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Sustained Glucocorticoid Exposure Recruits Cortico-limbic CRH Signaling to Modulate Endocannabinoid Function

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Abstract

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Contributors

JMG, CDW, TTL, INK and MNH performed data acquisition and analysis; QJP, JMD, CJH, BSM, JS, INK, SP and MNH provided technical expertise and/or tools to perform the research; JMG and MNH wrote the initial draft of the manuscript together and all other authors provided critical feedback and revisions and approved the final draft of this manuscript.

Sustained exposure to stress or corticosteroids is known to cause changes in brain endocannabinoid (eCB) signaling, such that tissue contents of the eCBs *N*-arachidonyl ethanolamine (AEA) are generally reduced while 2-arachidonoylglycerol (2-AG) levels increase. These changes in eCB signaling are to be important for many of the aspects of chronic stress, such as anxiety, reward sensitivity and stress adaptation, yet the mechanisms mediating these changes are not fully understood. We have recently found that the stress-related neuropeptide corticotropin-releasing hormone (CRH), acting through the CRH type 1 receptor (CRHR1), can reduce AEA content by increasing its hydrolysis by the enzyme fatty acid amide hydrolase (FAAH) as well as increase 2-AG contents. As extra-hypothalamic CRH is upregulated by chronic corticosteroid or stress exposure, we hypothesized that increased CRH signaling through CRHR1 contributes to the effects of chronic corticosteroid exposure on the eCB system within the amygdala and prefrontal cortex. Male rats were exposed to 7 days of systemic corticosterone capsules, with or without concurrent exposure to a CRHR1 antagonist and examined eCB content. Consistent with previous studies in the amygdala, sustained corticosterone exposure increases CRH mRNA in the prefrontal cortex. As was shown previously, FAAH activity was increased and AEA contents reduced within the amygdala and prefrontal cortex following chronic corticosterone exposure. Chronic corticosterone exposure also elevated 2-AG content in the prefrontal cortex but not the amygdala. These corticosteroid-driven changes were all blocked by systemic CRHR1 antagonism. Consistent with these data indicating sustained increases in CRH signaling can mediate the effects of chronic elevations in corticosteroids, CRH overexpressing mice also exhibited increased FAAH-mediated AEA hydrolysis in the amygdala and prefrontal cortex compared to wild type. CRH overexpression increased 2-AG content in the amygdala, but not the prefrontal cortex. These data indicate that chronic elevations in CRH signaling, as is seen following exposure to chronic elevations in corticosterone or stress, drive persistent changes in eCB function. As reductions in AEA signaling mediate the effects of CRH and chronic stress on anxiety, these data provide a mechanism linking these processes together.

Keywords

2-arachidonoylglycerol(2-AG); glucocorticoid; fatty acid amide hydrolase(FAAH); restraint; corticotropin-releasing; hormone receptor 1 (CRHR1); HPA axis

1. Introduction

Over the last sixteen years, endocannabinoids (eCBs) have become widely appreciated as a neuromodulatory signaling system that is capable of dampening endocrine stress responses, decreasing activation of stress-sensitive brain circuits, and reducing the detrimental impacts of stress on mood and anxiety (see Hill and Tasker, 2012; Morena et al., 2015; Gray et al., 2014 for reviews). To better understand how eCBs exert these effects, recent studies have characterized eCB changes in response to acute and chronic stress in animal and human models (Morena et al., 2015). Although the scope of human studies is currently limited (see Hillard et al., 2012 for review), the accumulating wealth of animal studies indicates that eCB responses show similarities across a variety of stress models, suggesting the neural basis coordinating these changes is likely similar (Morena et al., 2015). A notable commonality across acute psychological stress and chronic stress paradigms is that the eCBs *N*-

arachidonylethanolamine (AEA) and 2-arachidonoylglycerol (2-AG) typically change in opposite directions. While decreases in AEA content are consistently observed in the prefrontal cortex (PFC), amygdala and hippocampus, and to a lesser extent in the hypothalamus (Patel et al., 2005; Rademacher et al., 2008; Hill et al., 2010b; McLaughlin et al., 2012; Hill et al., 2009; Dubreucq et al., 2012; Gray et al., 2015b; Jennings et al., 2016), these same regions show increases in 2-AG concentrations, which are generally most prominent during repeated and chronic stress conditions (Patel et al., 2005; Hill et al., 2010b; Dubreucq et al., 2012; Wang et al., 2012; Evanson et al., 2010; Hill et al., 2011; see Gray et al. 2014 and Morena et al., 2015 for review).

