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Remote CB1 receptor antagonist administration reveals multiple sites of tonic and phasic endocannabinoid neuroendocrine regulation

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ABSTRACT

Endogenous cannabinoids (endocannabinoids, eCB) are expressed throughout the body and contribute to regulation of the hypothalamo-pituitary-adrenal (HPA) axis and general stress reactivity. This study assessed the contributions of CB1 receptors (CB1R) in the modulation of basal and stress-induced neural and HPA axis activities. Catheterized adult male rats were placed in chambers to acclimate overnight, with their catheters connected and exteriorized from the chambers for relatively stress-free remote injections. The next morning, the CB1R antagonist AM251 (1 or 2 mg/kg) or vehicle was administered, and 30 min later, rats were exposed to loud noise stress (30 min) or no noise (basal condition). Blood, brains, pituitary and adrenal glands were collected immediately after the procedures for analysis of c-*fos* and CB1R mRNAs, corticosterone (CORT) and adrenocorticotropin hormone (ACTH) plasma levels. Basally, CB1R antagonism induced c-*fos* mRNA in the basolateral amygdala (BLA) and auditory cortex (AUD) and elevated plasma CORT, indicating disruption of eCB-mediated constitutive inhibition of activity. CB1R blockade also potentiated stress-induced hormone levels and c-*fos* mRNA in several regions such as the bed nucleus of the stria terminalis (BST), lateral septum (LS), and basolateral amygdala (BLA) and the paraventricular nucleus of the hypothalamus (PVN). CB1R mRNA was detected in all central tissues investigated, and the adrenal cortex, but at very low levels in the anterior pituitary gland. Interestingly, CB1R mRNA was rapidly and bidirectionally regulated in response to stress and/or antagonist treatment in some regions. eCBs therefore modulate the HPA axis by regulating both constitutive and activity-dependent inhibition at multiple levels.

1. Introduction

The endogenous cannabinoid (eCB) system contributes to the regulation of psychoemotional states and the reactivity of the hypothalamic-pituitary-adrenal (HPA) axis under basal, acute and repeated stress conditions (Hill et al., 2010a, 2012; Hillard et al., 2012, 2016; Lutz et al., 2015; Micale and Drago, 2018; Morena et al., 2016; Patel and Hillard, 2008; Valverde, 2005). Given the widespread expression of eCB ligands and cannabinoid receptors throughout the body (Cota, 2007; Herkenham et al., 1991; Lynn and Herkenham, 1994; Mackie, 2008), the eCB system is in a position to modulate several aspects of stress reactivity independently and cooperatively.

We previously reported that systemic pharmacological antagonism of CB1 receptors (CB1R) with AM251 potentiated noise stress-induced neural and HPA reactivity in rats, as indexed by the immediate early gene c-*fos* in multiple limbic regions, anterior pituitary gland activity and plasma adrenocorticotropic hormone (ACTH) levels (Newsom et al., 2012). These findings are in agreement with previous research indicating widespread phasic eCB activity in limiting the magnitude of HPA axis reactions to psychological stress (Finn, 2010; Hill and McEwen, 2010; Lutz, 2009; Patel et al., 2005; Riebe and Wotjak, 2011; Valverde, 2005). In the same study, CB1R antagonism alone robustly induced c-*fos* mRNA in multiple neural regions and elevated basal plasma corticosterone (CORT). These findings additionally suggest the contribution of the eCB system in constitutive CB1R activity that mediates a constraining tonic inhibition of neural activities in some central and peripheral regions regulating the HPA axis. Interestingly, this CB1R antagonist-mediated induction of basal activity appeared to be independent and orthogonal to the observed elevation in neural and endocrine activity measured following stress exposure.

A possible explanation for the increased basal activity measured after AM251 administration is the stressful nature of the injection procedure itself, which mildly induces stress reactions (Ryabinin et al., 1999) and could be potentiated and/or sustained by AM251 treatment (Ginsberg et al., 2010). However, this possibility does not address the apparent differential neural regulation observed during basal and stress-induced activity by CB1R antagonism. For example, while CB1R antagonism prior to stress potentiated activity in some limbic structures, the same treatment given basally did not elevate activity in these regions (Newsom et al., 2012). Another perplexing result (Newsom et al., 2012) is the incongruence of basal AM251-induced elevation in PVN activity (as indexed with c-*fos* mRNA induction) together with unchanged anterior pituitary activity, given that these measures are typically highly correlated (Burow et al., 2005). The basal elevation in

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CORT measured after AM251 administration raises the possibility that the adrenal gland is under local CB1R-dependent constitutive inhibitory regulation to an extent similar to the neural structures that were stimulated by AM251 injection (Newsom et al., 2012). Involvement of CB1 receptors in tonic inhibition of the HPA axis has been suggested, but it is currently unclear whether both its central and peripheral components may be responsible for this inhibition (Cota, 2007). CB1 receptors and mRNA have been detected in adrenal and pituitary gland tissues in some species (Buckley et al., 1998; Pagotto et al., 2001; Ziegler et al., 2010), and have been explored to limited extent, but have been hypothesized (Hillard et al., 2016) and demonstrated (Surkin et al., 2018) to contribute to inhibitory regulation of HPA axis activities. Peripheral CB1R activity warrants further investigation for roles in HPA axis regulation, which may have implications for potential therapeutic target strategies. Indeed, peripheral CB1R antagonists are of recent therapeutic interest in metabolic regulation (Bowles et al., 2015; Di Marzo et al., 2011). It would therefore be important to determine if peripheral CB1R antagonist administration leads to significant elevation of circulating CORT due to effects at adrenal or pituitary locations, which could be beneficial or detrimental (Sapolsky, 2000).

Dose-dependent effects of similar CB1R antagonist rimonabant (SR141716A) on CORT levels have been reported (Patel, 2004), but it is unknown whether this effect is due to modification of central or peripheral activity (Cota, 2007; Hill and Tasker, 2012; Newsom et al., 2012), or how reported pharmacological CB1R inverse agonist-like and neutral antagonist-like effects relate to normal CB1 receptor activity (Di et al., 2013; Hill and Tasker, 2012; Ho et al., 2010; Newsom et al., 2012). The current study was designed to more carefully assess and investigate the contributions of CB1R in inhibitory regulation of basal compared to stressor-induced, and central compared to peripheral, activities in stress-reactive neural regions and the HPA axis using measures of c-*fos* mRNA and plasma hormones (ACTH and CORT). Additionally, two doses of CB1R antagonist AM251 were included in all comparisons and remotely administered drug treatment through intraperitoneal (i.p.) catheters was employed to minimize the potential confounds or interactions of handling and injection stress on basal and stress-related measures as well as to allow for potential distinction between tonic and phasic inhibitory regulation by CB1R when co-occurring in the same tissues. Finally, considering theoretical and known eCB system involvements in recovery from acute stress (Riebe and Wotjak, 2011), and modulation of reactivity to repeatedly experienced stressors, which may involve stimulation or stress-dependent alterations in CB1R activity (Hill et al., 2010a; Newsom et al., 2019; Patel and Hillard, 2008; Riebe et al., 2012), we also measured CB1R mRNA in central and peripheral tissues. The use of systemic administration of AM251 and measures of multiple central and peripheral components demonstrating in vivo tonic and phasic activities from the same subjects importantly allowed for analysis of their relative contributions to neural and neuroendocrine activity regulations.

