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Suppression of Amygdalar Endocannabinoid Signaling by Stress Contributes to Activation of the Hypothalamic–Pituitary–Adrenal Axis

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Endocannabinoids inhibit hypothalamic–pituitary–adrenal (HPA) axis activity; however, the neural substrates and pathways subserving this effect are not well characterized. The amygdala is a forebrain structure that provides excitatory drive to the HPA axis under conditions of stress. The aim of this study was to determine the contribution of endocannabinoid signaling within distinct amygdalar nuclei to activation of the HPA axis in response to psychological stress. Exposure of rats to 30-min restraint stress increased the hydrolytic activity of fatty acid amide hydrolase (FAAH) and concurrently decreased content of the endocannabinoid/CB_1 receptor ligand *N*-arachidonylethanolamine (anandamide; AEA) throughout the amygdala. In stressed rats, AEA content in the amygdala was inversely correlated with serum corticosterone concentrations. Pharmacological inhibition of FAAH activity within the basolateral amygdala complex (BLA) attenuated stress-induced corticosterone secretion; this effect was blocked by co-administration of the CB_1 receptor antagonist AM251, suggesting that stress-induced decreases in CB_1 receptor activation by AEA contribute to activation of the neuroendocrine stress response. Local administration into the BLA of a CB_1 receptor agonist significantly reduced stress-induced corticosterone secretion, whereas administration of a CB_1 receptor antagonist increased corticosterone secretion. Taken together, these findings suggest that the degree to which stressful stimuli reduce amygdalar AEA/CB_1 receptor signaling contributes to the magnitude of the HPA response.
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INTRODUCTION

The hypothalamic–pituitary–adrenal (HPA) axis governs the neuroendocrine adrenocortical response to aversive stimuli. Corticotropin releasing hormone (CRH) neurosecretory cells within the paraventricular nucleus of the hypothalamus (PVN) integrate input from other regions of the nervous system and activation of these cells is the initiating step of the adrenocortical response to stress. The endpoint of HPA axis activation is the release of glucocorticoid hormones, such as corticosterone, from the adrenal cortex into the general circulation. Among other effects, glucocorticoids promote glucose mobilization and redirect energy stores necessary for rapid, adaptive responses to stress (Pecoraro *et al*, 2006). Although the HPA axis is ubiquitously activated in response to aversive stimuli, the up-stream neural circuits mediating this activation depend on the nature of the stressor. 'Physiological' stressors, which evoke disturbances in internal homeostasis, activate the HPA axis through a bottom–up circuit in which brainstem nuclei recruit the HPA axis directly, whereas 'psychological' stressors elicit a neuroendocrine response through top–down processes (Pecoraro *et al*, 2006; Sawchenko *et al*, 2000; Herman *et al*, 2003). In this circuit, psychological stressors activate corticothalamic sensory systems, which convey information to cortical and limbic integrative areas where information is processed and aversive salience is determined. These integrative limbic areas, particularly the amygdala, then regulate the HPA axis through trans-synaptic relays, including those within the bed nucleus of the stria terminalis and local hypothalamic nuclei (Herman *et al*, 2005).

Recent data indicate that the endocannabinoid system negatively regulates the neuroendocrine response to

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psychological stress (see Gorzalka *et al*, 2008; Steiner and Wotjak, 2008). The endocannabinoid system is composed of two neuroactive signaling lipids, *N*-arachidonylethanolamine (anandamide; AEA) and 2-arachidonoylglycerol (2-AG), which bind to the cannabinoid type 1 (CB_1) receptor localized to axonal processes (Freund *et al*, 2003). Both endocannabinoid ligands and the CB_1 receptor are expressed within the amygdala (Herkenham *et al*, 1991; Bisogno *et al*, 1999), and endocannabinoid signaling within the amygdala is known to modulate both excitatory and inhibitory neurotransmission (Katona *et al*, 2001; Azad *et al*, 2003, 2004; Zhu and Lovinger, 2005; Domenici *et al*, 2006). Additionally, tissue content of AEA and 2-AG within the amygdala is modulated in response to psychological stressors (Patel *et al*, 2005c; Hill *et al*, 2008; Rademacher *et al*, 2008).

At a systems level, genetic or pharmacological disruption of endocannabinoid signaling increases CRH transcription within the PVN, enhances both basal and stress-induced corticosterone secretion and impairs glucocorticoid-mediated negative feedback regulation of the HPA axis (Patel *et al*, 2004; Cota *et al*, 2007; Steiner *et al*, 2008a). On the other hand, pharmacological augmentation of endocannabinoid neurotransmission attenuates the neuroendocrine response to psychological stressors (Patel *et al*, 2004). The neural circuitry subserving the inhibitory effects of endocannabinoids on the HPA axis is not well characterized; in particular, the influence of endocannabinoid signaling within limbic structures on activation of the HPA axis by psychological stress is not known. In this regard, the aim of this study was to use biochemical analyses of endocannabinoid signaling and local microinjections of cannabinoid ligands to determine the contributions of stress-induced modulation of endocannabinoid signaling within discrete nuclei of the amygdala to the activation of the HPA axis. Our findings reveal that endocannabinoid/CB_1 receptor signaling in the amygdala is disrupted by acute exposure to psychological stress, which in turn contributes to stress-induced activation of the HPA axis.

METHODS

Subjects

Seventy-day-old male Sprague–Dawley rats (300 g; University of British Columbia Breeding Center) were used in this study. The rats were pair housed (except after surgical procedures, when they were individually housed) in standard maternity bins lined with contact bedding. Colony rooms were maintained at 21°C, and on a 12 h light/dark cycle, with lights on at 0900 h. All rats were given ad libitum access to Purina Rat Chow and tap water. All protocols were approved by the Canadian Council for Animal Care and the Animal Care Committee of the University of British Columbia. All studies occurred during the first third of the light cycle, during the daily nadir of HPA axis activity.

Biochemical Studies

For biochemical studies, animals were randomly assigned to either basal or stress conditions. For stress conditions, rats were put into a polystyrene tube (diameter 6 cm, length 20 cm) with breathing holes. Tubes were long enough to completely encase the rat and too narrow for turning or other large movements. Rats were left in the tubes for 30 min, then removed and immediately decapitated. Basal animals remained in their home cage until they were decapitated. The amygdala was dissected as described earlier (Hill *et al*, 2006), frozen in liquid nitrogen within 5 min of decapitation and stored at −80°C until analysis. Trunk blood was collected at the time of decapitation for the analysis of serum corticosterone. One cohort of animals ($n=10$) was used to determine brain regional endocannabinoid contents and serum corticosterone levels. Membrane fractions were isolated from brain regions of another cohort of animals ($n=4$) and were used for CB_1 receptor radioligand binding, CB_1 receptor-mediated GTPγS binding and fatty acid amide hydrolase (FAAH) activity.

Membrane Preparation

Brain sections were homogenized in 10 volumes of TME buffer (50 mM Tris-HCl, pH 7.4; 1 mM EDTA and 3 mM $MgCl_2$). Homogenates were centrifuged at 18 000 *g* for 20 min and the resulting pellet, which constituted the membrane fraction, was resuspended in 10 volumes of TME buffer. Protein concentrations were determined by the Bradford method (Bio-Rad, Hercules, CA, USA).

