mPFC in WT and CB1 receptor KO mice. Bands shown are representative of one sample obtained from one animal per group. A. Western blot for TH in protein extracts from the LC in WT and CB1 receptor KO mice. TH expression is significantly higher in CB1 receptor KO mice compared with WT mice. B. Western blot analysis of β1-adrenoceptor expression in the mPFC of WT and CB1 receptor KO mice. β1-Adrenoceptor expression is significantly lower in CB1 receptor KO mice compared with WT mice. C. Western Blot analysis of α2-adrenoceptor expression in the mPFC in WT and CB1r-KO mice. α2-Adrenoceptor is significantly lower in CB1 receptor KO mice compared with WT. Data are presented as mean ± SEM of change in band intensity. β-Actin immunoblotting was used as a control to verify equal protein loading. Data were analyzed using paired Student's *t*-test. \* $P < 0.05$  vs WT mice, \*\* $P < 0.01$  vs WT mice.



**Fig. 3.** A. Electron photomicrograph showing the absence of CB1 receptor immunolabeling in mPFC in a representative tissue section obtained from a CB1 receptor KO mouse incubated with CB1 receptor primary antibody and processed for silver enhancement to visualize immunogold silver labeling. B. Electron photomicrograph showing the absence of HA- $\alpha$ 2A-adrenoceptor immunogold-silver labeling in mPFC of a representative tissue section obtained from an HA epitope-tagged HA- $\alpha$ 2A-adrenoceptor knock-in mouse incubated without HA- $\alpha$ 2A-adrenoceptor primary antibody and processed for immunoperoxidase detection. C. Electron photomicrograph showing the absence of HA- $\alpha$ 2A-adrenoceptor immunogold-silver labeling in mPFC of a representative tissue section obtained from a non-HA epitope-tagged mice incubated with HA- $\alpha$ 2A-adrenoceptor primary antibody and processed for immunoperoxidase detection. ud: unlabeled ut: unlabeled terminal. Scale bars, 0.5  $\mu$ m.

CB1 receptor deletion, a mouse model is necessary. Moreover, due to the lack of availability of specific antibodies directed against adrenoceptor subtypes for anatomical analysis in perfused brain tissue (Jensen et al., 2009; Bohmer et al., 2014), an HA-epitope tagged wild-type  $\alpha$ 2-adrenoceptor knock-in mice was used to determine the anatomical relationship between the  $\alpha$ 2a-adrenoceptor and CB1 receptor in the mPFC.

Approaches that combine immunohistochemical detection of an antibody with electron microscopy offer certain advantages. Specifically, the pre-embedding immunohistochemistry maintains morphological