The mechanisms by which stress modulates eCB signaling are not entirely understood. We have recently demonstrated the importance of the stress-related neuropeptide, corticotropin-releasing hormone (CRH), acting through the CRH type 1 receptor (CRHR1), in mediating the rapid decline in amygdalar AEA tissue contents by increasing AEA hydrolysis by the enzyme fatty acid amide hydrolase (FAAH; Gray et al., 2015b). While these acute AEA/FAAH amygdala effects appear to be independent of corticosterone (CORT) changes, acute stress-induced CORT increases are required for elevations in 2-AG content in the PFC (Hill et al., 2011), hippocampus (Wang et al., 2012) and the hypothalamus (Evanson et al., 2010). Following chronic stress, however, reductions in tissue levels of AEA and elevations of 2-AG in limbic structures, including the amygdala, become amplified and both of these effects are recapitulated by sustained elevations in corticosteroids (Patel et al., 2005; Dubreucq et al., 2012; Hill et al., 2005; Bowles et al., 2012). The inability of acute CORT exposure to induce the same AEA changes found following chronic CORT exposure (Hill et al., 2010a), or chronic stress suggest that the effects of chronic CORT on AEA are likely indirect, and could involve secondary signaling mechanisms downstream of glucocorticoid receptor activation.

In this regard, a hallmark feature of clinical studies examining stress-related disorders, such as major depression, is a significant increase in central CRH levels (Nemeroff et al., 1984). Rodent models of chronic stress also show an increased capacity for central and extra-hypothalamic CRH signaling to modulate neuronal function. These studies have consistently described CORT-dependent CRH mRNA increases in the amygdala and bed nucleus of the stria terminals (BNST) following sustained exposure to glucocorticoid elevations (Swanson and Simmons, 1989; Makino et al., 1994a, 1994b) and the facilitation of acute stress-induced CRH release in the amygdala and PFC using microdialysis approaches (Merali et al., 2008). Similarly, repeated restraint stress also increases CRH mRNA levels in the amygdala, which is thought to be due to CORT-dependent upregulation (Makino et al., 1999; Gray et al., 2010). Given the role of CRH in the acute regulation of FAAH activity and AEA content by stress, these data suggest that the progressive recruitment of CRH signaling by sustained CORT elevations could also mediate the effects of chronic stress on the eCB system. The aim of the current study was to determine the necessity of CRH signaling in the effects of chronic CORT exposure on the eCB system, and determine if CRH overexpression alone is sufficient to precipitate the eCB changes associated with chronic stress and prolonged exposure to glucocorticoid elevations.

2. Methods and materials

2.1. Animals

Adult male Sprague Dawley rats (200-225 g) from Charles River Laboratories (Kingston, NY) were used. Rats were pair housed under standard conditions of light (lights on at 0900 h and off at 2100 h) and temperature ($22 \pm 2^\circ\text{C}$) and given one week of acclimatization to the animal facility upon arrival. Rats were provided Purina Rodent Chow (Labdiet 5012, Wilkes-Barre, PA) and tap water *ad libitum*.

Adult male C57BL/6J mice bred at the Max Planck Institute of Psychiatry were used to study the effects of centrally restricted CRH overproduction. Breeding details for the generation of homozygous mice that conditionally overexpress CRH in the brain (CRH-COE-Nes) have been previously described (Lu et al., 2008). In brief, the CRH-COE-Nes mice were generated by first inserting a single copy of the murine CRH cDNA, preceded by a *loxP*-flanked transcriptional terminator into the ubiquitously expressed ROSA26 (R26) locus, to produce a subset of homozygous *R26^{flopCrh/flopCrh}* mice (Dedic et al., 2012). These animals were crossed with mice expressing Cre under the nestin promoter ((Nes)-Cre mice) to ensure CRH overproduction is limited to the central nervous system. This approach permits CRH expression to be conditionally activated throughout the brain as early as embryonic day 10.5, when nestin expression is initiated. This mouse model, unlike other CRH overexpressing mouse lines which are associated with peripheral corticosteroid elevations, does not display elevations of basal adrenocorticotrophic hormone (ACTH) or CORT which allows the effects of CRH overproduction to be assessed independent of changes in circulating corticosteroids under resting conditions (Lu et al., 2008).

Male wild-type (WT) and CRH overexpressing mice (CRH-OE) were group housed 2-4 per cage under standard conditions of light (lights on at 0700 h and off at 1900 h), temperature ($22 \pm 2^\circ\text{C}$) and provided with food and tap water *ad libitum*. All mice were 2-3 months old at the time of tissue collection.