CB_1 Receptor Radioligand Binding Assay

CB_1 receptor radioligand binding was performed using a Multiscreen Filtration System with Durapore 1.2-μM filters (Millipore, Bedford, MA) as described earlier (Hillard *et al*, 1995a). Incubations (total volume = 0.2 ml) were carried out using TME buffer containing 1 mg/ml bovine serum albumin (TME/BSA). Membranes (10 μg protein per incubate) were added to the wells containing 0.25, 0.5, 1.0, or 2.5 nM [^{3}H]CP 55 940, a cannabinoid CB_1 receptor agonist. Ten μM Δ^9-tetrahydrocannabinol was used to determine nonspecific binding. K_d and B_{max} values were determined by nonlinear curve fitting to the single site binding equation using GraphPad Prism (San Diego, CA, USA).

CB_1 Receptor-Mediated GTPγS Binding Assay

The assay for [^{35}S]GTPγS binding was performed as described earlier by Kearn *et al* (1999). Membranes (final concentration, 5 μg of protein per incubation mixture) were added to TME buffer containing 0.1% fatty acid-free bovine serum albumin, 10 μmol/l GDP, and 150 mmol/l NaCl. [^{35}S]GTPγS (final concentration, 0.65 nmol/l) was added, and the incubation was continued for 30 min at 37°C using the Multiscreen Filtration System with Durapore filters (pore size, 1.2 μm; Millipore, Bedford, MA, USA). Nonspecific binding was determined in the presence of 10 μmol/l Gpp(NH)p and accounted for $<15\%$ of the total binding. Bound [^{35}S]GTPγS was separated from free [^{35}S]GTPγS by filtration followed by washing the filters four times with cold TME buffer containing NaCl and GDP. The cannabinoid CB_1 receptor agonist WIN 55 212 was added in 1 μl of dimethyl sulfoxide at concentrations of 0, 0.1, 0.3, 0.6, 1, 2, 3, 6, 10, 20, and 30 μmol/l. In each experiment, the agonist-dependent [^{35}S]GTPγS binding was calculated as a percentage of agonist-independent binding. The EC_{50} values and maximal agonist-induced increase in binding (E_{max}) of [^{35}S]GTPγS were determined by fitting the data to a sigmoidal concentration–response curve using nonlinear regression (Prism; GraphPad, San Diego, CA, USA).

FAAH Activity Assay

FAAH activity was measured as the conversion of AEA labeled with [^{3}H] in the ethanolamine portion of the molecule ([^{3}H]AEA; Omeir *et al*, 1995) to [^{3}H]ethanolamine preparations as reported earlier (Hillard *et al*, 1995b). Membranes were incubated in a final volume of 0.5 ml of TME buffer (50 mM Tris-HCl, 3.0 mM $MgCl_2$, and 1.0 mM EDTA, pH 7.4) containing 1.0 mg/ml fatty acid-free bovine serum albumin and 0.2 nM [^{3}H]AEA. Isotherms were constructed using eight concentrations of AEA at concentrations between 10 nM and 10 μM. Incubations were carried out at 37°C and were stopped with the addition of 2 ml of chloroform/methanol (1 : 2). After standing at ambient temperature for 30 min, 0.67 ml of chloroform and 0.6 ml of water were added. Aqueous and organic phases were separated by centrifugation at 1000 rpm for 10 min. The amount of [^{3}H] in 1 ml each of the aqueous and organic phases was determined by liquid scintillation counting and the conversion of [^{3}H]AEA to [^{3}H]ethanolamine was calculated. The K_I and V_{max} values for this conversion were determined by fitting the data to a single site competition equation using Prism.

Endocannabinoid Extraction and Analysis

Brain regions were subjected to a lipid extraction process as described earlier (Patel *et al*, 2003). Tissue samples were weighed and placed into borosilicate glass culture tubes containing 2 ml of acetonitrile with 84 pmol of [$^{2}H_8$]anandamide and 186 pmol of [$^{2}H_8$]2-AG. Tissue was homogenized with a glass rod and sonicated for 30 min. Samples were incubated overnight at −20°C to precipitate proteins, then centrifuged at 1500 *g* to remove particulates. The supernatants were removed to a new glass tube and evaporated to dryness under N_2 gas. The samples were resuspended in 300 μl of methanol to recapture any lipids adhering to the glass tube, and dried again under N_2 gas. Final lipid extracts were suspended in 20 μl of methanol, and stored at −80°C until analysis. The contents of the two primary endocannabinoids AEA and 2-AG within lipid extracts in methanol from brain tissue were determined using isotope-dilution, liquid chromatography–mass spectrometry as described earlier (Patel *et al*, 2005a).

Radioimmunoassay of Serum Corticosterone

After collection, blood was allowed to settle for 1 h before centrifugation. Samples were centrifuged at 3000 *g* for 20 min after which serum was removed and stored at −80°C until analysis. Serum corticosterone (5 μl) was measured using commercial RIA kits (MP Biomedicals, Costa Mesa, CA), as described earlier (Bingham and Viau, 2008). Briefly, for corticosterone analysis, the serum samples were diluted 1 : 100 and 1 : 200 for basal and stress conditions, respectively, to render hormone detection within the linear part of the standard curve. [^{125}I]-labeled corticosterone was used as trace; the corticosterone antibody cross-reacts slightly with desoxycorticosterone (0.34%) and testosterone and cortisol (0.10%).

Microinjection Studies

For microinjection studies, animals were subjected to stereotaxic surgery. Rats were anesthetized with 100 mg/kg of ketamine hydrochloride and 7 mg/kg xylazine, and implanted with bilateral 23-gauge stainless-steel guide cannulae. Separate cohorts of animals were generated with implantations of cannulae into the basolateral amygdala complex (BLA; flat skull anterior/posterior (AP) = −3.1 mm from bregma, medial/lateral (ML) = ± 5.0 mm from midline, dorsal/ventral (DV) = −6.1 mm from dura), the medial amygdala (MeA; AP = −2.6 mm; ML = ± 3.4 mm; DV = −7.4 mm) or the central nucleus of the amygdala (CeA; AP = −2.2 mm; ML = ± 4.1 mm; DV = −6.5 mm; Paxinos and Watson (1998)). Four steel screws and dental acrylic were used to permanently affix the guide cannulae to the skull. Stainless-steel stylets (30-gauge) were inserted into the guide cannulae until the time of infusion. Immediately after surgery, antibiotic ointment was applied to the skull and surrounding incision. All rats were individually housed during recovery and were given 1 week of recovery before testing.