2. Methods and materials

2.1. Subjects

Forty-seven adult male Sprague Dawley rats (Harlan, Indianapolis IN) weighing 300 − 350 grams were used. Animals were housed in polycarbonate tubs containing wood shavings, with wire lids providing rat chow and water ad libitum. Conditions in the animal colony were controlled to constant humidity and temperature, with a 12:12 h light/dark cycle (lights on at 7:00 am). Testing was performed between 8:00 am and 11:00 am during the circadian nadir of HPA axis activity (Spencer and Deak, 2016). All procedures were reviewed and approved by the Institutional Animal Care and Use Committee of the University of Colorado and conformed to the United States of America National Institute of Health Guide for the Care and Use of Laboratory Animals. All efforts were made to minimize animal suffering and the number of animals used.

2.2. Surgery

Following a week of acclimation to the colony and daily handling, all rats were surgically implanted with intra-peritoneal (i.p.) catheters to allow for remote administration of drug and vehicle treatments on testing days. This administration method was used to circumvent the potential confounds of handling and injection stress on basal and stress-induced measures. The surgical procedure was performed as previously described (Day and Akil, 1999; Day et al., 2005) with a minor alteration. Catheters were externalized at the center of the upper back (Bachtell and Self, 2009) rather than being mounted to the skull. Following surgery, dust caps were attached to the externalized opening of the catheters, and rats were individually housed. A recovery period of 5–8 days was allowed before each rat's single testing day.

2.3. Experimental design

Rats were randomly assigned to receive one of three drug treatments (vehicle, 1.0 or 2.0 mg/kg AM251) and acute noise stress or no noise control treatment (3 × 2 factorial design, 7–9 per group) to allow for examination of effects of antagonism of CB1R on basal and stress-induced neural and HPA axis activity. Due to the remote drug administration procedures, 8 or fewer rats were tested each experimental day. At approximately 5:00 pm on the day before testing days, the entire home cage of each rat was placed into ventilated acoustically attenuating chambers (described in detail in Day et al., 2009) and a saline filled length of polyethylene (PE) tubing within a stainless-steel flexible connector (Plastics One, Roanoke VA) was connected to the i.p. catheters and exteriorized from the acoustic chamber. Catheter extensions were attached to a fluid swivel that was mounted on additional cage tops to allow for free movement throughout the cage. Water bottles and rat chow were transferred to these cage tops for continued access. Lighting in the acoustic chambers was controlled to an intensity similar to the colony room, and a timer was used to maintain light/dark cycle in accordance with the colony schedule. Leading up to the single testing day, rats were habituated, by repeated presentations, to the mildly stressful procedures of travel from colony to testing location, handling necessary to affix PE tubing to cannula, and time in the acoustic chambers.

The next morning (on the day of testing), during the circadian trough of HPA axis activity, rats were remotely administered AM251 (1.0 or 2.0 mg/kg) or vehicle (Tween80, DMSO, saline at 1:1:8, 1 ml/kg) through the externalized PE tubing using sterile 1 cc syringes via blunted needles. An additional predetermined amount (0.2 ml) of saline was slowly flushed through the tubing following the drug-containing and vehicle solutions to clear the PE tubing and ensure administration of the entire volume. Thirty-minutes after drug administration, rats were exposed to 30 min of loud noise (95 dBA sound pressure level -SPL, A scale) stress. Non-stressed control rats remained in the acoustically attenuating chambers for the same amount of time without loud noise exposure (minimal background noise of fans at approximately 57 dBA). Treatment initiation was staggered by five-minute intervals to ensure precise standardization of procedure timing. Immediately following cessation of noise or no noise exposures, rats were unhooked from their catheters and transported to an adjacent room where they were rapidly euthanized by decapitation (see summary of test day procedure in Fig. 1A). Trunk blood was collected in (anticoagulant) EDTA-coated containers for later quantification of plasma ACTH and CORT. Brains, pituitary glands, and adrenal glands were rapidly excised and frozen for later sectioning and analysis.