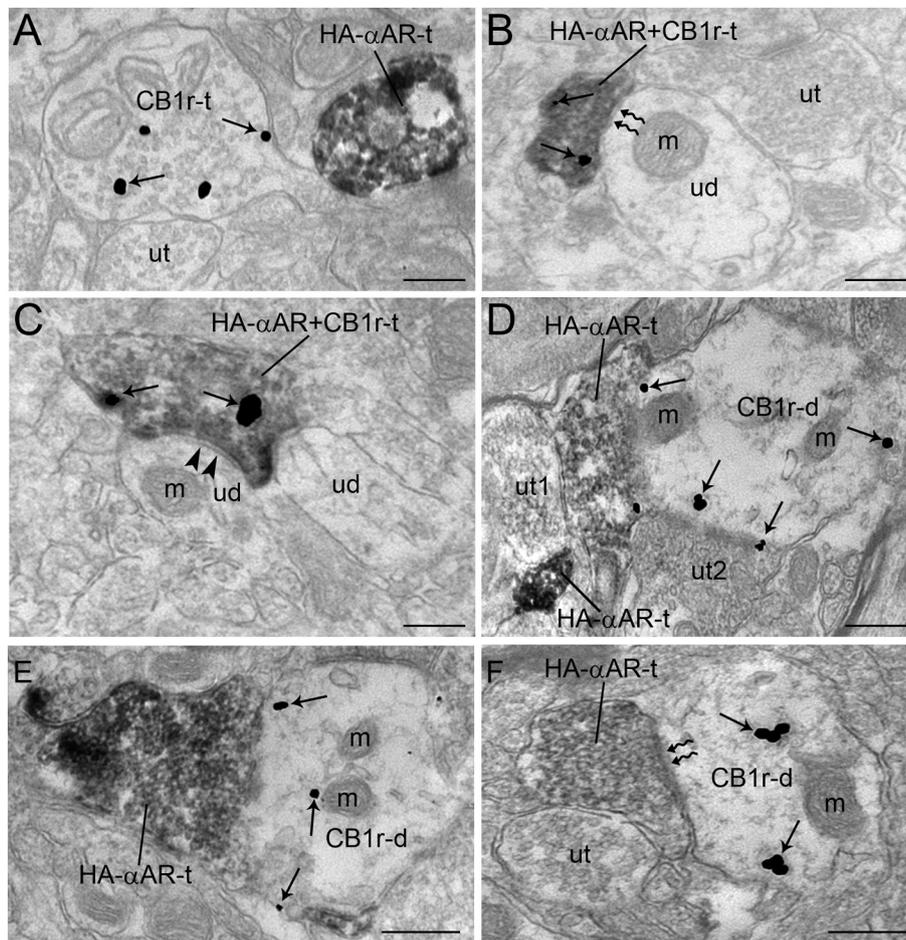
preservation of the neuropil while allowing detection of proteins that are present in low levels. A potential limitation associated with the pre-embedding immunohistochemical technique is the limited and/or differential penetration of immunoreagents in thick Vibratome sections (Leranth and Pickel, 1989; Chan et al., 1990). In order to circumvent this caveat, analysis of ultrathin section was carried out exclusively near the tissue-plastic interface where penetration of immunoreagents is maximal. Another caveat associated with the procedure is the specificity of immunogold-silver labeling. This was controlled by quantifying profiles that contained a minimum of two immunogold-silver particles (Carvalho et al., 2010a, 2010b; Cathel et al., 2014; Oropeza et al., 2007; Reyes et al., 2006; Reyes et al., 2015; Scavone et al., 2010; Van Bockstaele and Pickel, 1993).

Western blot analysis presents some experimental caveats that must be considered when interpreting data. These caveats include the accuracy of sampling of the region of interest and the comparison of equal protein quantities across treatment groups. In order to circumvent variability in tissue excision, a single investigator obtained the brain samples for each experiment. Moreover, to ensure equivalent loading of protein, blots were re-probed with  $\beta$ -actin and results were normalized to this internal standard.  $\beta$ -Actin expression was comparable across treatment groups examined.

#### 4.2. Desensitization of cortical $\alpha$ 2-adrenoceptor under conditions of CB1 receptor deletion

Several studies have reported that CB1 receptor KO mice exhibit angiogenic-like and depressive-like phenotypes (Haller et al., 2002; Viveros et al., 2005; Thiemann et al., 2009; Valverde and Torrens, 2012) with no changes in locomotor function (Haller et al., 2002). Administration of the CB1 receptor antagonist SR141716A in CB1 receptor-KO mice reduced anxiety-like behavior (Haller et al., 2002) while the CB1 receptor agonist AM251 did not induce any significant alterations in anxiety-like behavior in CB1 receptor-KO mice (Thiemann et al., 2009). Our present findings that show an increase TH and decrease in  $\alpha$ 2-AR and  $\beta$ 1-AR expression levels in CB1 receptor-KO mice may suggest involvement of a non-CB1 receptor and adrenergic receptors including vanilloid type 1 receptors (TRPV1) and GPR55 (Hong et al., 2009; Laricchiuta et al., 2013; Biernacki and Skrzydlewska, 2016; Marichal-Cancino et al., 2016; Zhou et al., 2016). It has been shown that eCBS can act to TRPV1 and GPR55 (Chavez et al., 2010; Hong et al., 2009; Laricchiuta et al., 2013; Biernacki and Skrzydlewska, 2016; Marichal-Cancino et al., 2016; Zhou et al., 2016). Our electrophysiology data in WT mice confirmed findings from earlier studies in rats indicating that stimulation of  $\alpha$ 2-adrenoceptors enhances the excitability of mPFC pyramidal neurons coupled with an increase in cellular input resistance (Andrews and Lavin, 2006; Carr et al., 2007; Reyes et al., 2012; Cathel et al., 2014). These effects are consistent with an inhibition of a HCN channel-mediated inward current in mPFC pyramidal neurons (Carr et al., 2007). Although we acknowledge that inter-species difference is critical (Ferreira et al., 2012; Lu et al., 2009), using rat (Cathel et al., 2014) and mice (present study) tissue with similar conditions demonstrate that we have obtained consistently similar data regardless of using mouse or rat tissue.  $\alpha$ 2-Adrenoreceptors on mPFC pyramidal dendrites play a key role in the synaptic integration function of these neurons.  $\alpha$ 2-Adrenoreceptor activation suppresses an HCN current that has two effects in rats: slight hyperpolarization of the pyramidal cell but increased temporal summation of distally evoked excitatory synaptic inputs that enhances the cell's excitability (Carr et al., 2007). The net effect of this  $\alpha$ 2-HCN interaction could explain the ability of norepinephrine to promote attentional mechanisms through enhancement of the signal-to-noise function of mPFC neurons (Sawaguchi et al., 1990; Li et al., 1999; Aston-Jones and Cohen, 2005; Wang et al., 2007; Carr et al., 2007).