Animals received bilateral infusions of either the CB_1 receptor agonist HU-210 (2.5 μg), the CB_1 receptor antagonist AM251 (2.5 μg), the FAAH inhibitor URB597 (0.1 or 1.0 μg) or vehicle (DMSO). For studies determining the CB_1 receptor dependency of HU-210 and URB597, mixtures of HU-210 and AM251 or URB597 and AM251 were infused using the same doses as above. These doses were chosen based on earlier data showing efficacy and selectivity for the target (McLaughlin *et al*, 2007; Rubino *et al*, 2008; Lin *et al*, 2006). A 30-gauge injection cannula extending 0.8 mm below the tips of the guide cannulae was used for infusions. Drug solutions or vehicle were delivered at a rate of 0.5 μl/72 s using a microsyringe pump (Sage Instruments Model 341). Injection cannulae were left in place for an additional 1 min to allow for diffusion. After infusions, animals were returned to their home cages. For stress induction, animals in studies using HU-210 or AM251 were left in their cages for 10 min before being put into restrainers, whereas animals for studies using URB597 were left in their cages for 20 min (to allow time for enzyme inhibition to occur) before being put into restrainers. At the conclusion of the 30-min restraint stress session, a small nick was made at the tip of the tail from which 300 μl of blood was collected for corticosterone analysis. Blood from a tail nick was also collected from the nonstressed rats at 40 or 50 min after bilateral infusions, depending on the drug infused. All rats were killed in a carbon dioxide chamber 24 h after testing. Brains were removed and fixed in a 4% formalin solution. The brains were frozen and sliced in 50 μm sections and mounted. Placements were verified with reference to the atlas of Paxinos and Watson (1998)

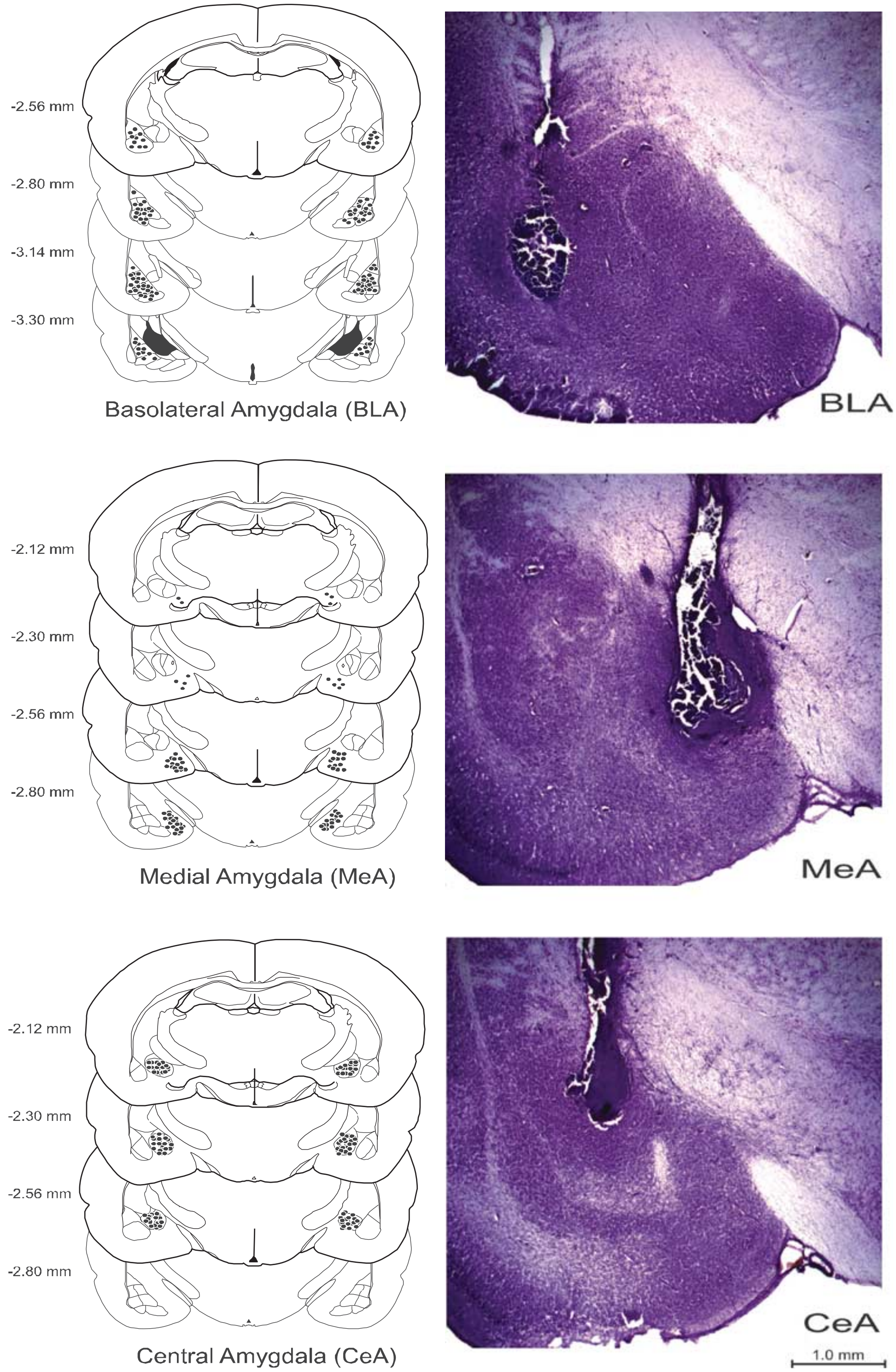

Figure 1 Schematic of coronal sections of the rat brain showing the placements of the tips of the cannulae for all rats that received infusions of HU-210, AM251 or URB597 into the basolateral nucleus of the amygdala, the central nucleus of the amygdala and the medial amygdala. Representative histological pictures of infusions into the amygdala nuclei are adjacent to placement diagrams.

and histological analysis showed that approximately 85% of cannula placements were in boundaries of the nuclei of interest (see Figure 1). Subjects with cannulae outside of the desired structure were excluded from subsequent analysis.

Statistics

Analyses of the effects of restraint stress on the tissue contents of AEA and 2-AG, CB_1 receptor binding parameters and CB_1 receptor-mediated GTPγS binding were performed using independent *t*-tests. Examination of the effects of URB597 on stress-induced corticosterone secretion was performed using a one-way analysis of variance (ANOVA), whereas the effects of HU-210 and AM251 on basal and stress-induced corticosterone secretion were analyzed using a univariate ANOVA with drug (HU-210 or AM251) and stress as fixed factors. For all neuroendocrine studies, *post hoc* analysis was performed using a Tukey's test. All analyses used $P<0.05$ as an indication of significance.

RESULTS

Psychological Stress Dampens Endocannabinoid Signaling Within the Amygdala

Restraint stress resulted in a significant reduction in tissue content of AEA within the amygdala [$t(18)=2.35$, $P<0.03$; Figure 2], whereas there was no effect of stress on amygdalar 2-AG content [$t(18)=0.85$, $P>0.05$; Figure 2]. The reduction in amygdalar AEA content after stress is likely attributable to an enhancement of hydrolysis as stress robustly increased the V_{max} of FAAH for AEA hydrolysis [$t(6)=2.59$, $P<0.05$; Table 1]. Stress did not affect the K_m of FAAH for AEA [$t(6)=1.58$, $P>0.05$; Table 1].