2.2. Experimental Methods

2.2.1. CRH mRNA *in situ* hybridization—Adjacent series of tissue from each rat were used for *in situ* hybridization and morphological analysis. *In situ* hybridization was performed using a ^{35}S -labeled (Amersham Biosciences Inc., Arlington, IL, USA) antisense CRH cRNA probe. Techniques for riboprobe synthesis are described in greater detail elsewhere (Makino et al., 1994b). A thorough description of tissue preparation and the *in situ* hybridization protocol can also be found elsewhere (Kinlein et al., 2015). Based on the strength of autoradiographic signal on test slides exposed to X-ray film (Kodak BioMax MR film, Sigma, St. Louis, MO, USA), hybridized slides containing the PFC were exposed to film for 2 days to optimize the detection of possible treatment differences. Films were then digitized with a scanner and semi-quantitative densitometric analysis of relative levels of CRH mRNA was performed. Optical densities were determined bilaterally and averaged across 3 adjacent coronal sections for each rat. Every measurement of optical density was corrected by background subtraction. Film images were analyzed using MCID-M4 software

(Imaging Research Inc., St. Catharines, Canada), which were then exported to Adobe Photoshop (v.10.0) for final figure assembly.

2.2.2. CORT plasma analysis—Trunk blood samples were immediately centrifuged upon collection at 10,000 rpm for 20 min at 4°C, then stored at –20°C until later testing. Plasma CORT was measured using the enzyme immunoassay (EIA) kit from Cayman Chemicals (#500655; Michigan, USA). Samples were tested in duplicate and diluted 1:1000 to ensure stress-induced levels fit on the linear portion of the standard curve. The detection limit of the assay was 30 pg/ml at 80% binding. The intra-assay and inter-assay coefficients of variation were < 14%.

2.2.3. Lipid extractions from tissue samples—Lipid extraction from dissected tissue was carried out as previously described (Gray et al., 2015b), with the homogenization volume spiked with 50 pmol d⁸-2-AG and 84 pmol d⁸-AEA per sample.

2.2.4. Analysis of eCB levels—Samples were quantified using LC-MS/MS on a Quantum triple-quadrupole mass spectrometer in positive-ion mode using selected reaction monitoring. Detection of eCBs was performed as previously described (Hermanson et al, 2013).

2.2.5. FAAH activity—Membrane fractions were prepared from homogenized tissue and samples normalized to protein content (10 µg) and FAAH mediated AEA hydrolysis was performed as previously described (Hill et al., 2009). In brief, samples were incubated, in duplicate, with 0.2 nM ³H-AEA and varying concentrations of cold AEA (0.05, 0.1, 0.25, 0.5, 0.75, 1.0 and 1.5 µM) for 10 min. The total amount of ethanolamine formed at each AEA concentration was determined and maximal hydrolytic activity (V_{max}) values were calculated using nonlinear regression to fit the data to the Michaelis-Menton equation using Prism software (GraphPad).

2.3. Experimental design

All protocols were approved by the Institutional Animal Care and Use Committee at Rockefeller University, and conducted in accordance with the Guide for the Care and Use of Laboratory Animals from the Government of Bavaria, Germany. All manipulations were performed between 0900 h and 1100 h to minimize potential circadian effects.

2.3.1. Experiment 1: The effects of sustained CORT exposure on CRH mRNA in the PFC—Multiple reports have described elevations in CRH mRNA in extra-hypothalamic structures such as the amygdala and BNST following sustained exposure to corticosteroids (Swanson and Simmons, 1989; Makino et al., 1994a, 1994b), but few studies have examined whether a similar CORT-dependent effect occurs in the PFC. In the present study, in situ hybridization was used to compare the effects of sustained CORT exposure on CRH mRNA in the PFC of male rats (n=8/group) receiving either a slowing-releasing CORT capsule (200 mg, 60 day release, Innovative Research of America, Toledo, OH, USA) or a placebo capsule containing cholesterol (Innovative Research of America, Toledo, OH, USA). This dose of CORT was chosen to provide consistent concentrations of plasma

CORT equivalent to elevations typically observed during psychological stress (>100 ng/ml) (Gray et al., 2015a). Adult male rats (270 – 300 g) were anesthetized with a mixture of 100 mg/kg ketamine hydrochloride and 7 mg/kg xylazine, then implanted with subcutaneous capsules at the base of the neck. Following surgery, rats remained pair-housed and were weighed daily prior to testing. Seven days after capsule implantation, rats were rapidly decapitated, brains were extracted, then immediately flash frozen on dry ice and stored at -80°C prior to slicing. Two-three days following collection, coronal sections (20 μm) were sliced on a cryostat, mounted onto slides, and stored at -80°C . At the time of decapitation, trunk blood samples were collected for CORT analysis. One sample was compromised during processing and so was not used in the in situ analysis.

