

REVIEW

ANTAGONISM OF CANNABINOID 1 RECEPTORS REVERSES THE ANXIETY-LIKE BEHAVIOR INDUCED BY CENTRAL INJECTIONS OF CORTICOTROPIN-RELEASING FACTOR AND COCAINE WITHDRAWAL

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Abstract—The endocannabinoid (eCB) system is an important regulator of the stress response and mediates several stress-related behaviors, including anxiety. Despite anatomical evidence that eCBs interact with the principle stress peptide, corticotropin-releasing factor (CRF), few data exist that address functional interactions between these systems. Accordingly, we examined the effects of the CB1 receptor antagonist, AM251, on behavioral anxiety induced by (1) exogenous CRF, and (2) withdrawal from chronic cocaine exposure (mediated by CRF). After behavioral testing, we collected blood and assessed plasma corticosterone levels. In Experiment 1, male Long-Evans rats were pretreated with AM251 (0, 10, 100, or 200 μg , i.c.v.), followed by CRF (0 or 0.5 μg , i.c.v.), before testing for anxiety-like behavior in the elevated plus maze (EPM). In Experiment 2, rats were exposed to cocaine (20 mg/kg, i.p.) or saline for 14 consecutive days. Forty-eight hours following cocaine exposure, rats were pretreated with AM251 (0, 10, or 100 μg , i.c.v.) and tested in the EPM. AM251 produced an anxiogenic response at the highest dose, but reversed the behavioral anxiety induced by CRF and withdrawal from chronic cocaine in a dose-dependent manner. AM251 also increased plasma corticosterone levels, but did so irrespective of CRF treatment or cocaine preexposure. Our findings suggest that the anxiogenic effects of CRF and cocaine withdrawal are mediated, at least in part, by CB1 receptor transmission, and provide evidence in support of eCB-CRF interactions that are independent of the hypothalamic-pituitary-adrenal axis.

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Abbreviations: AEA, anandamide; AM251, CB1 receptor antagonist; BLA, basolateral amygdala; BNST, bed nucleus of the stria terminalis; CB, cannabinoid; CeA, central nucleus of the amygdala; CRF, corticotropin-releasing factor; eCB, endocannabinoid; EPM, elevated plus maze; HPA, hypothalamic-pituitary-adrenal; PFC, prefrontal cortex; PVN, paraventricular nucleus of the hypothalamus; SR141716, CB1 receptor antagonist; 2-AG, 2-arachidonoylglycerol.

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The neuropeptide, corticotropin-releasing factor (CRF), plays an important role in the central regulation of stress and anxiety. In addition to its well-characterized ability to activate the hypothalamic-pituitary-adrenal (HPA) axis (Dunn and Berridge, 1990), CRF functions as a neurotransmitter at extrahypothalamic brain sites to mediate physiological and behavioral responses to stress (Sawchenko et al., 1993; Sarmay et al., 2001; Rodaros et al., 2007). For example, i.c.v. injections of CRF induce behavioral anxiety (e.g. Baldwin et al., 1991; Spina et al., 2002) that is unaltered by manipulations of HPA activity, such as

hypophysectomy or dexamethasone pretreatment (Eaves et al., 1985; Britton et al., 1986; Berridge and Dunn, 1989).

Similarly, withdrawal from several drugs of abuse induces anxiety that is mediated by extrahypothalamic, or more specifically, amygdalar CRF transmission. For example, heightened anxiety-like responses in the elevated plus maze (EPM) after 24–48 h withdrawal from repeated daily cocaine injections (Sarnyai et al., 1995) are blocked by i.c.v. pretreatment with the CRF receptor antagonist, α -helical CRF_{9–41} (DeVries and Pert, 1998). These behavioral effects are associated with reduced CRF-like immunoreactivity and receptor binding (Sarnyai et al., 1995; Zorrilla et al., 2001; Ambrosio et al., 1997) and enhanced CRF mRNA and dialysate content (Zhou et al., 1996; Erb et al., 2003; Maj et al., 2003; Richter and Weiss, 1999) in the amygdala. Furthermore, CRF receptor antagonists injected directly into the central nucleus of the amygdala (CeA) attenuate withdrawal-induced conditioned place aversion in morphine-dependent rats (Heinrichs et al., 1995) and reverse the anxiogenic effects of ethanol withdrawal in rats (Rassnick et al., 1993).