2.4. Drug treatment

The CB1 antagonist/inverse agonist AM251 (Ascent Scientific,

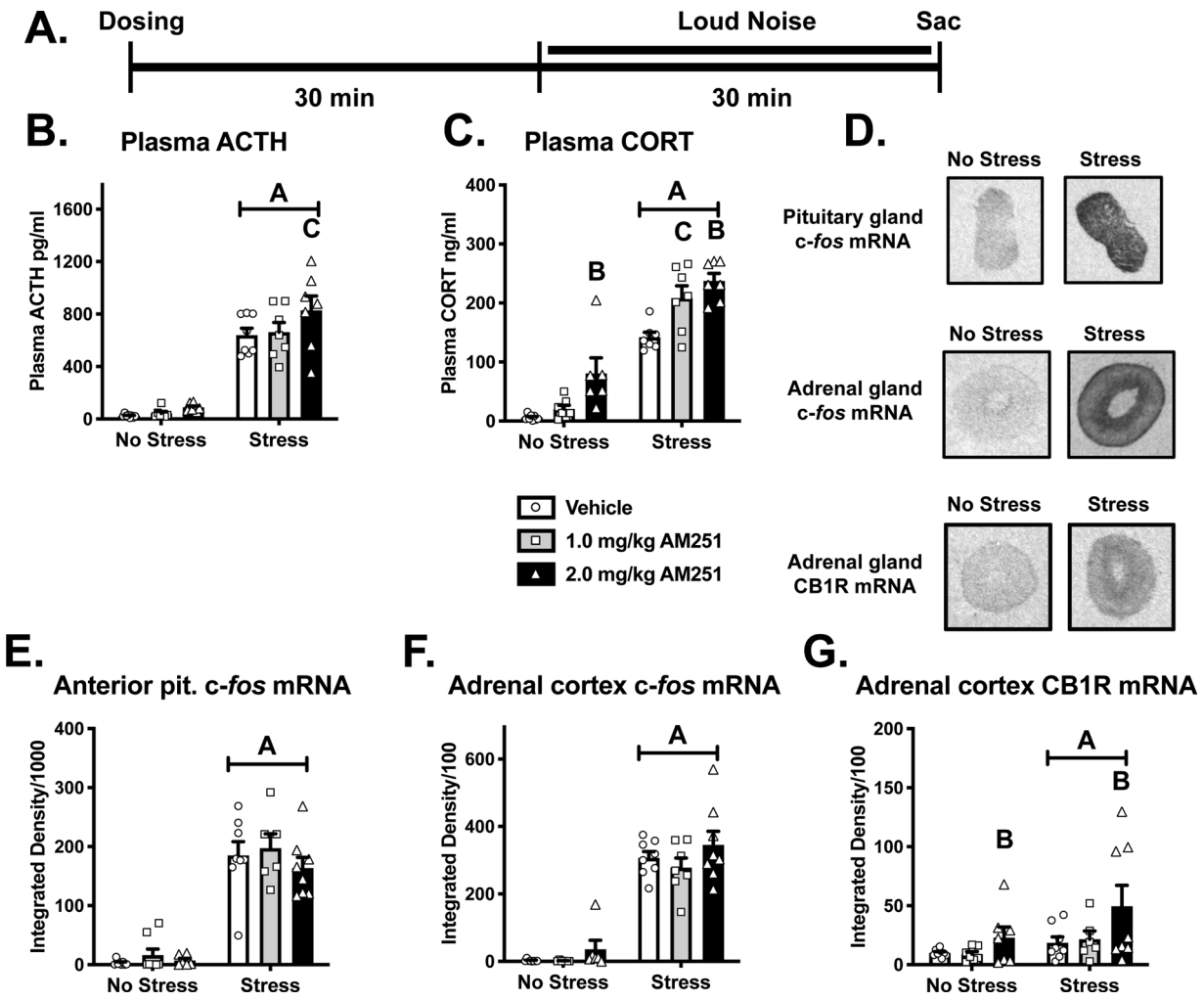

Fig. 1. Peripheral HPA axis and CB1R mRNA measures A. Experimental timeline for testing day. Sac = sacrifice. **B.** Mean plasma ACTH (+ 1 standard error) obtained following stress and/or AM251 treatments; Significant effect of stress (A, $p < 0.001$), significant potentiation of stress response by 2.0 mg/kg AM251 (C, $p < 0.05$). **C.** Mean plasma CORT (+1 standard error) obtained following stress and/or AM251 treatments; Significant effect of stress (A, $p < 0.001$), significant elevation of basal and stress-induced CORT by 2.0 mg/kg AM251 (B, $p < 0.05$), significant potentiation of stress-induced CORT response by 1.0 mg/kg AM251 (C, $p < 0.05$). **D.** Representative autoradiographs of c-*fos* and CB1R mRNA in pituitary and adrenal glands. **E.** Semi-quantitative results of anterior pituitary gland c-*fos* mRNA (+1 standard error) obtained following stress and/or AM251 treatments; Significant effect of stress (A, $p < 0.001$). **F.** Semi-quantitative results of adrenal cortex c-*fos* mRNA obtained following stress and/or AM251 treatments; Significant effect of stress (A, $p < 0.001$). **G.** Semi-quantitative results of adrenal cortex CB1R mRNA after stress and/or AM251 treatments; Significant effect of stress (A, $p < 0.05$), significant increase by 2.0 mg/kg AM251 compared to vehicle and 1.0 mg/kg AM251 treatments in basal and stress-induced conditions (B $p < 0.05$). (N = 47).

Princeton, NJ) was used to assess the normal involvement of the endogenous cannabinoid system in regulation of plasma ACTH and CORT levels, as well as limbic, pituitary and adrenal c-*fos* and CB1R mRNA expression in basal non-stressed conditions and in responses to acute loud noise exposure. AM251 was dissolved in dimethyl sulfoxide (DMSO) upon arrival, and added to Tween 80, and physiological (0.9%) saline (in a 1:1:8 ratio, respectively). Systemic doses of AM251 (1.0 and 2.0 mg/kg) were chosen based on previous results demonstrating 2.0 mg/kg to robustly induce plasma CORT increase and neural activity indicated by c-*fos* mRNA induction in several regions of the brain and to potentiate stress-induced increases in ACTH and c-*fos* mRNA in several other regions (Newsom et al., 2012). A lower dose (1.0 mg/kg) was included for examination of possible dose-dependency of these responses including avoidance of ceiling effects and putative interactions between drug and stress-induced neural and HPA axis measures.

2.5. Corticosterone enzyme linked ImmunoSorbent assays (ELISA)

The corticosterone assay was performed according to the manufacturer's instructions (kit #K014 H5–Arbor Assays, Ann Arbor, MI) using 10 microliters of previously frozen (−80 °C) plasma. Concentrations were quantified on a BioTek Elx808 microplate reader and calculated against a standard curve generated concurrently.

2.6. Adrenocorticotropic hormone assay

Plasma (200 ul) was assayed for the determination of ACTH concentrations using an Immunoradiometric Assay kit (Diasorin, Stillwater, MN, USA), according to the manufacturer's instructions. Briefly, frozen plasma (−80 °C) was thawed on ice and incubated overnight with a ^{125}I-labelled monoclonal antibody specific for ACTH 1–17, a goat polyclonal antibody specific for ACTH 26–39, and a polystyrene bead coated with a mouse anti-goat antibody. Only ACTH 1–39 in the sample bound both antibodies to form an antibody complex. Beads were washed to remove unbound radioactivity, counted with a gamma counter, and the concentrations of ACTH determined by comparison with a standard curve generated concurrently. All samples from this study were quantified in the same assay.

2.7. In situ hybridization

The method for in situ hybridization histochemistry has been previously described (Day and Akil, 1996). Briefly, 12-μm sections were cut on a cryostat (Leica model 1850), thaw-mounted on polylysine-coated slides and stored at −80 °C. ^{35}S- UTP-labeled riboprobes against c-*fos* mRNA (680 mer; courtesy of Dr. T. Curran, St Jude Children's Hospital, Memphis TN) and CB1 receptor mRNA (984 mer from the coding region [580–1563] of rat CB1 receptor, NM012784.4 produced by Dr. Heidi Day) were generated using standard transcription methods. Sections were fixed in 4% paraformaldehyde (1 h), acetylated in 0.1 M triethanolamine with 0.25% acetic anhydride (10 min.) and dehydrated through graded alcohols. Sections were hybridized overnight at 55 °C with a [35S]- UTP-labeled riboprobe diluted in hybridization buffer containing 50% formamide, 10% dextran sulfate, 2× saline sodium citrate (SSC), 50 mM PBS, pH 7.4, 1× Denhardt's solution, and 0.1 mg/ml yeast tRNA. The following day, sections were treated with RNase A, 200 ug/ml at 37 °C (1 h), and washed to a final stringency of 0.1 × SSC at 65 °C (1 h). Dehydrated sections were exposed to X-ray film (BioMax MR; Eastman Kodak, Rochester, NY) for structure-appropriate times (1–3 weeks) and the films analyzed as described below.

2.8. Semi-quantitative x-ray film analysis

Levels of c-*fos* and CB1 receptor mRNAs were analyzed by computer-assisted optical densitometry. Anatomical landmarks were based on the white matter distribution of unstained tissue sections, according to a standard rat brain atlas (Paxinos and Watson, 1998). Brain sections were captured digitally (CCD camera, model XC-77; Sony, Tokyo, Japan), and the relative optical density of the x-ray film was determined using Scion Image version 4.0 for PC. A macro was written (Dr. S. Campeau) that enabled signal above background to be determined automatically. For each section, a background sample was taken over an area of white matter, and a signal threshold was calculated as mean gray value of background + 3.5 standard deviation. The section was automatically density sliced at this threshold value, so that only pixels with gray values above the computed threshold were included in averages that were employed in the analysis. Regions of interest were chosen primarily due to the results of a previous study demonstrating them to have apparent sensitivity to CB1R antagonism alone or in combination with stress, unique expression patterns, as well as to better determine peripheral compared to central endocannabinoid system involvement in HPA axis regulation (Newsom et al., 2012). Regions of interest and quantification templates used are indicated in Fig. 2.

2.9. Statistical analyses

Prism (v 6.0, GraphPad Software Inc.) was used for all statistical analyses, which included two-way analyses of variance (ANOVA) for all measures using drug (vehicle, 1.0 and 2.0 mg/kg AM251) and stress treatments (acute noise stress, no noise control) as fixed factors. Given that inverse agonist effects of CB1R antagonists have been reported to be dose-dependent (Patel, 2004; Trezza et al., 2012), significant ANOVA effects were followed with Fisher's LSD post hoc analyses of all comparisons, for sensitivity. Significance for all tests was established at a $P = 0.05$. All data presented in the figures are listed as mean values +/− 1 standard error. Outlier values were identified as those being greater than 2 standard deviations from the group mean when included in the dataset, and were excluded without mean replacement. Additionally, some variation in degrees of freedom reflects sample loss during processing.

3. Results

3.1. Peripheral HPA axis and CB1R mRNA measures

Plasma CORT and ACTH were measured to assess contribution of eCB signaling to tonic inhibition and stress-reactivity (Fig. 1). Two-way ANOVA on plasma ACTH values revealed a significant main effect of stress ($F_{(1,38)} = 198.6$, $p < 0.001$) indicating that acute loud noise stress resulted in significant elevation of ACTH in all treatment groups (Fig. 1B). There was not a significant main effect of drug ($F_{(2,38)} = 2.86$, $p = 0.07$) or significant overall interaction for all treatment groups ($F_{(2,38)} = 0.77$, $p = 0.47$). However, post hoc analyses indicated that 2.0 mg/kg AM251 treatment significantly increased stress-induced plasma ACTH compared to vehicle treated rats ($p < 0.05$), while this dose did not significantly increase basal ACTH. This effect indicates potentiation of this index of stress-induced HPA axis stimulation by CB1R antagonist AM251. However, 1.0 mg/kg AM251 treatment did not increase basal or stress-induced ACTH compared to vehicle treatment ($p > 0.05$).