2.3.2. Experiment 2: The effects of CRHR1 signaling on CORT-dependent eCB changes—To examine whether the effects of sustained CORT on eCB contents require activation of CRHR1 receptors, four groups of male rats were studied. Half of the rats were implanted subcutaneously with a 200 mg CORT capsule (described above); the other half with a cholesterol containing control capsule. Half of each group was also implanted with a slow-release capsule containing 60 mg of the CRHR1 antagonist antalarmin (Innovative Research of America, Toledo, OH, USA), while the other half of each group received cholesterol capsules. Rats remained paired housed following surgery and were handled daily prior to euthanasia. Seven days after capsule implantation (postnatal day PND 62–65), rats were rapidly decapitated, brains were removed and PFC and amygdala were dissected using the previously described anatomical parameters (Gray et al., 2015b). Once removed, tissues were quickly flash frozen on dry ice and stored at -80°C . Among the pool of frozen samples, some samples were later used to test eCB levels using liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis, whereas other samples were used to test enzymatic activity for the eCB degrading enzyme fatty acid amide hydrolase (FAAH). Three amygdala samples were compromised during processing and did not have data analyzed from them for analysis.

2.3.3. Experiment 3: The effects of sustained CRH elevations on corticolimbic eCB levels—Having shown in experiment 2 that central eCB levels are susceptible to regulation by CRHR1 signaling, we next tested if chronic elevations in CRH signaling alone would exert the same effects. For this experiment we employed a genetically altered line of mice that centrally overexpress CRH (Lu et al., 2008). At PND 65–70, transgenic or control adult male mice were rapidly decapitated and brains were extracted. Freshly dissected tissue was taken from the PFC and amygdala as described above, flash frozen on dry ice, then stored at -80°C for later testing of eCB levels and FAAH enzymatic activity. Two amygdala samples were compromised during extraction and could not be included in analysis.

2.4. Statistical analysis

The data were analyzed with GraphPad Prism 6.02 (California, USA) using two-way ANOVAs where appropriate, and unpaired t-tests to examine CORT responses following capsule implantation and CRH mRNA levels in overexpressing mice. Post hoc analysis was performed with the Bonferroni method for specific group comparison. For all data $p < 0.05$ was used to indicate statistical significance.

3. Results

3.1. Corticosterone elevations are associated with CRH mRNA increases in the PFC

Animals implanted with 200 mg CORT capsules for 7 days showed a significant increase in plasma CORT, $t(14) = 6.12$, $p < 0.0001$; **Figure 1A**, when compared to vehicle controls, indicating this procedure was sufficient to elevate circulating CORT concentrations above baseline, and within a range typically observed during psychological stress (Gray et al., 2015a). It has been well established that chronic stress or exposure to CORT can increase CRH mRNA production within the amygdala and extended amygdala (Swanson and Simmons, 1989; Makino et al., 1994a, 1994b), however, little is known about if similar changes occur in the PFC. To this extent, we examined the effects of sustained exposure to CORT on CRH mRNA levels within the PFC. Animals implanted with CORT capsules also showed a significant increase in relative levels of CRH mRNA optical density within the PFC, $t(13) = 3.12$, $p = 0.008$; **Figure 1B**, as a semi-quantitative measure of changes in CRH mRNA expression (see **Figure C & D** for representative images).

3.2. Corticosterone relies on CRHR1 signaling to modulate PFC eCBs

To test the effect of implanted capsules containing CORT and/or CRHR1 antagonist on eCB content in the PFC, eCB contents were measured and analyzed using a two-way ANOVA with the first factor CORT exposure (vehicle, CORT; between subjects), and the second factor CRHR1 antagonism (vehicle, CRHR1 antagonist; between subjects). This analysis revealed a significant interaction between CORT exposure and CRHR1 antagonism on PFC levels of AEA, $F(1,28) = 4.39$, $p = 0.045$, **Figure 2A**; 2-AG, $F(1,28) = 4.56$, $p = 0.041$, **Figure 2B**; and FAAH enzymatic activity, $F(1,12) = 4.75$, $p = 0.049$, **Figure 2C**. No main effects or interactions were observed for FAAH binding affinity (K_m), data supplied in **Table 1**. Bonferroni post hoc tests indicated that CORT exposure significantly decreased AEA in the PFC ($p < 0.05$), increased PFC 2-AG ($p < 0.05$), and increased the V_{max} for FAAH ($p < 0.05$). In all cases, the effects of CORT changes did not occur with CRHR1 antagonism, suggesting that both AEA and 2-AG are sensitive to glucocorticoid-induced CRHR1 activation in the PFC.