In recent years, endocannabinoids (eCB), including anandamide (AEA) and 2-arachidonoylglycerol (2-AG), have emerged as critical regulators of behavioral and neuroendocrine responses to stress, primarily via their actions at presynaptic CB1 receptors. Behavioral findings demonstrate that pharmacological enhancement of CB1 receptor transmission is generally anxiolytic (e.g. Patel and Hillard, 2006; Rubino et al., 2007; but see Griebel et al., 2005), whereas pharmacological suppression of CB1 transmission is generally anxiogenic (e.g. Haller et al., 2004; Rodgers et al., 2005; but see Lafenêtre et al., 2007). These CB1-mediated effects, however, tend to be region-specific. Indeed, local injections of CB1 receptor agonists into the prefrontal cortex (PFC), ventral hippocampus, and CeA reduce anxiety-like behavior in the EPM (Rubino et al., 2008a,b; Zarrindast et al., 2008; but see Onaivi et al., 1995), whereas agonists injected into the dorsal hippocampus and basolateral amygdala (BLA) are anxiogenic (Roohbakhsh et al., 2007; Rubino et al., 2008a). Neuroendocrine studies reveal that stress-induced reductions in tonic AEA signaling, as seen after exposure to restraint stress in the rodent PFC (Hill et al., 2007), hippocampus (Hill et al., 2007), and amygdala (Patel et al., 2005b; Rademacher et al., 2008; Hill et al., 2009), may promote activation of the HPA axis (Hill et al., 2009). In contrast, stress-induced mobilization of 2-AG in the PFC, hippocampus, and hypothalamus, as demonstrated following restraint (Hill et al., 2007; see Hill and McEwen, 2010 for discussion), appears to play a role in terminating HPA activity (Di et al., 2003; Evanson et al., 2007; Hill and McEwen, 2010).

The eCB system has also been shown to mediate some behavioral effects of stress, including various drug- and ethanol-related behaviors. For example, CB1 receptor-deficient mice with a history of ethanol self-administration failed to show an increase in ethanol consumption following exposure to footshock stress, relative to their wild-type counterparts (Racz et al., 2003). More recently,

administration of the CB1 receptor antagonist, SR141716, before testing in the EPM reverses the reduction in time spent in the open arms by rats withdrawn from chronic exposure to ethanol (Rubio et al., 2008; Onaivi, 2008), diazepam, or cocaine (Onaivi, 2008). Taken together, these and other findings suggest that stress-induced mobilization of eCBs may mediate some effects of stress on behavior (Hill and McEwen, 2010).

Given the key roles that CRF and eCBs play in regulating anxiety, we hypothesized that these systems interact to mediate anxiety-like responses. Consistent with this idea, *in situ* hybridization studies reveal a high degree of colocalization between the mRNA of CB1 receptors and both CRF and CRF1 receptors within cortical and limbic brain regions known to be important for stress regulation (Hermann and Lutz, 2005; Cota et al., 2007). However, apart from evidence of an eCB-mediated suppression of hypothalamic CRF neurons (Di et al., 2003; Evanson et al., 2007, 2010), a direct functional interaction between eCB and CRF systems is largely unexplored. As such, we examined the effects of the CB1 receptor antagonist, AM251, on behavioral anxiety induced by (1) i.c.v. injections of CRF, and (2) withdrawal from chronic exposure to cocaine (mediated by CRF). Also, we measured plasma corticosterone levels to assess the possible involvement of the HPA axis in the behavioral effects.

EXPERIMENTAL PROCEDURES

Subjects

Male Long–Evans rats (Charles River, Montreal, QC, Canada; $N=162$; 275–300 g initial weight) were used in the experiments. Rats were individually housed in plastic cages in a temperature (21 ± 1 °C) and humidity-controlled vivarium and maintained on a reverse light-dark schedule (lights on 1900–0700) with free access to water and standard laboratory rat chow. All procedures were performed in accordance with Canadian Council of Animal Care guidelines and were approved by the University of Toronto animal care committee.

Surgery

Under isoflurane anesthesia (3–5% in O₂; Benson Medical, Markham, ON, Canada), rats were implanted with a 22-gauge cannula (Plastics One, Roanoke, VA, USA). The cannula was aimed 1 mm above the right lateral ventricle, according to the following stereotaxic coordinates: A/P: –1.0 mm from bregma; M/L: –1.4 mm from bregma; D/V: –2.7 mm from dura (Paxinos and Watson, 1997). The i.c.v. cannula was embedded in dental cement and anchored to the skull with jeweler's screws. At the end of the surgery, a stainless steel blocker, extending 1 mm beyond the cannula tip, was inserted into the cannula. Animals were given a 7-day recovery period before the start of any behavioral procedures.