CB1 receptor KO mice showed a reduction of basal excitability of mPFC neurons, consistent with our anatomical data indicating reduced



**Fig. 4.** A. Electron photomicrograph showing immunoperoxidase labeling of HA- $\alpha$ 2A-adrenoceptor (HA- $\alpha$ AR-t) and immunogold-silver labeling (arrows) of CB1r in separate axon terminals in the mPFC of an HA epitope-tagged HA- $\alpha$ 2A-adrenoceptor knock-in mouse. B. An immunoperoxidase-labeled axon terminal contains dense immunoreactivity for HA- $\alpha$ 2A-adrenoceptor and immunogold-silver labeling for CB1 receptor (HA- $\alpha$ AR + CB1r-t). The dually labeled terminal forms an asymmetric type synapse (zigzag arrows) with an unlabeled dendrite (ud) that contains a mitochondria (m) and is apposed by an unlabeled terminal (ut). C. A dually labeled HA- $\alpha$ -adrenoceptor + CB1r-t axon terminal forms a symmetric type synapse (arrowheads) with an unlabeled dendrite (ud) containing a mitochondria (m). D. A densely peroxidase labeled HA- $\alpha$ AR-t is shown contacting an unlabeled axon terminal (ut1) that contacts another HA- $\alpha$ AR-t that in turn contacts a CB1 receptor-labeled dendrite (CB1r-d) containing several mitochondria (m). Arrows point to immunogold-silver labeled particles. The CB1r-t also receives a synapse from another unlabeled terminal (ut2). E. HA- $\alpha$ AR-t is shown contacting a CB1r-d with several mitochondria (m). F. A HA- $\alpha$ AR-t forms an asymmetric synapse (zigzag arrows) with a CB1r-d and is directly apposed by an unlabeled terminal (ut). m: mitochondria; ut: unlabeled terminal. Scale bars, 0.5  $\mu$ m.