Exposure of rodents to 30 min of restraint stress did not significantly affect the maximal binding site density [B_{max}; $t(6)=1.10$, $P>0.05$; Table 2] or the binding affinity of [^{3}H]CP55940 for the CB_1 receptor [K_d; $t(6)=1.27$, $P>0.05$; Table 2]. Similarly, there was no effect of restraint stress on the maximal stimulation of CB_1 receptor-mediated GTPγS binding [$t(6)=0.53$, $P>0.05$; Table 2], but there was a trend for stress to increase the EC50 of WIN 55212-2, suggestive of reduced agonist sensitivity of the CB_1 receptor to induce GTP exchange [$t(6)=1.94$, $P=0.10$; Table 2].

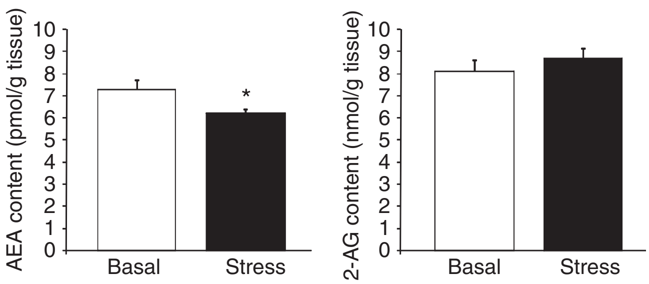

Figure 2 Acute psychological stress modulates endocannabinoid content in the amygdala. The effect of 30-min restraint stress on the tissue content of the endocannabinoid ligands *N*-arachidonylethanolamide (anandamide; AEA) and 2-arachidonoylglycerol (2-AG) within the amygdala. Values are denoted as means ± SEM. *Significant differences ($P<0.05$) between animals under basal and stress conditions.

Table 1 Acute Stress Rapidly Induces Activation of Fatty Acid Amide Hydrolase Activity Within the Amygdala

	Basal	Stress
Amygdala		
V_{max} (pmol/mg protein)	1212.1 ± 145.3	3395.0 ± 829.2*
K_m (nM)	0.47 ± 0.15	0.89 ± 0.22

Exposure to 30-min restraint stress resulted in a significant enhancement of the maximal hydrolytic activity (V_{max}), while not significantly altering the binding affinity (K_m) of fatty acid amide hydrolase for anandamide within the amygdala. For both treatment conditions, $n=4$. Data are presented as means ± SEM. *Significant differences ($P<0.05$) between basal and stress conditions.

Suppression of AEA/CB_1 Receptor Signaling Within the BLA Contributes to Stress-Induced Activation of the HPA Axis

As anticipated, restraint stress increased serum corticosterone levels [$t(18)=20.7$, $P<0.001$; Figure 3]. To determine whether the changes in amygdalar endocannabinoid content after stress were related to the magnitude of the HPA axis response, we examined the correlation between serum corticosterone concentrations and both AEA and 2-AG, under stress conditions. Although amygdalar 2-AG tissue content did not exhibit a significant correlation with serum corticosterone ($r=-0.18$, $P>0.05$; Figure 3), amygdalar AEA content was significantly and negatively correlated with corticosterone levels ($r=-0.72$, $P<0.02$; Figure 3), indicating that higher levels of serum corticosterone after stress were associated with lower levels of AEA content within the amygdala.

To further explore the relationship between amygdalar FAAH activity, AEA content and serum corticosterone, we determined the effect of local inhibition of FAAH activity within the BLA, CeA, and MeA on stress-induced corticosterone secretion. Administration of the FAAH inhibitor URB597 blocks AEA hydrolysis by FAAH and increases AEA content (Kathuria *et al*, 2003), providing an ideal tool to examine this relationship. Within the BLA there was a significant effect of infusion of the FAAH inhibitor, URB597, on stress-induced corticosterone secretion [$F(4,26)=65.7$, $P<0.001$; Figure 4], with *post hoc* analysis revealing that administration of 0.1 μg URB597 into the BLA significantly reduced stress-induced increases in corticosterone secretion ($P<0.05$). The fact that 1 μg of URB597 into the BLA did not mimic the effects seen with the lower dose is consistent with earlier work showing that URB597 is optimally effective at a dose of 0.1 μg, presumably due to the fact that greater increases in AEA are believed to lose selectivity for the CB_1 receptor and begin to saturate TRPV1

Table 2 The Effects of Acute Psychological Stress on CB_1 Receptor Binding and Signaling

	Basal	Stress
Amygdala		
B_{max} (pmol/mg protein)	0.11 ± 0.01	0.17 ± 0.05
K_d (nM)	0.33 ± 0.04	1.09 ± 0.60
E_{max} (% baseline)	188.0 ± 6.3	181.7 ± 10.1
EC50 (nM)	81.0 ± 8.5	127.6 ± 22.5

There was no effect of 30-min restraint stress on the maximal binding (B_{max}) or the binding affinity (K_d) of the CB_1 receptor, nor the maximal stimulation (E_{max}) or the EC50 of CB_1 receptor-mediated ^{35}S-GTPγS binding within the amygdala. Values are denoted as means ± SEM. For all treatment conditions, $n=4$.

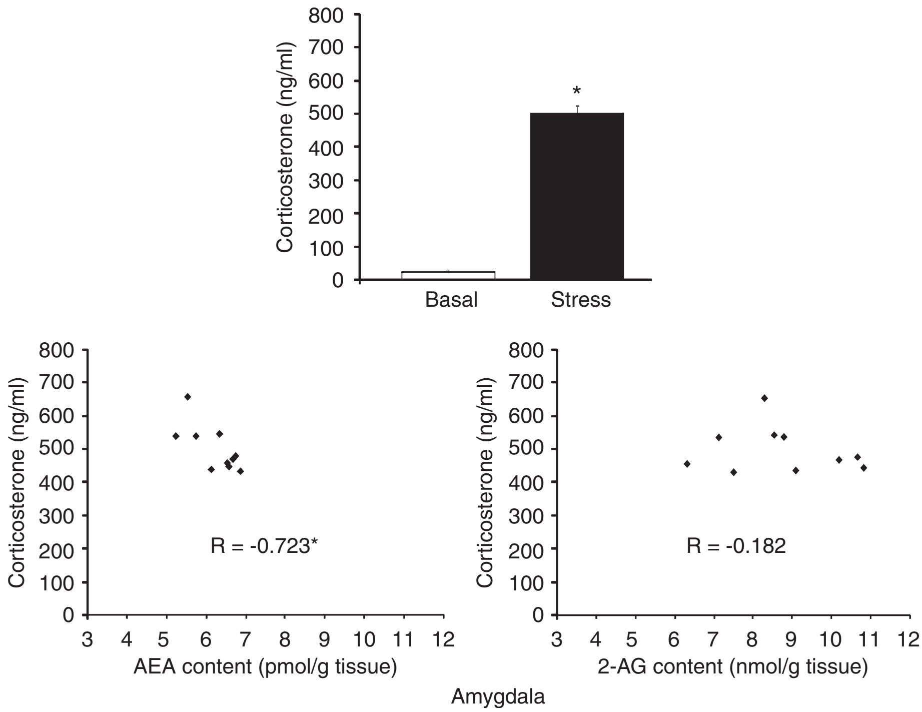

Figure 3 Acute stress-induced increases in corticosterone secretion: correlations with endocannabinoid content within the amygdala. The 30-min restraint stress resulted in a significant increase in circulating corticosterone. Values are denoted as means ± SEM. *Significant differences ($P<0.05$) in corticosterone levels between basal and stress conditions. Under stress conditions, the magnitude of corticosterone secretion correlated significantly and negatively with anandamide (AEA) content in the amygdala. There was no significant correlation between corticosterone and 2-AG content in the amygdala. *Significant correlation ($P<0.05$).