Plasma CORT was found to display a different pattern (Fig. 1C). Two-way ANOVA indicated significant main effects of stress ($F_{(1,36)} = 203.7$, $p < 0.001$) and drug ($F_{(2,36)} = 19.37$, $p < 0.001$), but not a significant overall drug x stress interaction ($F_{(2,36)} = 1.79$, $p = 0.18$). Post hoc comparisons revealed that 2.0 mg/kg, but not 1.0 mg/kg AM251 treatment significantly elevated basal CORT compared to vehicle treatment ($p < 0.05$). Acute stress increased CORT in all three groups ($p < 0.001$). Both doses of AM251 significantly elevated stress-induced CORT compared to vehicle treatment ($p < 0.05$). Significant elevation of stress-induced CORT by 1.0 mg/kg AM251 compared to vehicle treatment indicates a potentiation of stimulated HPA axis response by this dose of the CB1R antagonist.

Immediate early gene c-*fos* mRNA was analyzed as an indicator of recent cellular activity in pituitary and adrenal glands (Fig. 1D). Analysis of anterior pituitary values with two-way ANOVA revealed a significant effect of stress ($F_{(1,38)} = 29.77$, $p < 0.001$), indicating that stress treatment increased c-*fos* mRNA in all treatment groups (Fig. 1E). There was no main effect of drug treatment ($F_{(2,38)} = 0.15$, $p = 0.86$), or interaction between drug and stress ($F_{(2,38)} = 0.07$, $p = 0.93$). Similarly, adrenal cortex (Fig. 1 F) analysis with two-way ANOVA revealed a significant increase in c-*fos* mRNA from stress treatment ($F_{(1,38)} = 219.4$, $p < 0.001$) but not drug treatment ($F_{(2,38)} = 2.29$, $p = 0.11$), or interaction between the two ($F_{(2,38)} = 0.28$, $p = 0.76$).

We detected CB1R mRNA in the anterior pituitary gland and cortex of the adrenal gland. However, expression was very light and indistinguishable between groups in preliminary quantification in the anterior pituitary gland, even at the longest film exposure duration. These quantifications are therefore not presented herein. Adrenal cortex CB1R mRNA displayed patterns of sensitivity to stress and antagonist treatments similar to those of plasma CORT with increased expression resulting from both acute stress treatment ($F_{(1,39)} = 4.55$, $p < 0.05$), and CB1R antagonist ($F_{(2,39)} = 3.69$, $p < 0.05$), but no stress x drug interaction ($F_{(2,39)} = 0.53$, $p = 0.59$) (Fig. 1G). Post hoc analyses indicated that the significant elevation of CB1R mRNA by antagonist treatment was found only in the 2.0 mg/kg treatment groups compared to both vehicle and 1.0 mg/kg AM251 treatments, and was detected in both non-stressed and acute stress treatment conditions ($p < 0.05$ for all).

3.2. Neural activity in sensory and limbic regions

Stress-reactive and basal sensory and limbic neural activity was also assessed by c-*fos* mRNA expression in several neural regions. Example autoradiograph images from these mRNAs can be found in Fig. 2, and semi-quantitative results are presented in Fig. 3. In general, remote administration of CB1R antagonist AM251 increased c-*fos* mRNA basally and in stressed rats, in dose-dependent and regionally variable

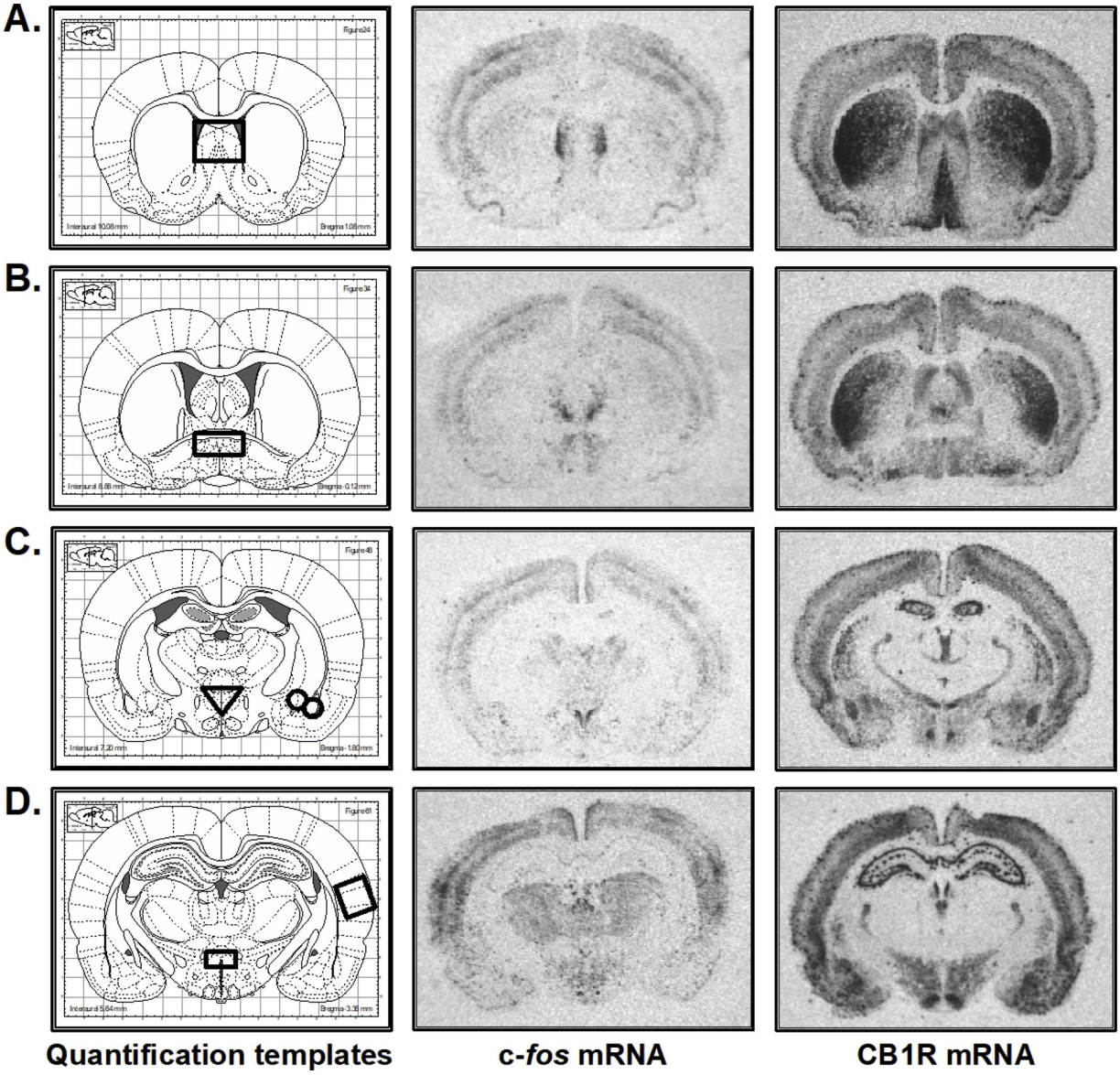

Fig. 2. Quantification templates from the Paxinos and Watson (1998) **Rat Brain Atlas (left column) and representative autoradiograms of c-fos mRNA (middle column) and CB1R mRNA (right column) A.** Lateral septum (LS) region **B.** Anteroventral bed nucleus of the stria terminalis (BSTav) region **C.** Paraventricular nucleus of the hypothalamus (PVN), central amygdala (ACe), basolateral amygdala (BLA) regions **D.** Rostral posterior hypothalamus (rPH) and Auditory cortex (AUD) regions. Representative autoradiograms of c-*fos* mRNA are all from acutely stressed rats. Representative autoradiograms of CB1R mRNA were selected independent of treatment conditions.

patterns.