3.3. Corticosteroids rely on CRHR1 signaling to modulate amygdala AEA contents, but not 2-AG

Analysis of the amygdala data using a two-way ANOVA found a significant interaction between rats exposed to capsules containing CORT and those exposed to CRHR1 antagonist on AEA levels in the amygdala, $F(1,25) = 4.69$, $p = 0.04$, **Figure 2D**. Post hoc analysis revealed that control animals receiving CORT capsules had significantly reduced AEA contents ($p < 0.05$), and this effect was blocked with CRHR1 antagonism. No interaction was found for amygdala 2-AG contents, $F(1,25) = 0.93$, $p = 0.34$, **Figure 2E**. Complementary to the observed changes in amygdala AEA, there was also a significant interaction between CORT exposure and CRHR1 antagonism for FAAH enzymatic activity, $F(1,12) = 5.56$, $p = 0.036$, **Figure 2F**. Consistent with the effects of CORT on AEA, post hoc tests revealed that rats receiving CORT capsules had significantly higher FAAH V_{max} for AEA ($p < 0.05$), and this increase was blocked with CRHR1 antagonism. There was a main effect of CORT to

increase the K_m , thus reduce the binding affinity, of FAAH for its ligand AEA $F(1,12) = 6.42$, $p = 0.02$, **Table 1**, but no interaction between CORT and CRHR1 antagonism $F(1,12) = 0.15$, $p = 0.71$, **Table 1**.

3.4. CRH overexpression is associated with decreased AEA content in the PFC, with no effect on 2-AG

To examine the effects of chronic CRH overexpression on eCB tone, genetically modified mice that conditionally overexpress CRH in the brain, but not the periphery were employed (Lu et al., 2008). Compared to wild-type control mice, CRH overexpression significantly decreased levels of AEA in the PFC, $t(17) = 2.12$, $p < 0.04$, **Figure 3A**, however there was no effect on PFC levels of 2-AG, $t(17) = 0.46$, $p = 0.64$, **Figure 3B**. Consistent with the AEA findings, CRH overexpression significantly increased FAAH V_{max} $t(4) = 2.58$, $p = 0.03$, **Figure 3C**, with no effect on K_m of AEA for FAAH, $t(4) = 1.82$, $p = 0.14$, data presented in **Table 2**. These data are complementary to our previous findings in the amygdala (Gray et al., 2015b), suggesting that CRH indirectly reduces AEA content by increasing hydrolytic activity of the degrading enzyme FAAH.

3.5. CRH overexpression is associated with decreased AEA levels in the amygdala, and increased 2-AG

We also determined the effects of CRH overexpression on amygdala resting levels of eCBs. Compared to wild-type control mice, CRH overexpressing mice displayed significantly decreased levels of AEA in the amygdala $t(15) = 2.47$, $p = 0.02$; **Figure 3D**, and significantly elevated amygdala levels of 2-AG $t(15) = 2.25$, $p = 0.04$; **Figure 3E**. CRH overexpression also significantly increased FAAH enzymatic activity (V_{max}) $t(8) = 2.78$, $p = 0.02$; **Figure 3F**, and increased the K_m of AEA for FAAH, $t(8) = 3.71$, $p = 0.006$, data presented in **Table 2**.

4. Discussion

These studies demonstrate that the recruitment of extra-hypothalamic CRH signaling by sustained CORT exposure mediates alterations in cortico-amygdala eCB content. Additionally, we found that chronic overexpression of CRH alone was sufficient to largely recapitulate these effects. This extends our recent findings that CRH acting at CRHR1 receptors rapidly triggers FAAH-mediated AEA hydrolysis in the amygdala (Gray et al., 2015b), and further demonstrates the importance of CRH/eCB interactions to conditions of chronic CORT exposure and, potentially, chronic stress. Inhibition of FAAH during chronic stress can prevent the decline in AEA signaling (Hill et al., 2013), and reverse stress-induced anxiety and anhedonia (Rossi et al., 2010; Hill et al., 2013; Lomazzo et al., 2015; Bortolato et al., 2007). Given that the induction of FAAH activity and depletion of AEA signaling in the amygdala has been linked to the anxiogenic effects of CRHR1 (Gray et al., 2015b), these data help inform a model for understanding how stress, CRH and eCB signaling interact to regulate emotional behavior. In the acute response, stress-induced release of CRH in the amygdala triggers FAAH activity and transiently impairs AEA signaling to promote the development of an anxious state and stress-induced peripheral CORT increases. The resultant and delayed increase in central CORT may, through distinct non-genomic actions

of CORT, contribute to the eventual normalization of AEA levels (Hill et al., 2010a; Gray et al., 2015b) and reduce excitability of the amygdala (Karst et al., 2010), thereby creating a closed loop mechanism that allows for both the induction of an anxious state and its termination during the stress recovery period. Following exposure to chronic stress, however, persistent CORT elevations appear to upregulate CRH in the amygdala and PFC, which in turn causes a sustained increase in FAAH activity and the development of an AEA deficient state. This steady state reduction in AEA signaling likely contributes to the development of an anxious phenotype during chronic stress, possibly through the regulation of structural plasticity within the basolateral amygdala (Hill et al., 2013; Gunduz-Cinar et al., 2013). This suggests that with respect to stress-induced regulation of FAAH and AEA, CRH is likely an integral mediator of eCB changes produced by both acute and chronic stress.