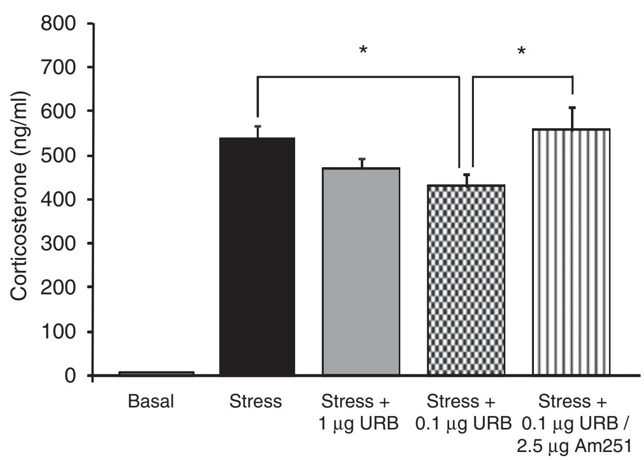

Figure 4 Inhibition of fatty acid amide hydrolase within the basolateral amygdala reduces stress-induced corticosterone secretion. Infusion of URB597 (0.1 and 1 μg), a pharmacological inhibitor of FAAH, into the basolateral amygdala significantly reduced stress-induced increases in corticosterone secretion. Values are denoted as means ± SEM. *Significant differences ($P<0.05$).

vanilloid receptors (Rubino *et al*, 2008). Animals that received an infusion of the CB_1 receptor antagonist AM251 in conjunction with URB597 exhibited no significant difference in stress-induced corticosterone secretion relative to vehicle-infused animals ($P>0.05$), supporting the hypothesis that URB597 administration into the BLA, at this dose, reduced HPA axis activation through increased activation of the CB_1 receptor by AEA. URB597 infusions into the CeA and MeA were without effect on stress-induced serum corticosterone levels (Table 3).

Table 3 The Effect of Infusion of the FAAH Inhibitor URB597 into the Central Nucleus and the Medial Nucleus of the Amygdala on Stress-Induced Corticosterone Secretion

	Serum corticosterone (ng/ml)
CeA	
Basal	43.1 ± 10.1
Stress	401.1 ± 85.5
Stress + 0.1 μg URB597	385.8 ± 34.8
Stress + 1 μg URB597	410.1 ± 52.7
MeA	
Basal	49.3 ± 6.1
Stress	443.5 ± 18.4
Stress + 0.1 μg URB597	427.1 ± 33.6
Stress + 1 μg URB597	446.8 ± 30.3

Infusion of the FAAH inhibitor URB597 (0.1 and 1 μg) into the central nucleus of the amygdala (CeA) and the medial nucleus of the amygdala (MeA) before 30-min restraint stress had no effect on the increase in corticosterone secretion after stress. Values are denoted as means ± SEM. For all treatment conditions, $n=4–5$.

These data suggest that decreased tonic CB_1 receptor activity within the BLA promotes HPA axis activation by

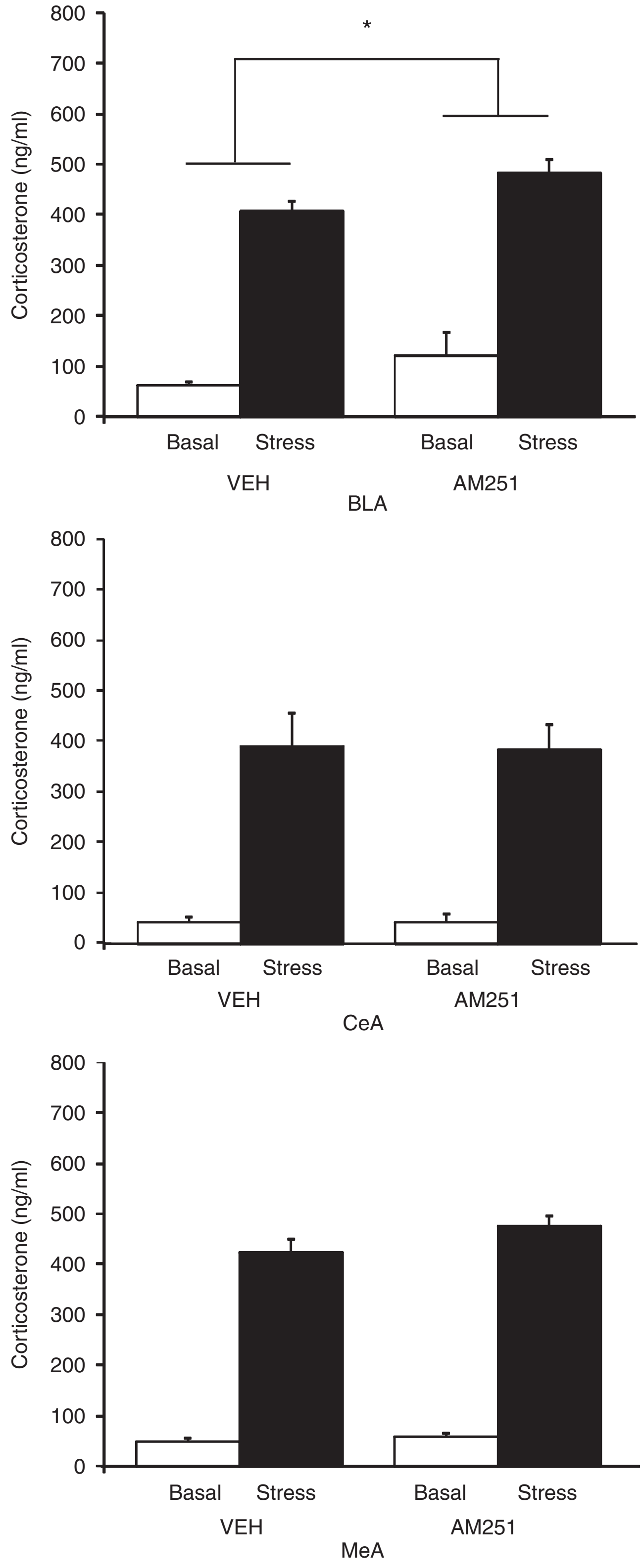

Figure 5 Antagonism of the CB_1 receptor within distinct amygdalar nuclei differentially affects stress-induced increases in corticosterone secretion. Infusion of the CB_1 receptor antagonist AM251 (2.5 μg) into the basolateral nucleus of the amygdala (BLA) significantly increased corticosterone secretion, whereas there was no effect of AM251 infusion into the central nucleus of the amygdala (CeA) or the medial amygdala (MeA). Values are denoted as means ± SEM. *Significant differences ($P<0.05$).

stress; to test this hypothesis, we examined the effects of local administration of AM251 into the BLA on both basal and stress-induced corticosterone secretion (Figure 5). There was a significant main effect of both stress [$F(1,15)=121.77$, $P<0.001$] and intra-BLA administration of AM251 [$F(1,15)=4.81$, $P<0.05$] on serum corticosterone but no significant interaction between AM251 administration and stress [$F(1,15)=0.14$, $P>0.05$]. AM251 administration into the BLA resulted in a moderate increase in serum corticosterone under both basal and stress conditions, but did not potentiate stress-induced corticosterone secretion, *per se*. Infusion of AM251 into the CeA and MeA did not affect stress-induced serum corticosterone (Figure 5).