Analysis of PVN c-*fos* mRNA indicated a significant increase by acute loud noise stress ($F_{(1,39)} = 118.6$, $p < 0.001$) and AM251 treatments ($F_{(2,39)} = 7.75$, $p < 0.01$), but no significant overall stress x drug interaction ($F_{(2,39)} = 1.05$, $p = 0.36$) (Fig. 3A). Fisher's LSD post hoc multiple means comparisons of basal neural activity in the PVN between 2.0 mg/kg AM251 and vehicle treatment failed to reach significance ($p = 0.08$). Post hoc comparisons did confirm that both doses of AM251 resulted in significantly higher neural responses to noise stress (1.0 mg/kg: $p < 0.05$, 2.0 mg/kg: $p < 0.001$). The absence of stimulation of basal PVN activity by 1.0 mg/kg AM251 ($p = 0.61$) most clearly indicates that the increase in stress-induced activity by this dose demonstrates significant potentiation of stress-induced PVN activity.

Two-way ANOVA of BLA c-*fos* mRNA values revealed significant increase by stress ($F_{(1,38)} = 8.20$, $p < 0.01$) and drug ($F_{(2,38)} = 8.80$, $p < 0.001$), but no significant overall stress x drug interaction ($F_{(2,38)} = 1.02$, $p = 0.37$). A similar dose-dependent pattern was significantly detected in the BLA as in the PVN (Fig. 3B). Post hoc analyses detected a significant stimulation of basal neural activity from 2.0 ($p < 0.05$), but not 1.0 mg/kg AM251 ($p = 0.33$). Both doses resulted in significantly higher c-*fos* mRNA induction from loud noise stress compared to vehicle controls (1.0 mg/kg: $p < 0.05$, 2.0 mg/kg: $p < 0.001$). Stress alone did not stimulate c-*fos* mRNA in vehicle-treated rats ($p = 0.62$), but antagonism of CB1R potentiated stress-induced neural activity in this region. This is visible in post hoc measures that indicate significant increase in stress-induced c-*fos* mRNA in 1.0 mg/kg AM251-treated rats compared to vehicle rats, given that this increase is absent in non-stressed conditions.

Auditory cortex c-*fos* mRNA (Fig. 3C) analysis indicated a significant effect of stress ($F_{(1,38)} = 77.6$, $p < 0.001$), but no significant overall effect of drug ($F_{(2,38)} = 2.73$, $p = 0.08$). Post hoc comparisons found 2.0 mg/kg AM251 treatment to significantly increase basal c-*fos* mRNA compared to vehicle-treated controls ($p < 0.05$).

Two-way ANOVA of BSTav c-*fos* mRNA (Fig. 3D) indicated

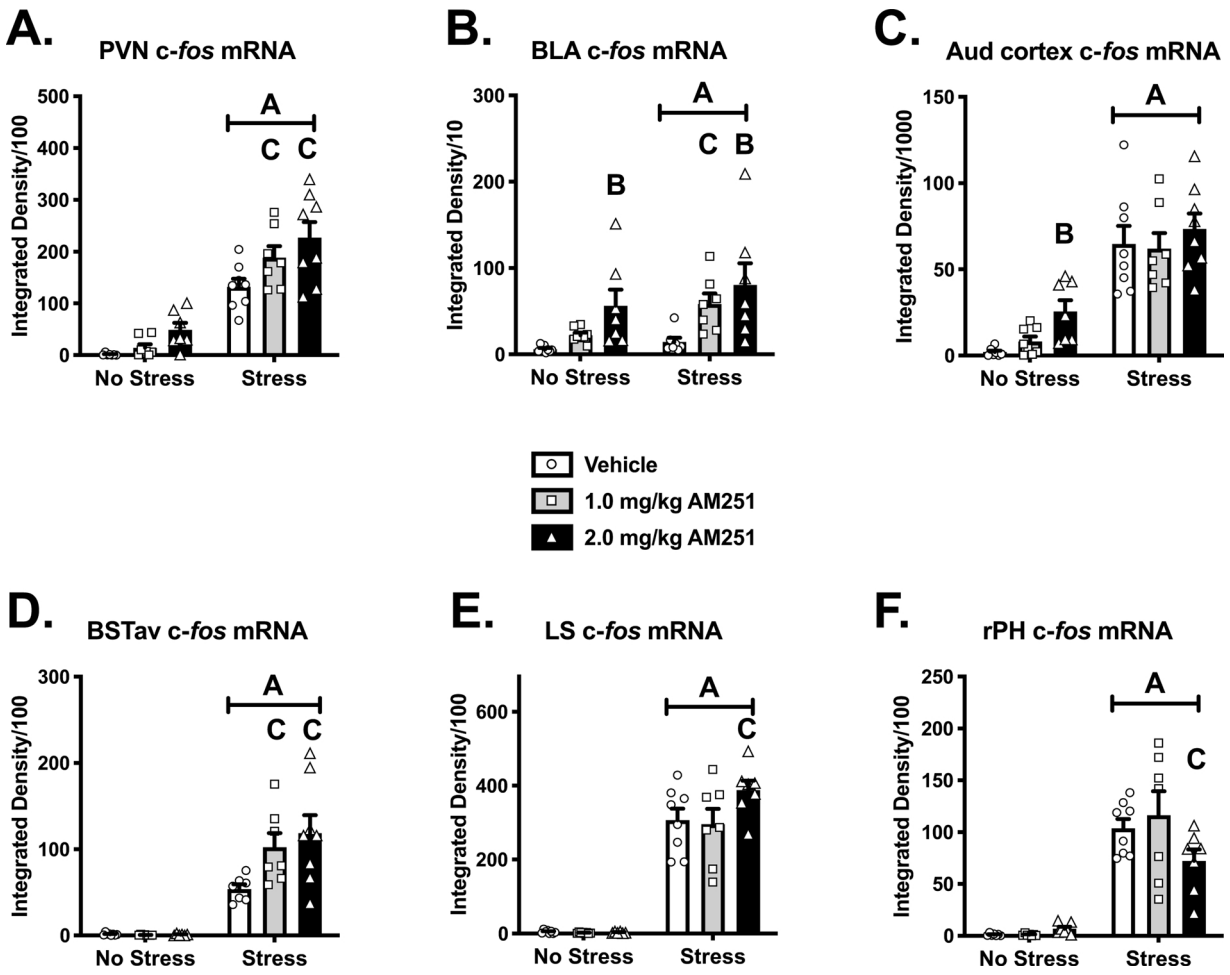

Fig. 3. Neural activity in sensory and limbic regions. Semi-quantitative results of c-*fos* mRNA presented as means (+1 standard error) following stress and/or AM251 treatments for: A. PVN region: Significant effect of stress (A, $p < 0.001$), potentiation of stress response by 1.0 and 2.0 mg/kg AM251 (C, $p < 0.05$). **B.** BLA region: Significant effect of stress (A, $p < 0.01$), significant increase in basal c-*fos* mRNA by 2.0 mg/kg AM251 (B, $p < 0.05$), significant potentiation of stress response by 1.0 mg/kg AM251 (C, $p < 0.05$). **C.** Auditory cortex region: Significant effect of stress (A, $p < 0.001$), significant increase in basal c-*fos* mRNA by 2.0 mg/kg AM251 (B, $p < 0.05$). **D.** BSTav region: Significant effect of stress (A, $p < 0.001$), significant potentiation of stress response by both doses of AM251 (C, 1.0 mg/kg: $p < 0.01$, 2.0 mg/kg: $p < 0.001$). **E.** LS region: Significant effect of stress (A, $p < 0.001$), significant potentiation of stress response (C, $p < 0.05$). **F.** rPH region: Significant effect of stress (A, $p < 0.001$) and significant inhibition of stress response compared to vehicle ($p < 0.05$) and 1.0 mg/kg AM251 ($p < 0.01$). (N = 47).