Drugs

AM251 [(N-(piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide] (Tocris BioScience, Burlington, ON, Canada) was dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich, Oakville, ON, Canada) at concentrations of 2.5, 25, and 50 $\mu\text{g}/\mu\text{l}$ and injected in a volume of 4 μl (10, 100, 200 μg , i.c.v.). DMSO was injected as the vehicle in the 0 μg AM251 dose condition. CRF (Sigma Aldrich) was dissolved in

saline at a concentration of 0.125 $\mu\text{g}/\mu\text{l}$ and injected in a volume of 4 μl (0.5 μg , i.c.v.). AM251 and CRF were infused using 28-gauge stainless steel injectors that extended 1 mm below the tip of the cannula to the site of injection. Infusions took place over a 2-min period; injectors were left in place for an additional 2 min following infusion to prevent backflow. Cocaine HCl (Medisca Pharmaceuticals, St. Laurent, QC, Canada) was dissolved in saline at a concentration of 20 mg/ml and injected in a volume of 1 ml/kg (20 mg/kg, i.p.).

Apparatus

The EPM was equipped with two open and two closed arms (each 50×10 cm), radiating at 90° angles from an elevated center platform (10×10 cm). The maze was elevated 115 cm above the floor and placed in a darkened room, with dim lighting provided below each of the two closed arms. A video camera was positioned to record the movement of the rat in the maze.

Procedures

Experiment 1: Effects of AM251 on CRF-induced anxiety and corticosterone. EPM testing. In order to acclimatize animals to the transport and injection procedures used during testing, on each of three days before testing in the EPM, rats ($N=108$) were transported in their home cages to a darkened room adjacent to the testing room, where they were left undisturbed for 30 min. Rats were then given two sham i.c.v. injections separated by 30 min and, after an additional 30-min period, were returned to the colony room.

On the test day, rats were again transported to the darkened room and left undisturbed for 30 min. They were then injected with AM251 (0, 10, 100, or 200 μg , i.c.v.) and, 30 min later, with CRF (0 or 0.5 μg , i.c.v.). After an additional 30 min, rats were placed individually onto the center platform of the EPM, facing towards a closed arm, and allowed to explore the maze freely for 5 min. Video footage of the testing was captured, and an observer blind to the treatments later scored the footage for entries made into and time spent in the open and closed arms, as well as stretch-attend behaviors into the open arms. Rats were considered within an arm when both front paws and at least one rear paw were within the boundaries of the arm. Following each test, the maze was cleaned using a 70% ethanol solution and allowed to dry.

Plasma collection and corticosterone radioimmunoassay. Twenty-five minutes following testing in the EPM, rats were anaesthetized with isoflurane and sacrificed by decapitation. Trunk blood (2 ml) was collected and chilled to 4 °C on ice. Samples were then centrifuged (6000 rpm; 4 °C) for 5 min and plasma was stored at –80 °C. Immediately following decapitation, brains were extracted, frozen in –40 °C isopentane, and stored at –80 °C. Accurate cannula placement was subsequently confirmed by histological analysis.

Plasma corticosterone was measured using a double-antibody ^{125}I radioimmunoassay (ImmuChem 07-120103, MP Bio-medicals, Orangeburg, NY, USA), which has been used extensively for rat plasma (e.g. Sithisarn et al., 2011). Further, using a plasma pool from animals in this study, parallelism was confirmed between the standard curve and serially diluted plasma. Manufacturer's directions were followed except that the volumes of all reagents were halved and plasma diluted 1:300 (0.16 μl of plasma per tube). Each sample was measured in duplicate. The lowest point on the standard curve was 3.1 pg corticosterone/tube. Intra-assay variation was 4.2%, and inter-assay variation was 6.1% (Low Control) and 8.4% (High Control).

Experiment 2: Effects of AM251 on cocaine withdrawal-induced anxiety and corticosterone. Cocaine/saline preexposure. Seven days after surgery, a different cohort of rats ($N=54$) was injected daily with cocaine (20 mg/kg, i.p.) or saline for 14 days. All

injections were given during the dark phase of the light cycle, within 1–2 h of the time of day that their subsequent testing was performed. Rats were handled for 2 min on each preexposure day to reduce their basal anxiety. On each of the three final preexposure days, rats were habituated to the transport and injection procedures used during anxiety testing. Specifically, rats were transported in their home cages to a darkened room adjacent to the testing room, where they were left undisturbed for 30 min. Rats were then given a sham i.c.v. injection. Thirty minutes later, they were given a cocaine or saline injection and, after an additional 30 min, were returned to the colony room.

EPM testing. Forty-eight hours after the last cocaine/saline injection, corresponding to high levels of withdrawal-induced anxiety (e.g. Sarnyai et al., 1995), rats were tested in the EPM. Rats were transported to the darkened room adjacent to the testing room and left undisturbed for 30 min. Rats were then injected with AM251 (0, 10, or 100 μg i.c.v.). Thirty minutes later, all rats were given a sham i.p. injection to simulate the procedures used during the preexposure phase. After an additional 30 min, rats were given a 5-min test in the EPM, as described in Experiment 1.

Plasma collection and corticosterone radioimmunoassay. Twenty-five minutes after testing in the EPM, trunk blood and brains were collected as described for Experiment 1. Accurate cannula placement was subsequently confirmed by histological analysis, and plasma corticosterone levels were measured as described in Experiment 1.