In the next set of studies, we examined the effects of intra-amygdalar infusion of a direct CB_1 receptor agonist on basal and stress-induced serum corticosterone. There was a significant interaction between administration of the CB_1 receptor agonist HU-210 into the BLA and stress on serum corticosterone [$F(1,15)=12.72$, $P<0.005$; Figure 6]. *Post hoc* analysis revealed that HU-210 administration into the BLA had no effect on serum corticosterone levels in nonstressed rats; however, administration of HU-210 into the BLA before stress resulted in a significant reduction of stress-induced corticosterone secretion compared with vehicle ($P<0.01$). When HU-210 was co-administered with the CB_1 receptor antagonist AM251 into the BLA (Figure 6), corticosterone levels after stress were no different than those of animals receiving a vehicle infusion before stress ($P>0.05$) and were significantly higher than those after HU-210 infusion alone ($P<0.05$).

There was a significant interaction between stress and administration of HU-210 into the MeA on serum corticosterone levels [$F(1,15)=6.72$, $P<0.03$; Figure 6] with subsequent analysis indicating that administration of HU-210 into the MeA before stress resulted in a significant facilitation of stress-induced corticosterone secretion ($P<0.02$ relative to vehicle-infused animals). There was no significant interaction between stress and infusion of HU-210 into the CeA [$F(1,13)=0.3$, $P>0.05$; Figure 6] nor a main effect of HU-210 administration into the CeA [$F(1,13)=0.2$, $P>0.05$] on serum corticosterone levels, whereas there was a main effect of stress to increase serum corticosterone [$F(1,13)=52.7$, $P<0.001$].

DISCUSSION

The results of this study show that exposure of rats to a psychological stressor evokes an increase in FAAH-mediated hydrolysis of AEA within the amygdala, which results in a suppression of AEA/CB_1 receptor signaling within the BLA. This reduction in AEA/CB_1 receptor signaling within the BLA determines the magnitude of the corticosterone response during the stress-induced activation of the HPA axis. On the basis of these and other findings, we propose that AEA/CB_1 receptor signaling in the rat amygdala is tonically active, and that it serves as a functional gatekeeper of HPA axis activation. Thus, after exposure to stress, FAAH activity increases, which results in a drop in AEA tone that promotes neuronal activation within the BLA and increases activation of the HPA axis.

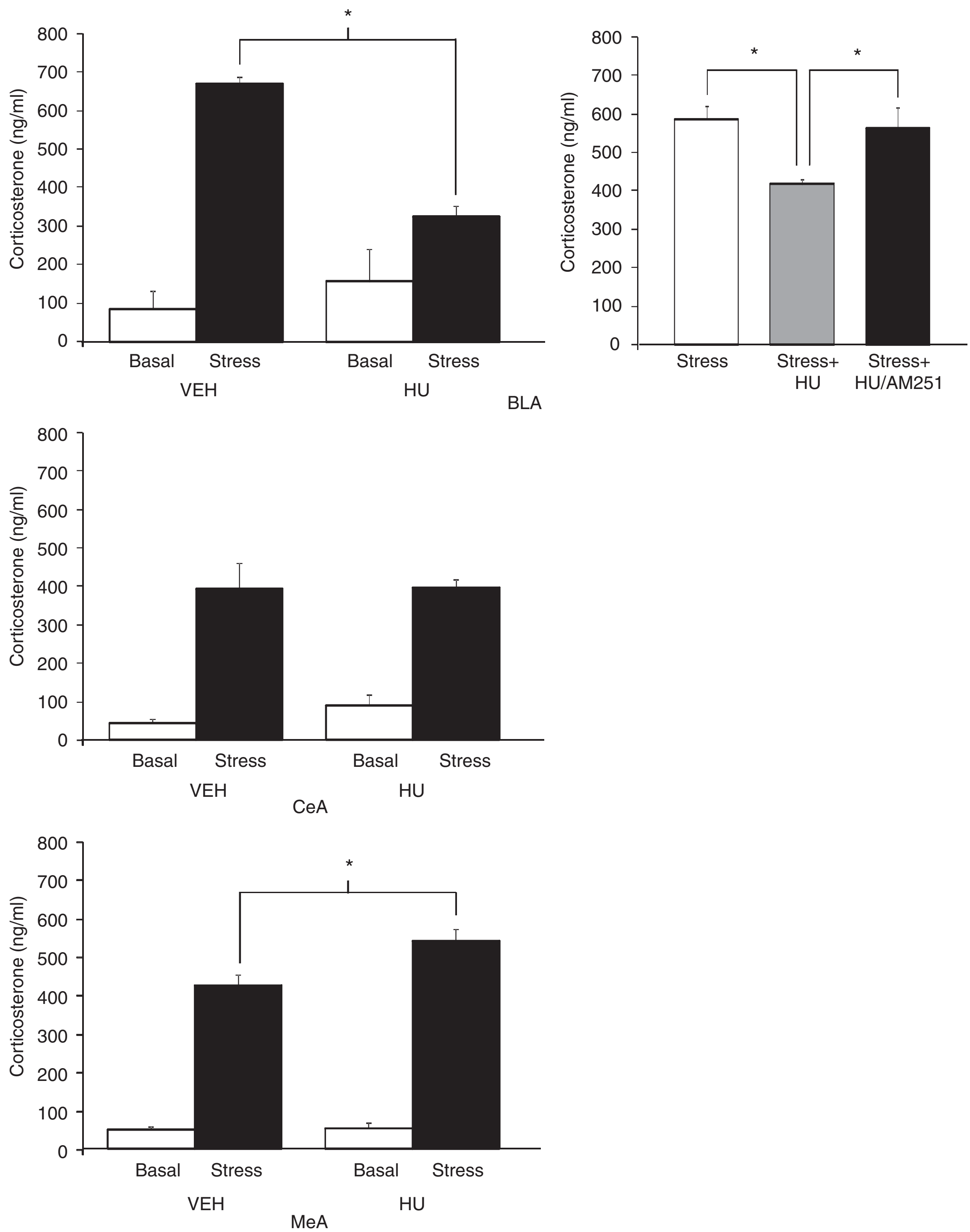

Figure 6 Pharmacological activation of the CB_1 receptor within distinct amygdalar nuclei differentially affects stress-induced increases in corticosterone secretion. Infusion of the CB_1 receptor agonist HU-210 (HU; 2.5 μg) into the basolateral amygdala (BLA) suppressed stress-induced corticosterone secretion, in a CB_1 receptor-dependent manner, whereas infusion of HU-210 into the medial amygdala (MeA) enhanced stress-induced corticosterone secretion. There was no effect of HU-210 infusion into the central nucleus of the amygdala (CeA). Values are denoted as means ± SEM. *Significant differences ($P<0.05$).