With respect to the PFC, these data present new findings demonstrating that AEA concentrations in the PFC are susceptible to CRHR1-dependent reductions, likely mediated by CRHR1-driven increases in FAAH enzymatic activity. Specifically, our data demonstrate that blockade of CRHR1 receptors had no effect on FAAH activity or AEA content, under resting levels. Following exposure to chronic CORT, however, this same dose of CRHR1 antagonist abrogated the CORT-mediated upregulation of CRH. Our earlier studies examining the acute effects of CRH signaling within the PFC did not indicate a consistent interaction between these systems (Gray et al., 2015b), which parallels the mixed findings obtained from acute stress studies examining AEA content in the PFC, (Rademacher et al., 2008; Hill et al., 2011; McLaughlin et al., 2012; Dubreucq et al., 2012; Gray et al., 2015b), and which likely reflects the large variations across studies in the time-points studied and the differences in stressor intensities used. However, the data reported herein show that under conditions of sustained CRH signaling, either produced by CRH overexpression or enhanced CRH production by the actions of chronic CORT elevations, CRHR1 activity in the PFC enhances FAAH-mediated AEA hydrolysis. These data suggest that the amygdala may be more sensitive to CRH-mediated regulation of FAAH than the PFC, and that the PFC may require longer lasting, sustained changes in CRH signaling before changes in FAAH activity occur.

Our previous work identified that CRH-regulation of FAAH activity and AEA signaling within the amygdala contributes to CRH-induced anxiety (Gray et al., 2015b). By now linking CRH and FAAH signaling processes in the PFC, this mechanism may also offer insight towards understanding CRH-dependent anxiogenic effects in the PFC (Jaferi and Bhatnagar, 2007; Miguel et al., 2014). Cannabinoid type 1 receptor (CB1) expression in the PFC is predominantly localized to gamma-aminobutyric acid (GABA)-positive interneurons in layers II/III and V of the prelimbic zone, where CB1 binding facilitates disinhibition of pyramidal cells and the promotion of PFC activation (Hill et al., 2011). CRHR1 is similarly situated in these same regions, but found postsynaptically on pyramidal neurons (Refojo et al., 2011), where our data suggests it could act as a neuromodulator gating the synaptic effects of eCBs. CB1-mediated regulation of PFC activity is a key component during processing and termination of stress responses (Hill et al., 2011), as CB1 binding reduces GABAergic inhibition, to promote PFC-activation of downstream inhibitory efferents mediating the inactivation of stress-responsive centers like the amygdala and hypothalamus

(McLaughlin et al., 2014). As activation of these same circuits also encourages the display of active coping responses to stress (Warden et al., 2012), eCB regulation of PFC output likely relates to stress-induced changes in emotional behavior and coping strategies as well (McLaughlin et al., 2012, 2014). In contrast, if CRHR1 activity increases enzymatic degradation of AEA, this could reduce basal eCB signaling on inhibitory terminals and result in compromised PFC activation of downstream inhibitory circuits (McLaughlin et al., 2014). Under conditions of chronic stress, PFC activity is reduced and structural changes are associated with impaired termination of the stress response (Mizoguchi et al., 2003), and deficits in higher order cognitive processing (Liston et al., 2006). Thus, future experiments should explore if changes in PFC function following chronic stress can be rescued through CRHR1 inhibition, or FAAH inhibition to amplify AEA signaling.