Data analyses

In Experiment 1, behavioral and corticosterone data were analyzed using two-way ANOVAs for the factors of AM251 (0, 10, 100, 200 μg) and CRF (0, 0.5 μg). In Experiment 2, two-way ANOVAs for the factors of AM251 (0, 10, 100 μg) and preexposure (PE; Saline [SAL] PE, Cocaine [COC] PE) were used. For EPM data, separate ANOVAs were carried out for the dependent measures of time spent in the open arms, number of open arm entries, number of closed arm entries, total number of arm entries (open plus closed), percent ratio of open to closed arm entries, and number of stretch-attend behaviors. For corticosterone data, ANOVAs were conducted on concentration (ng/ml) in trunk plasma. Significant effects were followed by LSD post hoc comparisons, where appropriate. Pearson correlation coefficients ($P<0.05$) were also calculated between each EPM measure and plasma corticosterone levels.

RESULTS

Experiment 1: Effects of AM251 on CRF-induced anxiety and corticosterone

EPM testing. Two-way ANOVA of time spent in the open arms of the EPM (Fig. 1A) revealed a significant interaction of AM251 × CRF ($F(3,108)=5.70$, $P<0.001$). CRF treatment reduced open arm time in rats pretreated with 0 μg , but not with 10, 100, or 200 μg AM251 ($P<0.001$). Rats pretreated with 100 or 200 μg AM251 prior to a CRF challenge spent significantly more time in the open arms than those pretreated with 0 μg AM251 ($P<0.01$). In addition, at the highest dose (200 μg), AM251 reduced time spent in the open arms ($P<0.05$). Analyses of the percent ratio of entries into the open relative to closed arms (Fig. 1B) revealed similar results, including a significant AM251 × CRF interaction ($F(3,106)=4.21$, $P<0.01$) and comparable significant post hoc comparisons ($P<0.05$). Collectively, these results indicate that AM251,

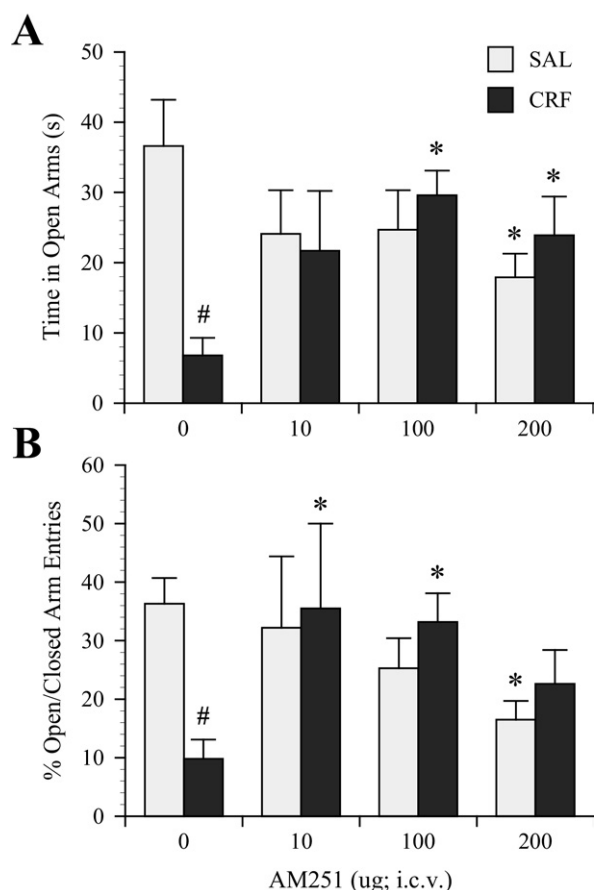


Fig. 1. Mean (\pm SEM) time spent in the open arms (A) and percent ratio of open to closed arm entries (B) during a 5-min test in the EPM by rats following administration of AM251 (0, 10, 100, or 200 μ g, i.c.v.) and CRF (0 [SAL] or 0.5 μ g, i.c.v.). [#] Different from SAL Condition, $P < 0.001$. * Different from 0 μ g AM251 condition, $P < 0.05$.

while anxiogenic at the highest dose, reversed the anxiety-like behavior induced by i.c.v. CRF.