significant increase by stress ($F_{(1,38)} = 91.5$, $p < 0.001$) and drug ($F_{(2,38)} = 4.09$, $p < 0.05$), and a significant stress x drug interaction ($F_{(2,38)} = 4.28$, $p < 0.05$). Importantly, post hoc testing revealed that neither dose of remotely injected AM251 increased basal, non-stressed activity (1.0 mg/kg: $p = 0.95$, 2.0 mg/kg: $p = 0.97$), but that both doses resulted in potentiation of stress-induced activity, measured as significant increase in stress-induced c-*fos* mRNA compared to vehicle treatment (1.0 mg/kg: $p < 0.01$, 2.0 mg/kg: $p < 0.001$).

A similar lack of stimulation of basal activity after remote CB1R antagonist administration was measured in LS tissue, as well as a potentiation of stress-induced activity in rats treated with the higher dose of AM251 (Fig. 3E). Two way ANOVA revealed significant effects of stress ($F_{(1,38)} = 284.2$, $p < 0.001$) but not drug ($F_{(2,38)} = 2.24$, $p = 0.12$), or significant stress x drug interaction ($F_{(2,38)} = 2.26$, $p = 0.12$). Post hoc analysis indicated a significant increase in stress-induced c-*fos* mRNA induction in 2.0 mg/kg AM251-treated rats compared to vehicle treated controls ($p < 0.05$), but no difference from this drug treatment in non-stressed rats ($p = 0.96$).

Analysis of rPH c-*fos* mRNA (Fig. 3F) found significant increase by stress ($F_{(1,37)} = 109.3$, $p < 0.001$), but this region was not found to be sensitive to AM251 treatment in main effect of drug ($F_{(2,37)} = 1.55$, $p = 0.23$), overall interaction ($F_{(2,37)} = 2.70$, $p = 0.08$). Post hoc analyses indicated 2.0 mg/kg AM251 resulted in significantly lower stress-induced c-*fos* mRNA compared to vehicle ($p < 0.05$) and 1.0 mg/kg AM251 ($p < 0.01$) treatments.

3.3. CB1R mRNA in stress reactive neural regions

CB1R mRNA alterations from acute stress have not been well explored, and may have implications for repeated stress and recovery as well as stress-related damage. Previous reports have largely been limited to HPA axis intrinsic tissues (Surkin et al., 2018). We found bidirectional stress and antagonist-related alterations in CB1R mRNA in multiple stress-reactive neural regions, which were often found to vary by dose (see Figs. 2 and 4).

PVN CB1R mRNA (Fig. 4A) was analyzed by two-way ANOVA, which revealed a significant interaction between stress and drug treatments ($F_{(2,38)} = 3.73$, $p < 0.05$), but no significant main effect of stress ($F_{(1,38)} = 0.01$, $p = 0.93$) or drug treatments ($F_{(2,38)} = 1.47$, $p = 0.25$). Post hoc comparisons indicated that acute loud noise stress significantly increased CB1R mRNA, as compared to the basal vehicle-treated condition ($p < 0.05$). Interestingly, this stress-induced increase is prevented in both AM251 treatment groups. A stimulatory effect of the higher 2.0 mg/kg dose of AM251 on basal CB1R mRNA failed to reach significance ($p = 0.13$).