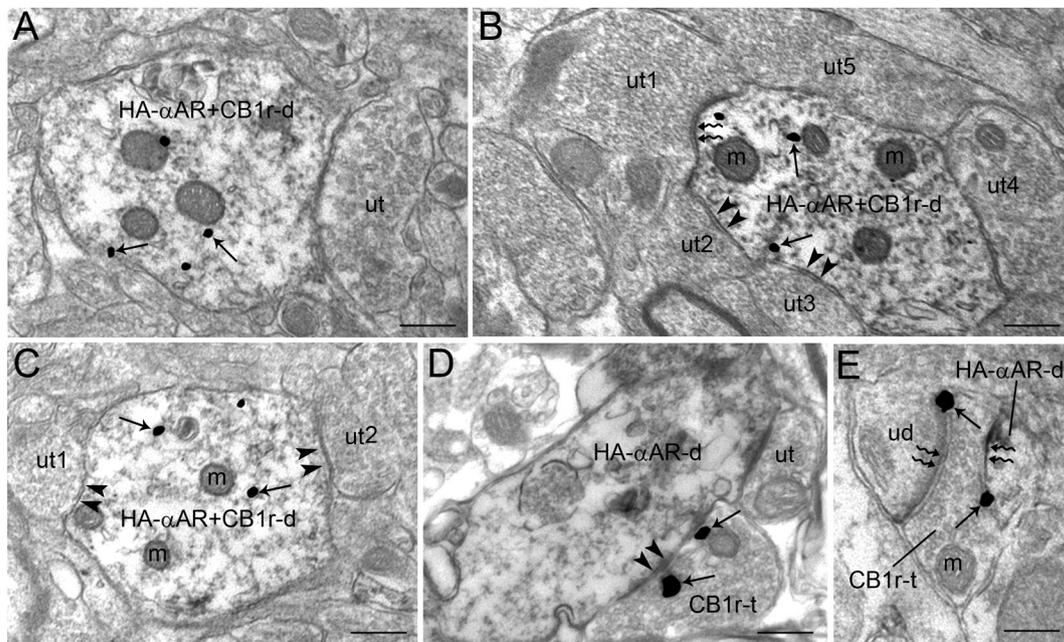
cortical  $\alpha$ 2-adrenoceptor expression. This reduced excitability likely reflects tonic adrenergic activity at  $\alpha$ 2-adrenoceptors, which normally maintains cortical cell excitability, an effect that is diminished in CB1 receptor KO mice. This reduction of cortical  $\alpha$ 2-adrenoceptors in CB1r KO mice further supports the finding that they do not show the normal  $\alpha$ 2-adrenoceptor response exhibited by WT controls. Indeed, the effects of clonidine in CB1 receptor KO mice is opposite of that observed in controls: an  $\alpha$ 2-adrenoceptor-mediated reduction of mPFC excitability coupled with a slight reduction of input resistance. Together, these results provide evidence for functional desensitization of cortical  $\alpha$ 2-adrenoceptors in CB1 receptor KO mice and more broadly, evidence of cannabinoid regulation of cortical  $\alpha$ 2-adrenoceptor-HCN interactions using a transgenic mouse model.

Interestingly, it has been previously reported that CB1 receptor activation using chronic WIN 55,212-2 treatment decreases cortical  $\beta$ 1-adrenoceptor levels (Page et al., 2007) and binding site density (Hillard and Bloom, 1982; Reyes et al., 2009) as well as completely abolishing the clonidine-induced excitation of mPFC neurons (Reyes et al., 2012). Our present results indicate that CB1 receptor deficient mice show a decrease in  $\alpha$ 2- and  $\beta$ 1-adrenoceptor expression levels and  $\alpha$ 2-adrenoceptor desensitization to clonidine exposure. Considering that these are two opposite manipulations (CB1 receptor activation and deletion) which seem to induce equivalent consequences with

regard to expression and function of adrenoceptors, it is possible that chronic WIN 55,212-2 treatment downregulates CB1 receptors and that this effect may somehow contribute to adrenoceptor downregulation which CB1 receptor deficient mice may mimic producing similar effect on the NE system. It is also likely that the constitutive genetic deletion of CB1 receptors may produce some kind of developmental adaptation that have their own effects on NE neurotransmission, downregulating adrenoceptors. Chronic CB1 receptor agonism might be producing the same effect but via a different mechanism.

#### 4.3. Anatomical relationship of cortical $\alpha$ 2-adrenoceptor and CB1 receptor in mPFC

Endogenous and exogenous cannabinoids acting through the CB1 receptor have been implicated in a plethora of physiological functions (Busquets-Garcia et al., 2015; Wyrofsky et al., 2015). In humans and rodents, convergent studies suggest that exogenous cannabinoids and the eCB system is an essential regulator of executive functions, mood and emotions (Fagundo et al., 2013; McLaughlin et al., 2014). As an integral part of the reward system, the mPFC regulates executive and cognitive functions. Dysfunction of the mPFC leads to dysregulated neurotransmission in limbic regions including the hippocampus and amygdala. This demonstrates the vital role of the mPFC in the development of