Two-way ANOVA of open arm entries (Table 1) revealed a significant AM251 \times CRF interaction ($F(3,108) = 5.06$, $P < 0.005$). Comparable to the dependent measures depicted in Fig. 1, open arm entries were significantly reduced by CRF in rats pretreated with 0 μ g of AM251 ($P < 0.001$), and by the highest dose of AM251 in saline-treated rats ($P < 0.05$). Furthermore, pretreatment with 100 or 200 μ g AM251 blocked CRF-induced reductions in open arm entries ($P < 0.05$). In contrast, two-way ANOVA of

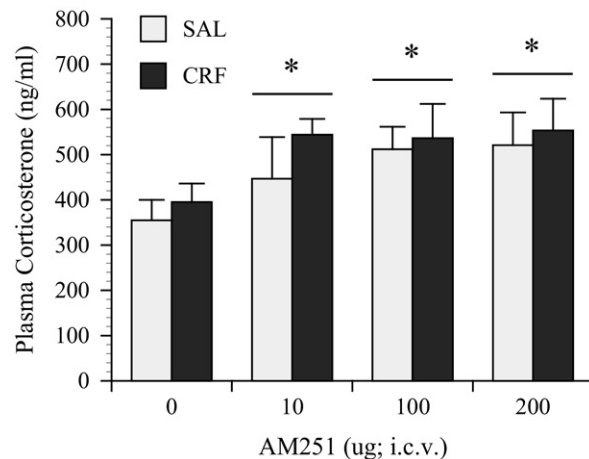


Fig. 2. Mean (\pm SEM) plasma corticosterone levels in rats following administration of AM251 (0, 10, 100, or 200 μ g, i.c.v.) and CRF (0 or 0.5 μ g, i.c.v.), and EPM testing. * Different from 0 μ g AM251 condition, $P < 0.05$.

closed and total arm entries (Table 1) revealed only overall reductions in rats given CRF (closed: $F(1,106) = 7.11$, $P < 0.01$; total: $F(1,106) = 9.47$, $P < 0.005$). All analyses of stretch-attend behaviors were nonsignificant (data not shown).

Corticosterone radioimmunoassay. Two-way ANOVA of plasma corticosterone levels (Fig. 2) revealed a significant main effect of AM251 ($F(3,59) = 3.07$, $P < 0.05$), attributable to elevated levels at all doses of AM251 relative to the 0 μ g condition ($P < 0.05$). Pearson correlations between each EPM measure and plasma corticosterone levels were all nonsignificant ($P > 0.05$).

Experiment 2: Effects of AM251 on cocaine withdrawal-induced anxiety and corticosterone

EPM testing. Two-way ANOVA of time spent in the open arms of the EPM (Fig. 3A) revealed a significant main effect of AM251 ($F(2,54) = 5.04$, $P < 0.01$). Rats given 10 μ g of AM251, relative to those given 0 or 100 μ g, spent more time in the open arms ($P < 0.05$). Although the AM251 \times preexposure interaction was not significant, inspection of Fig. 3A shows that the increased time spent in the open arms after AM251 pretreatment was apparent in cocaine, but not in saline, preexposed rats. Two-way ANOVA of the percent ratio of entries into the open relative to closed arms (Fig. 3B) revealed, in addition to a significant main

Table 1. Mean (\pm SEM) number of open, closed, and total arm entries made during a 5-min test in the EPM by rats following administration of AM251 (0, 10, 100, or 200 μ g, i.c.v.) and CRF (0 or 0.5 μ g, i.c.v.)

Treatment	Open arm entries				Closed arm entries				Total arm entries			
	0	10	100	200	0	10	100	200	0	10	100	200
SAL	4.0 (0.5)	2.7 (0.6)	2.9 (0.6)	1.7 (0.3)*	11.1 (0.8)	10.3 (1.1)	11.6 (0.7)	11.9 (0.8)	15.1 (1.0)	13.0 (1.0)	14.5 (1.0)	13.8 (0.9)
CRF	0.9 (0.3) [#]	2.4 (0.8)	2.7 (0.4)*	2.5 (0.7)*	9.9 (0.7)	9.1 (1.5)	8.6 (0.4)	10.9 (0.7)	10.8 (0.7)	11.6 (1.3)	11.4 (0.5)	13.4 (1.1)

[#] Different from SAL Condition, $P < 0.001$.

* Different from 0 μ g AM251 condition, $P < 0.05$.