Comparable to past studies, we found 2-AG levels in the PFC are sensitive to corticosteroid enhancement (Hill et al., 2011) and CRHR1 activation (Gray et al., 2015b), which is reminiscent of PFC 2-AG elevations typically observed following repeated stressor exposure (Rademacher et al., 2008; Dubreucq et al., 2012). We additionally showed for the first time a reliance of enhanced 2-AG content on CRHR1 signaling by showing CORT-mediated increases in basal 2-AG are blocked by CRHR1 antagonism. Based on our data that CRH expression in the PFC is sensitive to CORT-mediated upregulation, these data may explain why stress-induced 2-AG increases in the PFC become more prominent over the course of repeated stress (Rademacher et al., 2008; Dubreucq et al., 2012; see Gray et al., 2014 and Morena et al., 2015 for review). This theory is supported by previous studies showing that stressful stimuli trigger increases in CRH release within the PFC (Merali et al., 2008). Our previous work showed that CRHR1 activation is sufficient to increase 2-AG levels in the PFC when CRHR1 agonist is administered intracerebroventricularly (Gray et al., 2015b), and our current findings suggest that exposure to chronic stress or corticosteroid exposure also increases CRH signaling capacity in the PFC. As such, it appears enhanced CRH expression and possibly release in the PFC following exposure to chronic stress or corticosteroids could result in amplified 2-AG release. However, our current data indicate that CRH overexpression in the forebrain had no effect on PFC 2-AG concentrations when examined under resting state conditions. The inability of CRH overexpression to change 2-AG levels in the PFC within our current study could be due to CRHR1 desensitization in the PFC, as reduced CRH binding in the frontal cortex is a known feature of CRH-associated human disorders (Nemeroff et al., 1988). Similarly in rodent models, CRH overexpressing mice also exhibit CRHR1 internalization and desensitization in some regions such as the locus coeruleus (Bangasser et al., 2013), although others (hippocampus, amygdala) display increased levels of CRHR1 (Lu et al., 2008). If this hypothesis of reduced CRHR1 expression in the PFC is correct, it would also suggest that FAAH activity displays a greater sensitivity to CRH modulation than 2-AG, as PFC FAAH/AEA changes did prevail on the background of CRH overexpression. Alternatively, despite the general consistency between rats and mice, with respect to the effects of stress and stress-mediators on the eCB system, it is also possible that the sensitivity of the relationship between CRH and the eCB system differs somewhat between rats and mice, at least with respect to the manipulations performed herein. Regardless, these data do ultimately indicate, that CRHR1 signaling

mediates the ability of sustained exposure to glucocorticoids to increase 2-AG content in the PFC.

Unlike 2-AG regulation in the PFC, amygdala basal levels of 2-AG were not modulated by CORT in the present study. This is consistent with previous studies showing that repeated restraint stress (Hill et al., 2010b), chronic stress (Hill et al., 2013), and chronic exposure to CORT (Bowles et al., 2012) have no effect on basal levels of 2-AG within the amygdala. However, they are in contrast to a previous study in which we found that daily injections of CORT (which would produce a more pulsatile exposure to CORT as opposed to the steady state increases produced by the pellet implantation used in the current study) resulted in an increase in 2-AG within the amygdala (Hill et al., 2005). In addition to the difference in route of administration, the dose of CORT was also much higher (20 mg/kg/day) and the duration of the study was 21 days (compared to 7 days in the current study). Thus, there are several methodological differences between that study and the current procedure, which could contribute to the difference in effect on 2-AG. The data do suggest that chronic CORT can elevate amygdala 2-AG levels in some circumstances, although the specifics of this require further investigation. In the current study, however, given that sustained CORT exposure had no effect on amygdala 2-AG levels, but CRH overexpression did significantly increase amygdala 2-AG concentrations, it appears that certain biological changes associated with the CRH overexpressing mice may have contributed to these effects. The line of mutant mice we employed have higher levels of CRHR1 mRNA and protein expression in the basolateral amygdala (Lu et al., 2008; Silberstein et al., 2009), therefore a heightened CRHR1 signaling capacity may have contributed to the 2-AG differences observed in these mice.

We also previously reported that intracerebroventricular administration of CRH was capable of increasing 2-AG content within the amygdala, although the temporal nature of this effect (2 h from onset) was much more delayed than the CRH-induced effects on FAAH/AEA dynamics. These data suggest 2-AG amygdala levels could be regulated by CRHR1, but display a considerably lower sensitivity to CRHR1-modulation, and a delayed temporal onset than AEA/FAAH changes. Future work is required to disentangle the specific mechanisms by which stress, CORT and CRH specifically regulate eCB signaling, and why these effects exhibit regional and temporal variations.

In conclusion, the current experiments extend previous findings that CRHR1 signaling has a stimulatory effect on FAAH enzymatic activity in the amygdala (Gray et al., 2015b), as well as in the PFC. Importantly, this CRHR1-FAAH interaction appears to be a fundamental mechanism driving the development of regional AEA reductions following exposure to acute stress (Gray et al., 2015b) and chronic exposure to CORT (current data). We have also demonstrated that PFC and amygdala 2-AG levels are upregulated by CRHR1 activation, although the specific mechanisms involved require further investigation to determine under what specific conditions these effects are reliably observed. Based on our findings that CORT-dependent increases in CRH dramatically reduce basal levels of corticoamygdala AEA, these data present a putative mechanism whereby chronic stress could reduce AEA signaling (Patel et al., 2005; Rademacher et al., 2008; Hill et al., 2010b, 2013). Given that inhibition of FAAH can ameliorate stress-induced anxiety following repeated social defeat