Analysis of BSTav CB1R mRNA (Fig. 4B) revealed significant main

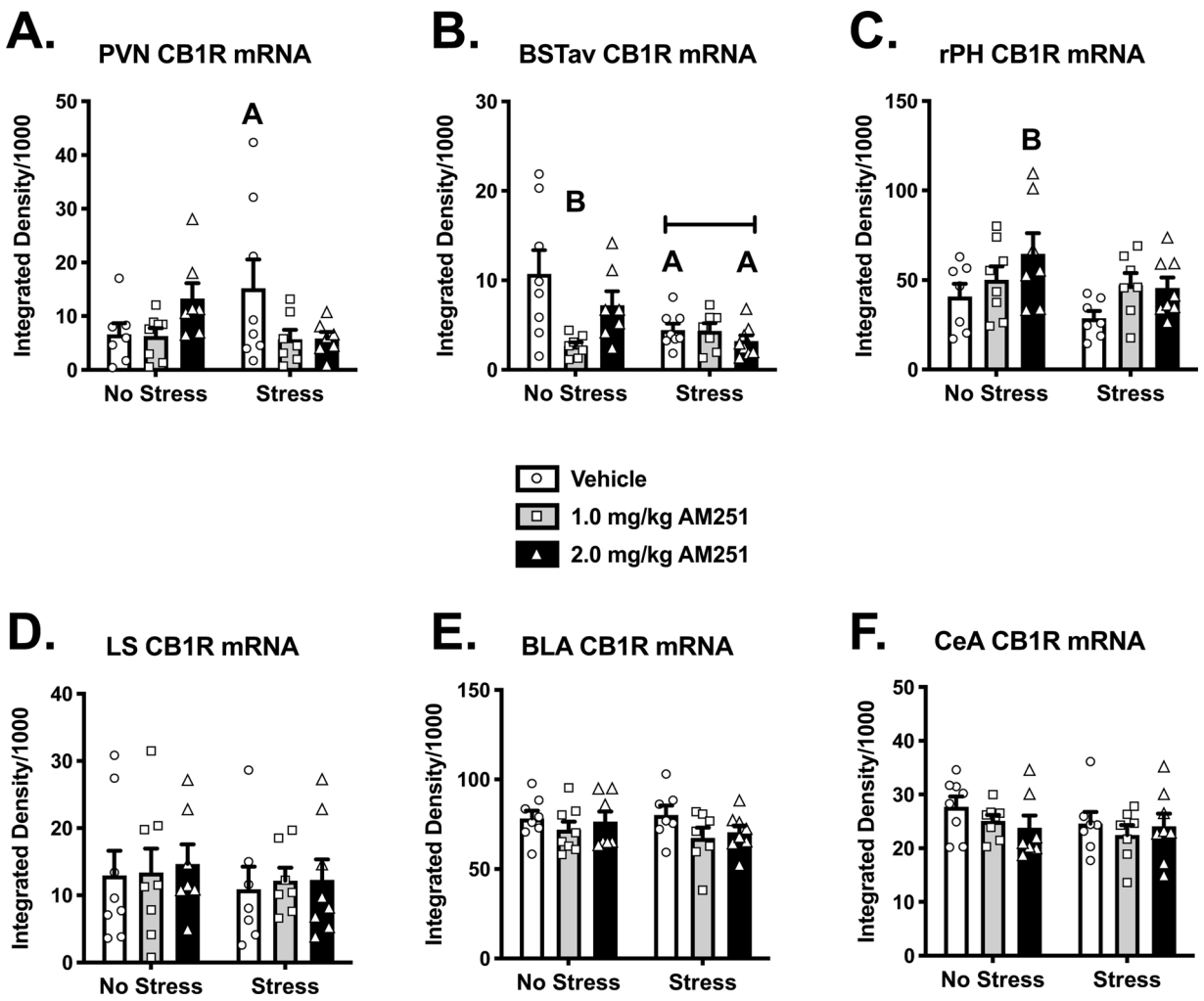

Fig. 4. CB1R mRNA levels in sensory and limbic regions. Semi-quantitative analysis of CB1R mRNA presented as means (+1 standard error) following stress and/or AM251 treatments for: **A.** PVN region: Significant increase by stress in vehicle-treated rats (A, $p < 0.05$), and prevention of stress response by CB1R antagonism. **B.** BSTav region: Significant decrease by stress in vehicle and 2.0 mg/kg AM251-treated rats (A, $p < 0.05$), significant decrease in basal expression by 1.0 mg/kg AM251 (B, $p < 0.05$), and no further change by stress treatment from this dose. **C.** rPH region: Significant increase in basal expression by 2.0 mg/kg AM251 (B, $p < 0.05$). Lack of sensitivity to stress and/or either dose of CB1R antagonist in: **D.** LS, **E.** BLA, and **F.** CeA regions. (N = 47).

effects of stress ($F_{(1,41)} = 6.82$, $p < 0.05$) and drug ($F_{(2,41)} = 4.63$, $p < 0.05$), and a significant stress x drug interaction ($F_{(2,41)} = 4.67$, $p < 0.05$). In vehicle treated rats, stress significantly decreased BSTav CB1R mRNA ($p < 0.01$). This stress-induced decrease was also measured in rats receiving the higher dose (2.0 mg/kg) of AM251 ($p < 0.05$). The lower dose (1.0 mg/kg) of AM251 significantly decreased basal CB1R mRNA compared to vehicle and 2.0 mg/kg AM251 ($p < 0.05$), and this level of expression was not changed by stress treatment ($p = 0.37$).

Rostral PH CB1R mRNA (Fig. 4C) was analyzed with two-way ANOVA, which trended toward a significant effect of stress ($F_{(1,38)} = 3.56$, $p = 0.07$). There was a significant effect of drug ($F_{(2,38)} = 3.91$, $p < 0.05$) but no significant stress x drug interaction ($F_{(2,38)} = 0.61$, $p = 0.55$). In post hoc testing, 2.0 mg/kg AM251 was found to increase basal CB1R mRNA in non-stressed rats ($p < 0.05$).

CB1R mRNA expression in the LS, BLA, and CeA was not significantly altered by stress or drug treatments ($p > 0.05$ for each, Fig. 4D–F). This lack of sensitivity to CB1R antagonism was visible at both treatment doses, and along with the absence of a stress-dependent effect, provides important contrast to the effects detected in the adrenal cortex and especially the unexpected bidirectional alterations concurrently measured in several adjacent neural regions using the same probe and in situ hybridization protocol. CB1R mRNA expression was not analyzed in the AUD due to moderate punctate expression of this mRNA in the cortex (see Fig. 2). This pattern is characteristic for in situ hybridization and autoradiographic expression of CB1R probe (see Vangopoulou et al., 2018) and is predictable in regions of high expression such as the hippocampus (see Fig. 2D) and specific layers of the cortex (see Fig. 2A–D). Analysis of CeA CB1R mRNA was added due to noticeably visible pattern of expression (in contrast to relative absence of c-*fos* mRNA in this region from loud noise stress -Campeau et al., 2002; Day et al., 2005), and as an adjacent region for comparison to the results obtained in the PVN.

4. Discussion

The results of this research are in agreement with the proposed role of the eCB system in affording inhibitory regulation of both basal and stimulated activities at multiple levels of the limbic hypothalamo-pituitary adrenal system. The stimulatory effects of CB1R antagonism indicates the normal inhibitory activities of eCBs binding at CB1R. Both main ligands of this system, N-arachidonylethanolamine (AEA, or "anandamide," Devane et al., 1992) and 2-arachidonylglycerol (2-AG, Sugiura et al., 1995), generally inhibit synaptic activity by interacting with CB1 receptors (Bedse et al., 2017), among others. As in previous reports (Patel et al., 2005; Hill et al., 2010a; Newsom et al., 2012),

CB1R antagonism directly or indirectly and dose-dependently enhanced neural and HPA axis activity both basally and in response to stress. The remote and relatively stress-free administration of AM251 in two doses more definitively established the basal inhibitory effect of CB1R antagonism in various central and peripheral regions as compared to our previous study (Newsom et al., 2012). Basal CB1R antagonist-mediated stimulatory effects were also observed together with stress-potentiating effects in some of the same regions. Finally, we report that this regulation coincided with rapid bidirectional alterations of CB1R mRNA in some of the same regions; acute stress and/or pharmacological antagonism increased or decreased CB1R mRNA in cooperative and/or competitive manners in a region-specific fashion. Whereas this mRNA regulation was likely too slow to effect CB1R function on the concurrent acute measures, it could contribute to the neural and neuroendocrine modifications to later basal, stress- or repeated stress exposure reactivity, as reported previously (Hill et al., 2010a; Newsom et al., 2019). These findings indicate that the eCB system regulates neural and neuroendocrine functions with some tissue specificity. Overall, the current study provides additional support for the view that CB1Rs generally constrain stress-induced reactivity as described in previous studies (Patel et al., 2005; Hill et al., 2010a; Newsom et al., 2012) and more direct evidence that these inhibitory effects generalize to basal activity in many of the same regions. The consistent pattern of system-wide inhibitory regulations by CB1R both centrally and peripherally may indicate the eCB system as an emerging target for systemic therapeutic strategy.

4.1. Peripheral HPA axis and CB1R mRNA measures

The CB1R antagonist-mediated potentiation of acute HPA axis response to loud noise stress was observed in both plasma ACTH and CORT measures. This pattern is similar to those detected in limbic regions and in the PVN, but multiple measures indicate that peripheral eCB effects contribute to basal HPA axis tone and the whole organism response to acute stress.

Adrenal cortex CB1R mRNA were rapidly (within 1 h) increased by high dose AM251 treatment in basal and stress conditions. The absence of anterior pituitary regulation of basal plasma ACTH and c-*fos* mRNA by the tested doses of AM251, along with lower expression of CB1R mRNA in this region compared to the adrenal glands, suggests distinct local eCB environments at different levels of the HPA axis, consistent with prior findings (Surkin et al., 2018). The antagonist-induced basal CORT elevation was measured during the circadian trough of HPA axis activity (Spencer and Deak, 2016), after relatively stress-free administration of AM251, suggesting an adrenal eCB-mediated constitutive inhibition at this circadian time point. This interpretation is notable given the more commonly observed pattern of eCB ligand production occurring “on demand” and resulting from acute cellular stimulation (Cota, 2007). This constitutive inhibition may relate to tonic anandamide activity at CB1R (Ziegler et al., 2010). As in the results of this study, inverse agonist-like effects of AM251 are increasingly reported from specific tissues rather than being uniformly present. Inverse agonist-like effects were recently related to disruption of inhibitory activities of tonic levels of eCB ligands binding at CB1R (amygdala: Hill et al., 2010b; adrenal/medial hypothalamus: Surkin et al., 2018). This interpretation is consistent with the view that it would be theoretically difficult to reverse the intracellular activity of the inhibitory g-protein coupled CB1 receptor (Pertwee, 2003, 2005). The latter mechanism would also likely be more ubiquitously expressed. This question warrants further investigation.