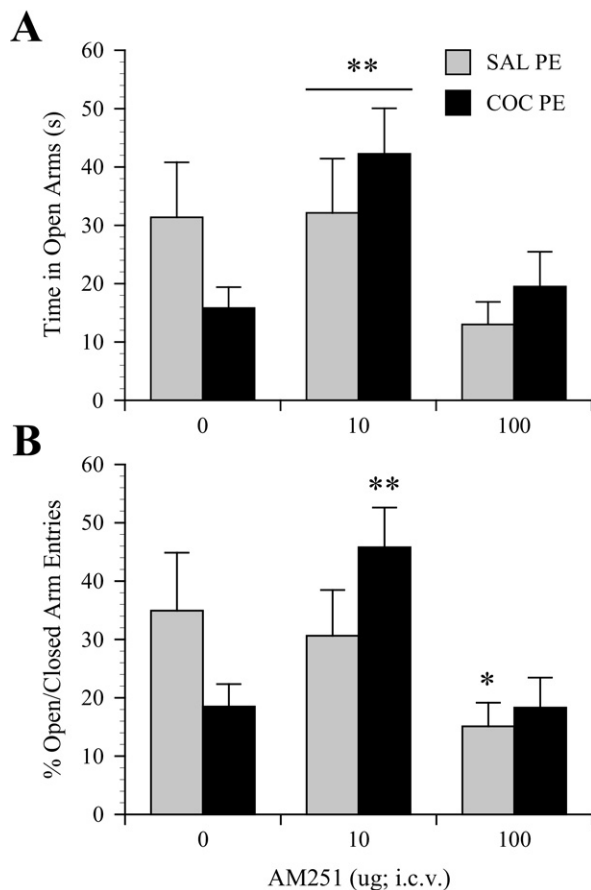


Fig. 3. Mean (\pm SEM) time spent in the open arms (A) and percent ratio of open to closed arm entries (B) during a 5-min test in the EPM by rats administered AM251 (0, 10, or 100 μ g, i.c.v.) 48 h following 14-d preexposure to saline or cocaine (20 mg/kg, i.p.). * Different from 10 μ g AM251 condition, $P < 0.05$. ** Different from 0 μ g and 100 μ g AM251 condition, $P < 0.05$ (A), $P < 0.005$ (B).

effect of AM251 ($F(2,54)=5.77$, $P < 0.01$), a significant interaction of AM251 \times preexposure ($F(2,54)=3.22$, $P < 0.05$). Rats preexposed to cocaine and pretreated with 10 μ g AM251 made proportionately more entries into the open relative to closed arms than did rats preexposed to cocaine and pretreated with 0 μ g or 100 μ g AM251 ($P < 0.005$). Consistent with the effects of AM251 pretreatment in Experiment 1, the highest dose of AM251 (in this case in saline preexposed animals) reduced the ratio of open to closed arm entries ($P < 0.05$). Collectively, these results indicate that AM251, despite having anxiogenic effects at

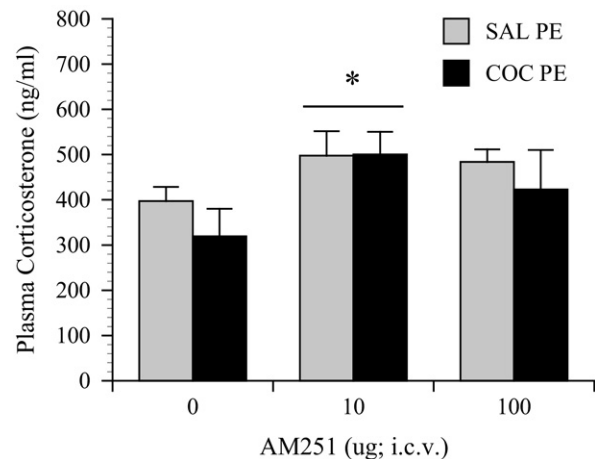


Fig. 4. Mean (\pm SEM) plasma corticosterone levels in saline and cocaine (20 mg/kg, i.p.) preexposed rats following AM251 (0, 10, or 100 μ g, i.c.v.) administration and EPM testing. * Different from 0 μ g AM251 condition, $P < 0.05$.

the highest dose, reversed the anxiety-like behavior induced by cocaine withdrawal.

Two-way ANOVA of open arm entries (Table 2) revealed a significant main effect of AM251 ($F(2,54)=3.89$, $P < 0.05$), attributable to increased entries by rats pretreated with 10 μ g, relative to 100 μ g AM251 ($P < 0.01$). In contrast, two-way ANOVA for closed and total arm entries (Table 2), as well as for stretch-attend behaviors (data not shown), revealed no significant effects.

Corticosterone radioimmunoassay. Two-way ANOVA of plasma corticosterone levels (Fig. 4) revealed a significant main effect of AM251 ($F(2,57)=3.50$, $P < 0.05$), attributable to elevated levels at the 10 μ g relative to 0 μ g dose ($P < 0.05$). Elevated levels of corticosterone in the group given 100 μ g AM251, relative to 0 μ g, approached significance ($P=0.062$). Pearson correlations between each EPM measure and plasma corticosterone levels were all nonsignificant ($P > 0.05$).

DISCUSSION

We report that the CB1 receptor antagonist, AM251, while anxiogenic in the EPM, reverses behavioral anxiety induced by CRF (Experiment 1) and withdrawal from chronic cocaine administration (Experiment 2) in a dose-dependent manner. Furthermore, although AM251 treatment elevated plasma corticosterone levels in both experiments, it

Table 2. Mean (\pm SEM) number of open, closed, and total arm entries made during a 5-min test in the EPM by saline or cocaine (20 mg/kg, i.p.) preexposed rats administered AM251 (0, 10, or 100 μ g, i.c.v.)