**Fig. 5.** Ultrastructural evidence for co-localization of HA- $\alpha$ -adrenoceptor and CB1 receptor in dendrites. A. An immunoperoxidase-labeled dendrite contains HA- $\alpha$ 2A-adrenoceptor immunoreactivity as well as immunogold-silver labeling for CB1 receptor (HA- $\alpha$ AR + CB1r-d). This dendrite is directly contacted by an unlabeled terminal (ut). B. A dually labeled HA- $\alpha$ AR + CB1r dendrite receives an asymmetric type synapse (zigzag arrows) from an unlabeled terminal (ut1) and a symmetric type synapse (arrowheads) from ut2 and ut3. A fourth unlabeled terminal (ut4) directly contacts the HA- $\alpha$ AR + CB1r dendrite. C. A dually labeled HA- $\alpha$ AR + CB1r dendrite receives two symmetric type synapses (arrowheads) from ut1 and ut2. D. A CB1 receptor labeled axon terminal (CB1r-t) forms a symmetric type synapse (arrowheads) with HA- $\alpha$ -adrenoceptor labeled dendrite (HA- $\alpha$ AR-d). The same HA- $\alpha$ AR-d is contacted by an unlabeled terminal (ut). E. A CB1r-t forms an asymmetric type synapse (zigzag arrows) with an unlabeled dendrite (ud) and a HA- $\alpha$ AR-d. m: mitochondria; ut: unlabeled terminal. Scale bars, 0.5  $\mu$ m.

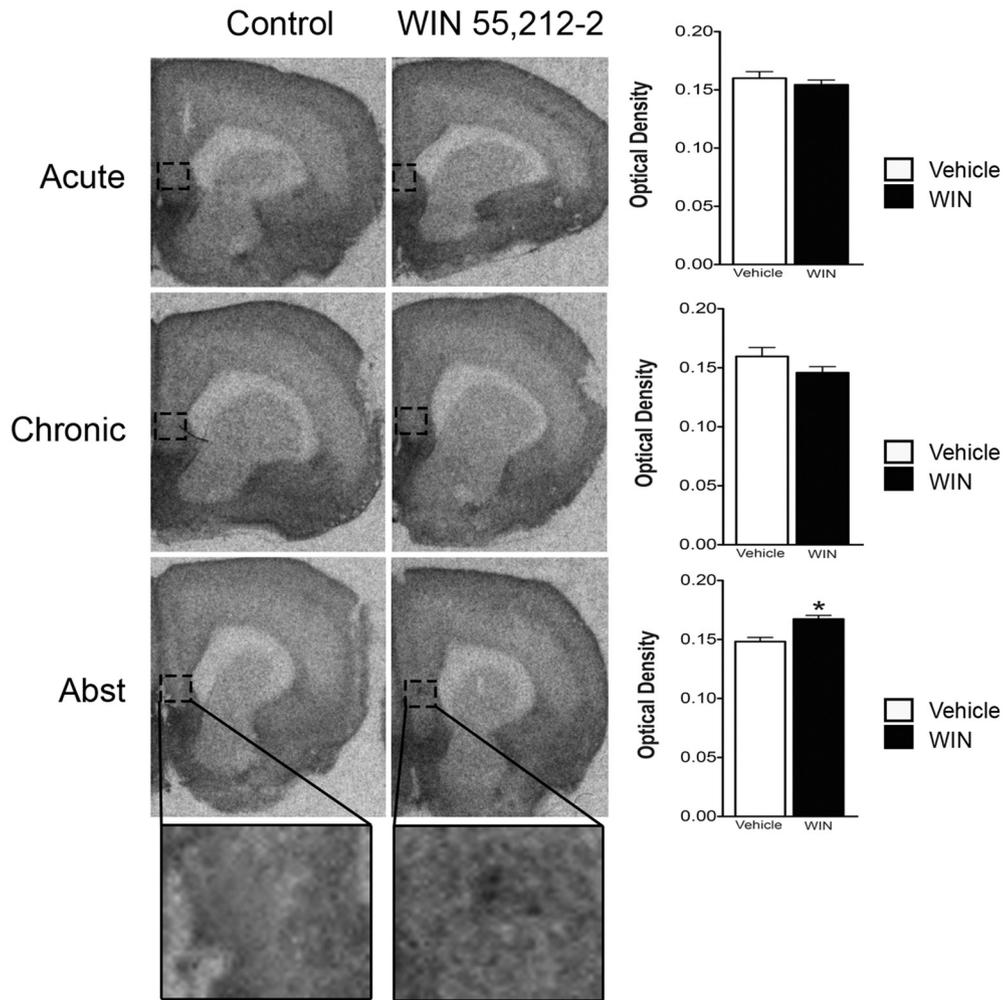
neuropsychiatric disorders. Likewise, the role of cannabinoids in neuropsychiatric disorders including anxiety or depression has been described (Cortes-Briones et al., 2015; Habibisaravi et al., 2015; Panlilio et al., 2015). Our data are consistent with others (Zarate et al., 2008; Richter et al., 2012) showing pre-synaptic localization of  $\alpha$ 2-adrenoceptor in the mPFC (Pudovkina et al., 2001; Flugge et al., 2004). Using electron microscopy, we had previously described the pre-synaptic distribution of  $\alpha$ 2-adrenoceptor and CB1 receptor in the mPFC, in independent studies (Oropeza et al., 2007; Cathel et al., 2014). We also used confocal triple immunofluorescence microscopy to reveal that HA- $\alpha$ 2-adrenoceptor immunoreactive fibers co-localized dopamine- $\beta$ -hydroxylase, a marker of NE axon terminals, and CB1 receptor in the mPFC (Cathel et al., 2014). To our knowledge, this is the first subcellular demonstration of co-existence between  $\alpha$ 2-adrenoceptor and CB1 receptor in common cortical axonal profiles. In addition, our immunoelectron microscopy results demonstrate different types of CB1r and  $\alpha$ 2-adrenoceptor interactions in the mPFC. CB1 receptor-containing axon terminals can be found pre-synaptic to  $\alpha$ 2-adrenoceptor-containing dendrites and CB1 receptor and  $\alpha$ 2-adrenoceptor can be found post-synaptically in common dendrites. Despite a low level of synaptic configurations (e.g. 15% of  $\alpha$ 2-adrenoceptor and CB1 receptor co-expression is symmetric), this interaction may still exert some significant behavioral consequences since small synaptic modifications applied to synapses in a given neuron disrupt the inhibitory and excitatory input and produce large effects for modifications induced by a single stimulus, and that few synaptic contacts may still exert a direct synaptic influence (Del Cid-Pellitero and Garzon, 2014; Yger et al., 2015).