(Rossi et al., 2010), chronic restraint stress (Hill et al., 2013), and chronic unpredictable stress (Lomazzo et al., 2015), these data create a framework to further explore the stress-linked mechanisms contributing to states of compromised AEA signaling, and understanding its possible application to anxiety-like disorders (Kathuria et al., 2003). This work also suggests that AEA decreases occurring in circumstances of chronic stress may play a fundamental role in promoting specific CRHR1-associated negative symptoms (Zobel et al., 2000), making eCB-based drugs a promising avenue for the treatment of stress-related anxiety disorders. Finally, as the current CORT regimen most closely parallels glucocorticoid-based therapeutic regimens for an array of medical conditions, particularly inflammatory disorders, these data may also be relevant to understanding the mechanisms by which alterations in emotional behavior and anxiety emerge following sustained glucocorticoid treatment (Judd et al., 2014).

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Abbreviations

ACTH	adrenocorticotrophic hormone
AEA	<i>N</i> -arachidonylethanolamine
CB1	cannabinoid type 1 receptor
CORT	corticosterone
CRH	corticotropin-releasing hormone
CRHR1	CRH receptor type 1
CRH-OE	CRH overexpressing mice
FAAH	fatty acid amide hydrolase
GABA	gamma-aminobutyric acid
PFC	prefrontal cortex
WT	wild-type
2-AG	2-arachidonoylglycerol

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Highlights

- Chronic elevations in corticosterone (CORT) modulate endocannabinoid levels
- CORT-induced changes in endocannabinoids are mediated by CRH signaling
- Chronic overexpression of CRH modulates endocannabinoids similar to chronic CORT

Figure 1.

Implantation of subcutaneous 200 mg corticosterone (CORT) capsules increase peripheral plasma CORT and CRH mRNA levels in the prefrontal cortex (PFC) following 7 days of exposure compared to vehicle (VEH) controls. Data are expressed as mean + SEM for (A) plasma CORT and (B) levels of relative optical density for CRH mRNA. $n=8/\text{group}$. Panels (C-D) depict representative images of PFC CRH mRNA expression among (C) vehicle controls (VEH), and (D) CORT-treated (CORT) subjects. *, $p < 0.05$

Figure 2.

Implantation of subcutaneous 200 mg corticosterone (CORT) capsules modulates AEA (A,D) and 2-AG (B,E) levels, as well as the hydrolytic activity of the enzyme fatty acid amide hydrolase (FAAH; C,F) in the prefrontal cortex (PFC; upper panel) and amygdala (lower panel) compared to vehicle (VEH) controls, and these effects are blocked by the CRHR1 antagonist antalarmin (R1-ANT). Data are expressed as mean + SEM, n=8 for AEA and 2-AG and n=4 for FAAH data. *: p < 0.05

Figure 3.

Overexpression of CRH (CRH-OE) in genetically altered mice changes AEA (A,D) and 2-AG levels (B,E), as well as the hydrolytic activity of FAAH (C,F) in the prefrontal cortex (PFC; upper panel) and amygdala (lower panel). Data are expressed as mean + SEM, n=9-10 for AEA and 2-AG and n=3-4 for FAAH data. *; $p < 0.05$

Table 1

Corticosteroid regulation of FAAH binding affinity (Km) in the prefrontal cortex (PFC) and amygdala.

Treatment	PFC Km		Amygdala Km	
	MEAN ± SEM		MEAN ± SEM	
VEH-VEH	0.43	0.05	0.55	0.10
VEH-CORT	0.71	0.12	0.72	0.08 [*]
VEH- CRHR1 Antagonist	0.48	0.09	0.45	0.06
CORT- CRHR1 Antagonist	0.41	0.04	0.69	0.07 [*]

Mean ± SEM levels of FAAH binding affinity (Km) in the PFC and amygdala, under basal conditions following 7 days of exposure to 200 mg corticosterone (CORT) subcutaneous capsules, or capsules containing 60 mg CRHR1 antagonist, relative to vehicle (VEH) controls. n=4/group.

^{*} Denotes a significant main effect of CORT, $p < 0.05$.

Table 2

Influence of central CRH overexpression on FAAH binding affinity (Km) in the prefrontal cortex (PFC) and amygdala.

Treatment	PFC Km		Amygdala Km	
	MEAN ± SEM		MEAN ± SEM	
WT	0.67	0.14	0.66	0.07
CRH-OE	1.34	0.34	0.99	0.05 *

Mean ± SEM levels of FAAH binding affinity (Km) in the PFC and amygdala for a conditional line of genetically altered mice which centrally overexpress CRH. All tissue samples were collected under basal conditions. n=3-5/group.

* Denotes a significant effect of CRH overexpression (CRH-OE) relative to wild-type (WT) controls, $p < 0.05$.