Treatment	Open arm entries			Closed arm entries			Total arm entries		
	0	10*	100	0	10	100	0	10	100
SAL PE	3.4 (1.0)	3.6 (1.0)	1.7 (0.5)	9.9 (0.9)	11.0 (0.7)	10.4 (0.9)	13.3 (1.4)	14.6 (1.6)	12.1 (1.3)
COC PE	2.1 (0.5)	4.3 (0.8)	2.1 (0.6)	11.7 (0.8)	9.3 (1.1)	11.6 (0.9)	13.8 (0.9)	13.5 (1.6)	13.8 (1.2)

* Different from 100 μ g AM251 condition, $P < 0.01$.

did so irrespective of whether animals were treated with i.c.v. CRF or were withdrawn from cocaine. Therefore, the effects of AM251 administration on plasma corticosterone did not parallel its effects on CRF- or cocaine withdrawal-induced anxiety, suggesting that the anxiogenic effects of CRF and cocaine withdrawal are mediated by CB1 receptor transmission, independent of HPA axis regulation.

The anxiogenic effects of i.c.v. CRF and cocaine withdrawal are in line with previous studies using similar dose and preexposure conditions (Spina et al., 2002; Sarnyai et al., 1995; Basso et al., 1999). Likewise, our findings are consistent with evidence that systemic administration of AM251 and other CB1 receptor antagonists induce anxiety-like behavior in the EPM (Navarro et al., 1997; Rodgers et al., 2005). Whereas the bed nucleus of the stria terminalis (BNST) (Sahuque et al., 2006) and BLA (Sajdyk et al., 1999) have been identified as regions important for mediating i.c.v. CRF-induced anxiety, and the CeA as critical for the expression of cocaine withdrawal-induced anxiety (Sarnyai et al., 1995; Zhou et al., 1996; Zorrilla et al., 2001; Erb et al., 2003; Maj et al., 2003), the brain sites involved in the effects of CB1 receptor agonists and antagonists on anxiety are unclear. Overall, local administration of CB1 receptor antagonists into specific brain nuclei (e.g. CeA, PFC) is largely without effect on anxiety (Zarrindast et al., 2008; Rubino et al., 2008b), whereas local CB1 receptor agonists induce bidirectional, region-specific anxiety effects (Roohbakhsh et al., 2007; Rubino et al., 2008a,b; Zarrindast et al., 2008).

The ability of AM251 to block the anxiogenic effects of CRF and cocaine withdrawal may seem at odds with a preponderance of evidence supporting an anxiolytic role for central eCB signaling (Patel and Hillard, 2006; Hill and McEwen, 2010). However, the present effects of AM251 are in fact consistent with several recent findings pointing to a facilitatory, rather than inhibitory, role for eCB signaling in some stress-related behaviors. Indeed, we have recently found that AM251 blocks CRF-induced reinstatement of drug seeking in rats with a history of cocaine self-administration (Kupferschmidt et al., 2010). Moreover, systemic pretreatment with the CB1 receptor antagonist, SR141716, reverses behavioral anxiety in the EPM induced by withdrawal from ethanol (Rubio et al., 2008; Onaivi, 2008), diazepam, or cocaine (Onaivi, 2008). It should be noted, however, that AM251, like many CB1 receptor antagonists, behaves under some conditions as an inverse agonist (Pertwee, 2005). In addition, AM251 has been shown to act as an antagonist at non-CB1 receptors (e.g. adenosine 1 receptors; Savinainen et al., 2003). Therefore, without clear evidence that i.c.v. CRF or cocaine withdrawal mobilizes eCBs, the possibility that AM251 reverses anxiety induced by these stressors independent of eCB activity cannot be ruled out.

We report here that AM251, but not CRF or cocaine withdrawal, reliably increased plasma corticosterone content. This effect of AM251 is consistent with the idea that eCB signaling negatively regulates activation of the HPA axis (Hill and McEwen, 2010). Specifically, CB1 receptor blockade may drive HPA activity by mimicking stress-

duced reductions in AEA content (Hill et al., 2009), and it prevents 2-AG signaling that would otherwise serve to terminate HPA activity (Di et al., 2003; Evanson et al., 2007, 2010; Hill and McEwen, 2010).

Although i.c.v. CRF (Dunn and File, 1987; Campbell et al., 2004) and acute cocaine withdrawal (Zhou et al., 2003, 2010) have been previously found to increase plasma corticosterone, our failure to detect such changes may be attributed to the fact that we collected blood from rats after testing in the EPM. Indeed, exposure to the EPM has, itself, been shown to increase plasma corticosterone levels (Suchecki et al., 2002). Therefore, it is possible that the EPM test sufficiently engaged the HPA axis so as to render any CRF- or cocaine withdrawal-induced corticosterone release undetectable.