The interaction of  $\alpha$ 2-adrenoceptor and CB1 receptor may have several functional consequences. Our previous electrophysiology findings of cortical pyramidal neurons showed that cannabinoid administration desensitized  $\alpha$ 2-adrenoceptor (Reyes et al., 2012; Cathel et al., 2014). Desensitization of presynaptic  $\alpha$ 2-adrenoceptor autoreceptors may result in the stimulation of NE efflux as removal of the agonist rapidly

restores receptor function (Hein and Kobilka, 1995). Desensitization does not seem to depend on protein degradation and as such no differences in the total receptor protein would be expected during desensitization and this is consistent with the previous results showing no difference in  $\alpha$ 2-adrenoceptor protein levels following chronic WIN 55,212-2 exposure (Reyes et al., 2009).  $\alpha$ 2-Adrenoceptor desensitization may occur through sequestration, downregulation and decreases in Gi that have been demonstrated following agonist exposure to  $\alpha$ 2-adrenoceptor (Jones et al., 1990; Jewell-Motz et al., 1998). Another possibility is that  $\alpha$ 2-adrenoceptor is phosphorylated by G-protein-coupled receptor kinases and/or second messenger-dependent kinases including protein kinases A and C (Milligan et al., 1995) because persistent activation of phospholipase C and protein kinase C signaling pathways induces firing (Carr et al., 2007). Alternatively, desensitization of  $\alpha$ 2-adrenoceptor may take place when it is sequestered to the intercellular compartment (Milligan et al., 1995). While our current and previous physiological evidence (Reyes et al., 2012; Cathel et al., 2014) suggests functional desensitization of  $\alpha$ 2-adrenoceptor in the mPFC through CB1 receptor agonism, future investigation is required to unravel the mechanism. Interestingly, while alterations of  $\alpha$ 2A-adrenoceptor binding in the mPFC occurred following abstinence from chronic WIN 55,212-2 exposure, the  $\alpha$ 2A-adrenoceptor mRNA expression was unchanged in the LC (data not shown) indicating that despite significant high densities of  $\alpha$ 2A-adrenoceptor in these two important nuclei (Probst et al., 1984), it is not dysregulated following acute or chronic WIN 55,212-2 exposure.