Stress-Induced Suppression of AEA/CB_1 Signaling Within the BLA Promotes Activation of the HPA Axis

The effect of restraint stress to reduce the tissue content of AEA within the amygdala of the rat is consistent with earlier reports in mice (Patel *et al*, 2005c; Rademacher *et al*, 2008). Within this study, the content of AEA within the amygdala determined at the termination of restraint stress was negatively correlated with the magnitude of the corticosterone response to stress within these same animals, indicating a relationship between amygdalar AEA and HPA axis activation by a psychological stressor. As pharmacological inhibition of FAAH activity within the BLA reduced the corticosterone response to psychological stress, it is likely that AEA content in the BLA has a function in constraining HPA axis activation, rather than the converse explanation, that the stress-induced reduction in amygdalar AEA is driven by increased levels of glucocorticoids. It is our hypothesis that stress-induced activation of FAAH within the amygdala is an early event in the cascade of signaling processes that ultimately regulate glucocorticoid secretion. This hypothesis is supported by the fact that we have seen reductions in AEA as rapidly as 5 min after the initiation of stress, which would preclude mediation by an increase in glucocorticoid secretion (MN Hill, RJ McLaughlin, BB Gorzalka and CJ Hillard, unpublished findings). Furthermore, despite the fact that tissue measurements of FAAH activity and AEA content were performed in sections of the entire amygdala, the pharmacological data would suggest that this increase in FAAH activity and reduction in AEA content is primarily occurring in the BLA. However, the mechanism by which stress rapidly induces FAAH activity has yet to be determined.

Consistent with this hypothesis, antagonism of the CB_1 receptor within the BLA increased HPA axis drive, whereas there was no effect of CB_1 receptor antagonism within the CeA or MeA on HPA axis output. Collectively, these data suggest that AEA/CB_1 receptor signaling in the BLA exerts tonic inhibition over the HPA axis. However, given that the independent effects of FAAH inhibition and CB_1 receptor antagonism within the BLA on stress-induced corticosterone secretion were modest, it appears that the role of endocannabinoid signaling within the BLA is modulatory in nature.

Pharmacological Activation of CB_1 Receptors Within the BLA Dampens HPA Axis Activation in Response to Stress

In agreement with the model that CB_1 receptor activation within the BLA limits activation of the HPA axis, local administration of the CB_1 receptor agonist HU-210 into the BLA reduced the stress-induced increase in circulating levels of corticosterone. This phenomenon was mediated through a CB_1 receptor-dependent process (as it was prevented by co-administration of the CB_1 receptor antagonist AM251), and the magnitude of this suppression was greater than what was seen after inhibition of AEA hydrolysis. In contrast, infusion of HU-210 into the CeA did not modulate the HPA axis response to restraint stress, whereas administration of HU-210 into the MeA resulted in an unexpected facilitation of stress-induced corticosterone secretion. These data show that the role of CB_1 receptor signaling in HPA axis regulation is functionally dissociable among amygdalar nuclei. Given that expression analysis of the CB_1 receptor reveals considerably higher densities in the BLA than in the CeA or MeA (Herkenham *et al*, 1991; Katona *et al*, 2001), it is not surprising that the most robust effects that were documented occurred through modulation of CB_1 receptor signaling in the BLA. Additionally, the fact that administration of a CB_1 receptor agonist into distinct amygdalar nuclei exerted different responses on stress-induced corticosterone secretion, confirms that the effects are due to CB_1 receptor activation within these restricted regions and not due to spillover of the infusion into surrounding amygdalar regions.

The mechanism by which CB_1 receptor activity within the BLA regulates HPA axis activity is not known. With respect to amygdalar nuclei, the predominance of research has focused on the roles of the CeA and MeA in the neuroendocrine response to stress, whereas the BLA has been largely neglected (Herman *et al*, 2003, 2005; Dayas *et al*, 1999). The BLA is the integration site within the amygdala that receives afferents from cortical, hippocampal and thalamic sites encoding external sensory and visceral information (McDonald, 1992; Sah *et al*, 2003). Immediate early gene studies have revealed that projection neurons of the BLA are activated in response to restraint stress (Patel *et al*, 2005b; Reznikov *et al*, 2008) and lesions of the BLA dampen neuroendocrine responses to psychological stressors, such as restraint or footshock (Bhatnagar *et al*, 2004; Goldstein *et al*, 1996). Similarly, direct stimulation of the BLA can increase HPA axis activity (Szafarcyk *et al*, 1986; Feldman *et al*, 1982, 1983). Moreover, overexpression of the SK2 potassium channel in the BLA attenuates stress-induced corticosterone secretion (Mitra *et al*, 2009). These data suggest that activation of BLA projection neurons positively contribute to HPA axis activation during psychological stress, likely through projection relays to the MeA or BNST, which, in turn, communicate directly to the PVN (Herman *et al*, 2003, 2005; Ulrich-Lai and Herman, 2009).

There is convincing evidence that the CB_1 receptor is present on both GABAergic and glutamatergic axons in the BLA. Immunohistochemical studies showed that CB_1 receptors are present on GABAergic synapses and function to inhibit GABAergic transmission (Katona *et al*, 2001; McDonald and Mascagni, 2001). Although it has been difficult to show the presence of CB_1 receptors on glutamatergic terminals using immunohistochemistry (Katona *et al*, 2001; McDonald and Mascagni, 2001), electrophysiological studies have established that activation of CB_1 receptors within the BLA inhibits excitatory synaptic transmission and the firing rate of BLA projection neurons (Pistis *et al*, 2004; Perra *et al*, 2008; Domenici *et al*, 2006; Azad *et al*, 2003). Furthermore, the ability of cannabinoids to reduce glutamatergic signaling overrides the suppression of GABAergic transmission, resulting in a net reduction in the excitability of BLA projection neurons (Azad *et al*, 2003). In line with this, immediate early-gene studies have found that systemic administration of a CB_1 receptor antagonist increases the neuronal activation of the BLA in unstressed animals (Singh *et al*, 2004; Patel *et al*, 2005b), supporting the hypothesis that endocannabinoid signaling tonically inhibits excitatory transmission in the BLA. Given that the primary source of excitatory inputs to BLA projection neurons are cortical and thalamic afferents transmitting sensory information regarding external conditions (Sah *et al*, 2003: McDonald, 1992), CB_1 receptor activation within the BLA likely attenuates HPA axis activity by gating excitatory sensory input afferents to the BLA, and subsequently dampens the firing rate of BLA projection neurons, which exert trans-synaptic relays to the PVN. This hypothesis is in agreement with recent transgenic data showing that endocannabinoid regulation of HPA axis responsivity is governed by CB_1 receptors on principal forebrain neurons, but not GABAergic interneurons (Steiner *et al*, 2008b).