As mentioned, our observed changes in HPA activity did not parallel or correlate with changes in behavioral anxiety; that is, the effects of AM251 on CRF- and cocaine withdrawal-induced anxiety were not reflected in plasma corticosterone content. These results suggest that while engagement of the HPA axis accounts, at least in part, for the anxiogenic effects of AM251 (which *did* lead to elevations in plasma corticosterone), the effects of AM251 on CRF- and cocaine withdrawal-induced anxiety are independent of HPA axis regulation. This interpretation is strengthened by evidence from several studies showing that CRF and CRF-mediated stressors can induce anxiety in animals with compromised HPA signaling (Britton et al., 1986; Berridge and Dunn, 1989; Pich et al., 1993). Moreover, the AM251-induced reversal of anxiety-like behavior is likely mediated via an interaction with extrahypothalamic CRF systems, given that CRF transmission in the extended amygdala mediates anxiety-like behavior induced by CRF and drug withdrawal (Walker et al., 2009; Rassnick et al., 1993; Sarnyai et al., 1995), and that eCB and CRF systems show pronounced overlap in extrahypothalamic brain regions known to be important for stress regulation (Herkenham et al., 1991; Sawchenko et al., 1993). Specifically, CB1 receptor mRNA is colocalized with the mRNA of both CRF (Cota et al., 2007) and the CRF1 receptor (Hermann and Lutz, 2005) in regions such as the PFC, BLA, CeA, and hippocampus. Thus, it is possible that AM251 modulates excitatory and inhibitory transmission in one or more of these regions (Auclair et al., 2000; Chiu et al., 2010; Katona et al., 2001; Domenici et al., 2006) to functionally block the effects of CRF and CRF-dependent cocaine withdrawal on anxiety-like behavior. One plausible scenario is that AM251 acts at CB1 receptors in the dorsal hippocampus and/or BLA, regions where CB1 receptor transmission induces behavioral anxiety (Roohbakhsh et al., 2007; Rubino et al., 2008a), to counter the anxiogenic effects of CRF and cocaine withdrawal. Interestingly, pretreatment with the CB1 receptor antagonist, SR141716, has been shown to attenuate BLA expression of Fos, a marker of neuronal activation, induced by restraint stress (Patel et al., 2005a).

AM251 may also interfere with CRF- and cocaine withdrawal-induced anxiety via its influence on transmitter systems downstream of CRF action, such as dopamine. For

example, both the BNST and CeA contain dopamine terminals that make direct synaptic contact with CRF neurons (Phelix et al., 1994; Eliava et al., 2003), and dopamine within these regions positively regulates CRF mRNA (Day et al., 2002) and peptide levels (Stewart et al., 2008). In turn, VTA dopamine neurons, innervated by CRF projections from the BNST and CeA (Rodaros et al., 2007), show an increased firing rate in response to CRF application *in vitro* (Wanat et al., 2008). Behaviorally, intra-amygdala infusion of dopamine D1-like receptor agonists and antagonists elicits anxiogenic and anxiolytic effects, respectively (de la Mora et al., 2010), and intra-BNST administration of the D1-like receptor antagonist, SCH23390, blocks CRF-enhanced startle responses (Meloni et al., 2006). Given that CB1 receptor antagonists suppress VTA dopamine cell firing (Pillolla et al., 2007), it is possible that AM251 induces its anxiolytic effects in CRF-treated and cocaine-withdrawn rats by suppressing dopamine release in the extended amygdala.

Finally, it warrants mention that there is recent evidence to suggest that socially isolated rats show altered eCB signaling and dysregulated endocrine and behavioral stress reactivity, relative to group-housed rats (Sciolino et al., 2010; Hermes et al., 2009; Toth et al., 2011). From this perspective, it is possible that our findings, obtained using animals that were singly housed, may not generalize to animals housed under other conditions. We believe, however, that this is unlikely to be the case given that anxiogenic effects of CRF (Meloni et al., 2006; Spina et al., 2002), CB1 receptor antagonists (Navarro et al., 1997; Arévalo et al., 2001), and cocaine withdrawal (Hall et al., 2010; Sarnyai et al., 1995) have been reliably demonstrated in both single- and group-housed rats.

CONCLUSION

In summary, we aimed to address functional interactions between eCB and CRF systems in mediating anxiety-like behavior. Our data suggest that CRF- and cocaine withdrawal-induced anxiety may add to an emergent collection of stress-related behaviors that are mediated by CB1 receptor transmission. Moreover, our findings suggest that the involvement of CB1 receptors in the anxiogenic effects of CRF and cocaine withdrawal is independent of HPA axis regulation, an observation that underscores the importance of future research aimed at exploring extrahypothalamic eCB-CRF interactions.

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