The increases in adrenal cortex CB1R mRNA by AM251 and acute stress are in the same direction as the elevations of basal and stress-induced plasma CORT levels. It is possible that the elevation in CORT serves as a signal in the regulation of CB1R mRNA, but additional studies will be required to address this question. The relatively larger increase in (2.0 mg/kg) antagonist-mediated induction of CB1R mRNA compared to that occurring in response to stress might have resulted from the longer treatment-to-sacrifice interval in the former condition. Potentiation of acute stress-induced plasma CORT was detected with the lower dose of AM251 (1.0 mg/kg) in the absence of increased plasma ACTH, suggesting a significant CB1R-mediated regulation of the HPA axis by stress at the level of the adrenal glands. Further in vivo examination of adrenal eCB regulation will benefit from the use of peripherally restricted CB1R agonists and antagonists.

4.2. Hypothalamic PVN c-fos and CB1R mRNA

The contribution of eCB activity at the level of the PVN has been examined basally and in response to stress (Evanson et al., 2010; Evanson and Herman, 2015; Hill and Tasker, 2012; Wamsteeker et al., 2010), but results have been inconclusive. In the current study, stress-induced PVN c-*fos* mRNA expression was potentiated in rats administered 1.0 mg/kg AM251. This effect is consistent with potentiated levels of stress-induced plasma CORT, and c-*fos* mRNA increases in several limbic regions directly or indirectly influencing HPA axis activity. Congruence with the latter makes this local measure of activity difficult to attribute to PVN-specific eCB-mediated regulation of stress reactivity. Localized PVN CB1R activity has been reported to primarily contribute to rapid glucocorticoid negative feedback (Evanson et al., 2010), which can constrain both the magnitude and duration of acute HPA axis stimulation (Osterlund et al., 2016). Expression of CB1R mRNA in the PVN was visibly pronounced and sensitive to both acute stress and CB1R antagonism, but distinct to that observed in the adrenal cortex. The stress-induced increase in PVN CB1R mRNA is consistent with findings of Surkin et al. (2018) and interestingly, was entirely prevented by both doses of AM251. This perhaps suggests that under normal healthy conditions, CB1R regulation induced by stress could contribute to later alterations in PVN neuron excitability and contribute to mechanisms of negative corticosteroid feedback in later, or repeated, stress conditions (McEwen, 1998). Future research should examine CB1R protein sensitivity to acute stress and its time course to directly examine functional consequences of mRNA regulation.

4.3. Limbic and cortical c-fos and CB1R mRNA

As in the PVN, the limbic and sensory region-wide increases in c-*fos* mRNA expression induced by CB1R antagonism indicated that the net effect of peripherally-administered CB1R antagonist is inhibitory in these regions. This is consistent with the general hypothesis that CB1R expressed on axon terminals normally reduce neural activation through retrograde negative feedback function of post-synaptic eCB ligands (Sullivan, 2002); thus, CB1R antagonist-mediated increases in c-*fos* mRNA induction could result from prolonged or enhanced neurotransmitter release from upstream or interconnected neural structures. Modulation of central activity by peripheral sensation cannot be excluded. More recently, CB1R localization on intracellular neuronal mitochondria has also been linked to regulation of neurotransmission (Djeungoue-Petga and Hebert-Chatelain, 2017), and may further contribute to general inhibitory modulation of cellular activities, such as those responsible for the induction of immediate-early genes.

The auditory cortex is stimulated by acute loud noise stress (Campeau et al., 2002), but lesion of this region does not interfere with HPA axis response to this stressor (Masini et al., 2012). Injection of 2.0 mg/kg AM251 increased basal but not acute noise stress-induced AUD c-*fos* mRNA. The patterns of c-*fos* mRNA measured in this region in the current study mirror those reported previously (Newsom et al., 2012), more clearly indicating that our initial findings were not mediated by the stress of the peripheral injections, supporting the interpretation that the eCB system affords constitutive inhibition of neural activity in this region.

The basolateral amygdala has been the focus of much research on eCB involvement in HPA axis regulation (Bedse et al., 2014; Gray et al.,

2015; Hill et al., 2009, 2010b; Ramikie and Patel, 2012). We previously reported systemic injection of 2.0 mg/kg AM251 to stimulate c-*fos* mRNA induction under basal conditions, but that this dose of the CB1R antagonist did not further potentiate acute response to loud noise stress (Newsom et al., 2012). The use of remote administration of AM251 in the current study strongly supports the interpretation that the eCB system contributes to constitutive inhibition of neural activity in this region, as suggested by others (Hill et al., 2009).

Acute loud noise stress induces c-*fos* mRNA in the BSTav and LS regions, which is positively correlated with both stressor intensity and acute neuroendocrine reactivity (Burow et al., 2005), and these limbic structures contribute to acute stress reactivity in general (Choi et al., 2008; Puente et al., 2011; Reis et al., 2011; Singewald et al;, 2011). In the current study both doses of AM251 potentiated acute loud noise stress-induced c-*fos* mRNA in the BSTav, as did 2.0 mg/kg in the LS. CB1R signaling in these regions may not only contribute to acute neuroendocrine reactivity to stress, but also regulation of behavioral (Crestani et al., 2013; Singewald et al., 2011), and sympathetic reactivity (Gomes-de-Souza et al., 2016), and may be involved in stress-related plasticity (Glangetas et al., 2013; Newsom et al., 2019). Interestingly, in both regions, neither doses of AM251 stimulated basal c-*fos* mRNA, compared to the basal stimulation observed in AUD and BLA. This pattern provides a distinct contrast to antagonist-evoked stimulation of the BLA, indicating some regional specificity of eCB signaling. The absence of basal LS and BSTav c-*fos* mRNA induction combined with drug-induced c-*fos* mRNA elevations in the BLA suggests that disruption of constitutive inhibitory tone does not produce a general stress response similar to that characteristic of stress exposure (Burow et al., 2005; Campeau et al., 2002; Newsom et al., 2012). In the BSTav, CB1R mRNA was reduced by acute stress and low dose AM251. This stress-induced reduction is in contrast to the elevations by acute loud noise stress measured in the adrenal cortex and PVN, as well as the stability of this mRNA expression in the LS, BLA, and CeA. The inhibition of basal BSTav CB1R mRNA by 1.0 mg/kg AM251 may or may not share a similar mechanism to the drug-induced reduction of CB1R mRNA induced by stress in the PVN.

The rPH contributes to regulation of neuroendocrine and autonomic response habituation to repeated psychological stress (Nyhuis et al., 2016), perhaps involving modulations of CB1R expression or sensitivity (Newsom et al., 2019). Surprisingly, however, the rPH only demonstrated a basal increase in CB1R mRNA at the higher dose AM251, but not with stress. The decrease in stress-induced c-*fos* mRNA expression by high dose CB1R antagonism contrasted to the general pattern of stimulation of basal and stress-related measures, and notably, did not interfere with it. Another region in which we have previously found this pattern is the medial geniculate (auditory) thalamus (Newsom et al., 2012). The inhibition of stress-induced rPH activity by CB1R antagonism in the current study may relate to noise stress-involved communication between the auditory thalamus and rPH (Day et al., 2009), and may have implications for modulation of reactivity to repeated stress.

4.4. Conclusions

Taken together, these results provide novel information and perspective that contribute to the understanding of a role of the eCB system in overall HPA axis regulation, which is largely homologous in inhibitory contributions to limbic and neuroendocrine basal and stress-stimulated activities. The exact location and mechanisms of eCB modulation in individual tissues and regions will require continued and targeted exploration but will need to be understood in the context of their contributions to whole organism patterns of stress reactivity. This perspective may be appropriate for further understanding the ways in which the eCB modulatory system can be best approached for systemic therapeutic strategy.

Declaration of Competing Interest

The authors report no conflicts of interest.

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