It is also possible that the effects of endocannabinoid signaling in the BLA on the HPA axis are not due to modulation of the CRH-ACTH pathway originating in the PVN, but instead are due to action on the autonomic arm of the stress axis. Specifically, it is possible that changes in amygdalar neuronal activity could affect principal autonomic relay nuclei, such as those in the brainstem and regions of the PVN that exhibit projections to autonomic spinal regions, which in turn could affect corticosterone secretion at a different peripheral level, either through changes in adrenal cortical cell sensitivity to ACTH or in hepatic blood flow to inhibit corticosterone clearance from the circulation. Although this pathway could account for the modest effect size that is seen after modulation of endocannabinoid signaling in the BLA, it seems less plausible given that the BLA, as well as the MeA, through which the BLA is believed to communicate under conditions of psychological stress (Herman *et al*, 2005; Ulrich-Lai and Herman, 2009), exhibit little to no projections to the primary autonomic output nuclei (Ulrich-Lai and Herman, 2009).

It should be noted, however, that we have found earlier using c-fos immunohistochemistry, that CB_1 receptor agonists (but not FAAH inhibitors) potentiate stress-induced neuronal activation of the CeA, but not the BLA, when administered systemically to mice (Patel *et al*, 2005b). It is our hypothesis that systemically administered agonists, which globally increase CB_1 receptor signaling throughout the brain, promote activation of incoming excitatory afferents to the amygdala through effects in an extra-amygdalar brain region. This pattern of activation could override the local suppressive effect AEA/CB_1 receptor activation within the BLA and concurrently promote activation of the CeA.

Activation of CB_1 Receptors Within the Medial or Central Nucleus of the Amygdala Exerts Differential Effects on Stress-Induced HPA Axis Activation

Administration of the CB_1 receptor agonist HU-210 into the MeA before restraint potentiated the corticosterone response to stress. Similarly, systemic administration of CB_1 receptor agonists can enhance stress-induced HPA axis activation (Patel *et al*, 2004; Jacobs *et al*, 1979; Hill and Gorzalka, 2006). The present data suggest that activation of CB_1 receptors within the MeA could contribute to the stress-potentiating effects of systemically administered cannabinoid agonists. Given that lesion studies have indicated that the MeA is recruited by psychological stressors to activate the HPA axis (Dayas *et al*, 1999; Figueiredo *et al*, 2003; Ma and Morilak, 2005; Herman *et al*, 2005), the current data suggest that CB_1 receptor activation within the MeA, when coupled with stressful stimuli, promotes the output neurons of this nucleus to increase the neuronal activation of the PVN. Our finding that neither AM251 nor URB597 infusions in the MeA affected the corticosterone response to stress suggests that this CB_1 receptor-mediated effect is not endogenously activated by stress.

Pharmacological modulation of CB_1 receptor signaling within the CeA did not alter the HPA axis response to acute restraint stress. This finding is not entirely unexpected, as the expression levels of FAAH and the CB_1 receptor within this part of the amygdala are relatively sparse (Katona *et al*, 2001; Gulyas *et al*, 2004). Furthermore, the CeA appears to be more important for HPA axis activation after physiological stressors, rather than psychological stressors, particularly restraint (Prewitt and Herman, 1997; Dayas *et al*, 1999, 2001).

Neurobehavioral Implications

The present findings likely generalize and extend beyond the regulation of the HPA axis. Amygdalar endocannabinoid signaling is important for adaptive emotional flexibility (Marsicano *et al*, 2002; Lin *et al*, 2006), such as fear extinction, which is disrupted by stressful stimuli (Izquierdo *et al*, 2006; Miracle *et al*, 2006; Holmes and Wellman, 2009; Baran *et al*, 2009). Specifically, AEA signaling within the amygdala mediates forms of synaptic plasticity, such as inhibitory long-term depression, which are conducive to fear extinction and emotional flexibility (Azad *et al*, 2004). Accordingly, it is possible that stress-induced reductions in AEA/CB_1 receptor signaling in the BLA contribute to the alterations in emotional reactivity and flexibility elicited by exposure to stressful stimuli.

A recent report has shown that endocannabinoid signaling within the BLA is also critical for the consolidation of aversive memories (Campolongo *et al*, 2009). Moreover, this research showed that endocannabinoid signaling in the BLA was also required in order for corticosterone to modulate aversive memory consolidation (Campolongo *et al*, 2009), suggesting that glucocorticoids were capable of inducing amygdalar endocannabinoid synthesis *in vivo* (Hill and McEwen, 2009). As the current data indicate that stress decreases amygdalar AEA content, collectively, these studies would suggest that stress and glucocorticoids differentially affect amygdalar endocannabinoid content, *per se*. That is, stress may decrease amygdalar endocannabinoid tone, through a glucocorticoid-independent mechanism of action, whereas glucocorticoids in the absence of stress may promote amygdalar endocannabinoid signaling. In a similar vein, unlike stress exposure, administration of glucocorticoid hormones can actually promote emotional flexibility and enhance fear extinction (de Quervain *et al*, 2009; Yang *et al*, 2006). Given the parallels between the effects of stress and glucocorticoids on fear extinction and amygdalar endocannabinoid signaling, the distinct possibility exists that endocannabinoid signaling is a direct mediator of these processes on adaptive emotional flexibility. Future research is required to address this question.

Conclusions

Psychological stress is hypothesized to activate the HPA axis through a top–down process wherein the HPA axis is activated by a complex network of afferents arising from the limbic forebrain (Pecoraro *et al*, 2006; Herman *et al*, 2003; Sawchenko *et al*, 2000). Within this circuit, the amygdala has been identified as a primary site providing excitatory drive to the HPA axis (Herman *et al*, 2005). The present findings suggest that endocannabinoid signaling is integrated into this circuit such that stress dampens AEA/CB_1 receptor signaling within the BLA, which in turn modulates the magnitude of HPA axis responses to stressful stimuli.

This process, and ability of CB_1 receptors to inhibit glutamate release from excitatory afferents, suggests a model by which tonic AEA within the BLA produces steady-state activation of the CB_1 receptor and results in tonic inhibition of corticothalamic sensory afferents to the BLA. The application of a psychological stressor results in increased FAAH activity, which reduces AEA/CB_1 receptor signaling, and disinhibits excitatory transmission in the BLA. As a result, the neural activity of BLA projection neurons that indirectly communicate with the PVN is increased. These data support the 'gatekeeper' hypothesis of endocannabinoid regulation of the HPA axis (Patel *et al*, 2004), but show that in addition to the PVN (Di *et al*, 2003), the BLA is an important structure involved in the suppression of the neuroendocrine response to stress by endocannabinoid signaling. Taken together, the present data indicate that stress-induced regulation of endocannabinoid signaling within the amygdala could be an important determinant in the neuroendocrine, and possibly emotional, responses to aversive, environmental stimuli.

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DISCLOSURE

The authors declare no conflict of interest.

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