#### 4.4. Adaptations in adrenoceptors following CB1 receptor agonist administration

Using Western blot analysis, we previously reported that WIN 55,212-2 administration affected the expression of cortical adrenoceptors (Reyes et al., 2009). We showed that chronic WIN 55,212-2 exposure, with or without abstinence, influences  $\beta$ 1-

mPFC  $\alpha$ 2A-AR binding

**Fig. 6.**  $\alpha$ 2-Adrenoceptor binding in mPFC of rats exposed to acute or chronic treatment with WIN 55,212-2. There was no change in the  $\alpha$ 2- adrenoceptor mRNA expression in the mPFC following acute or repeated administration of WIN 55,212-2 compared to control. However, following a period of abstinence from chronic WIN 55,212-2 exposure, there was a significant increase in  $\alpha$ 2-adrenoceptor mRNA binding in the mPFC. \* $P$  0.05 vs WIN 55,212-2. Bottom panels show boxed region at a higher magnification.

adrenoceptor and that abstinence also affects  $\alpha$ 2-adrenoceptor protein levels in the mPFC (Reyes et al., 2009). In the present study,  $\alpha$ 2-adrenoceptor binding was significantly increased following abstinence from WIN 55,212-2 exposure. The increase in  $\alpha$ 2-adrenoceptor binding in the present study supports a mechanism whereby WIN 55,212-2 administration activates LC neurons with a concomitant increase of NE efflux in the mPFC (Oropeza et al., 2005; Page et al., 2007). Increases in NE efflux may contribute to an increase  $\alpha$ 2-adrenoceptor binding as more NE binds to  $\alpha$ 2-adrenoceptor. Although  $\alpha$ 2-adrenoceptor functions as an autoreceptor to inhibit NE release (Richter et al., 2012), we and others have provided evidence for postsynaptic localization of  $\alpha$ 2-adrenoceptor (Cathel et al., 2014); thus it is possible that increases in  $\alpha$ 2-adrenoceptor binding could represent  $\alpha$ 2-adrenoceptor that are located postsynaptically. However, further investigation is needed to elucidate this. In addition, an increase in  $\alpha$ 2-adrenoceptor binding may occur as a response to altered NE level at the synapse (Page et al., 2007). After a period of abstinence increased  $\alpha$ 2-adrenoceptor binding may result as a consequence of a rebound in receptor expression similar to that reported after a sudden cessation of using a beta blocker (Pratt and Bowery, 1993). Following period of abstinence increases in  $\alpha$ 2-adrenoceptor expression might be a consequence of normalization of NE levels as it has been shown that anxiety-like behavior and NE levels

return to normal levels after chronic WIN 55,212-2 treatment followed by a cessation of drug use (Page et al., 2007).

#### 4.5. Functional implications

In summary, the results of our present study highlight the diversity of cellular interactions of the endocannabinoid and noradrenergic systems in the mPFC. Moreover, these anatomical results provide a cellular basis for the functional desensitization of cortical  $\alpha$ 2-adrenoceptors in CB1 receptor KO mice providing evidence of cortical  $\alpha$ 2-adrenoceptor regulation by the endocannabinoid system. Considering that nabilone's efficacy in the treatment of PTSD is attributed to the actions of cannabinoids on NE circuitry (Reyes et al., 2009; Villanueva et al., 2009; Carvalho et al., 2010a; Carvalho et al., 2010b) and that cortical NE neurotransmission is involved in cognitive and behavioral states including anxiety (Arnsten, 2007; Ramos and Arnsten, 2007; Arnsten and Pliszka, 2011), cannabinoid and adrenergic receptor interactions may underlie the regulation of cortical function by cannabinoids. Interestingly, recent studies have shown that CB1 and CB2 receptors also play a role in splenic contraction (Simkins et al., 2016), that both peripheral and central CB1 receptors control stress-induced impairment of memory consolidation (Busquets-Garcia et al., 2016) and that a memory

acquisition deficit induced by CB1 receptor agonist, arachidonylcyclopropylamide, involved  $\alpha 2$ -adrenoceptors in the mPFC (Beiranvand et al., 2016). Thus, these findings may have implications for advancing our understanding of the circuitry underlying  $\alpha 2$ -adrenoceptor and CB1 receptor interactions that may be targeted in the development of pharmacological treatment for neuropsychiatric disorders.

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