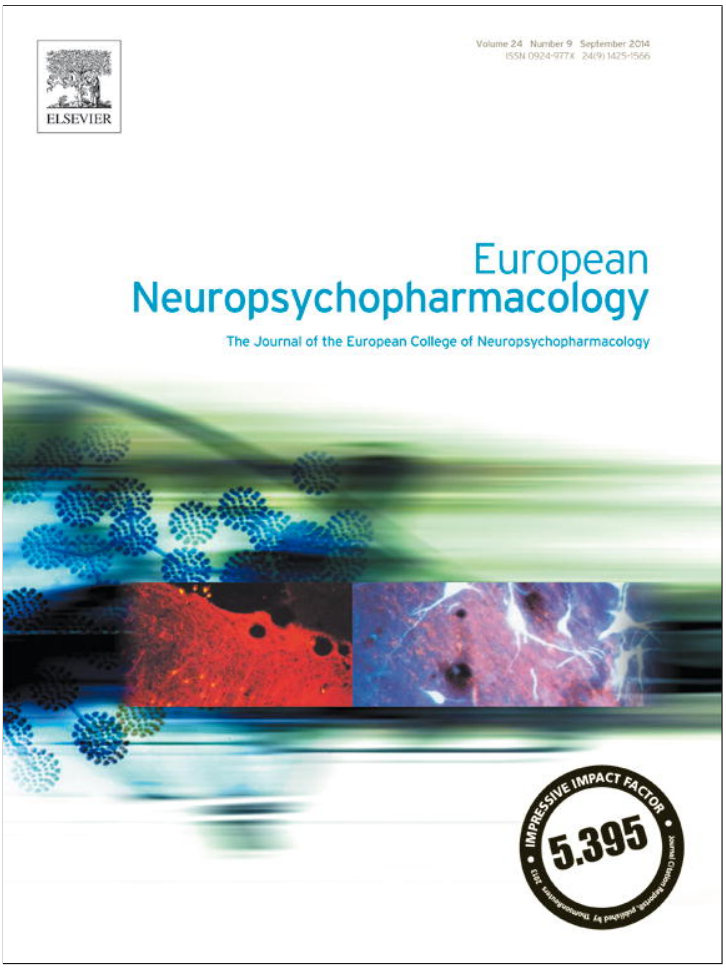

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Role of the basolateral amygdala in mediating the effects of the fatty acid amide hydrolase inhibitor URB597 on HPA axis response to stress

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Abstract
The endocannabinoid system is an important regulator of neuroendocrine and behavioral adaptation in stress related disorders thus representing a novel potential therapeutic target. The aim of this study was to determine the effects of the fatty acid amide hydrolase (FAAH) inhibitor URB597 on stress mediators of HPA axis and to study the role of the basolateral amygdala (BLA) in responses to forced swim stress.
Systemic administration of URB597 (0.1 and 0.3 mg/kg) reduced the forced swim stress-induced activation of HPA axis. More specifically, URB597 decreased stress-induced corticotropin-releasing hormone (CRH) mRNA expression in the paraventricular nucleus (PVN) of the hypothalamus, and pro-opiomelanocortin (POMC) mRNA expression dose-dependently in pituitary gland without affecting plasma corticosterone levels. URB597 treatment also attenuated stress-induced neuronal activation of the amygdala and PVN, and increased neuronal activation in the locus coeruleus (LC) and nucleus of solitary tract (NTS). Injection of the CB1 receptor antagonist AM251 (1 ng/side) in the BLA significantly attenuated URB597-mediated effects in the PVN and completely blocked those induced in the BLA.

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These results suggest that the BLA is a key structure involved in the anti-stress effects of URB597, and support the evidence that enhancement of endogenous cannabinoid signaling by inhibiting FAAH represents a potential therapeutic strategy for the management of stress-related disorders.

1. Introduction

Hypothalamic-pituitary-adrenal (HPA) axis is the main neuroendocrine system involved in the maintenance of homeostasis after stressful stimuli. The axis is composed of the paraventricular nucleus (PVN) of the hypothalamus, anterior pituitary gland and adrenal cortex (Herman and Cullinan, 1997). Stress stimulates parvocellular neurons in the PVN, to secrete the corticotropin-releasing hormone (CRH), which, in the anterior pituitary gland, drives the synthesis of adrenocorticotropic hormone (ACTH) from the precursor protein pro-opiomelanocortin (POMC). ACTH, released into the blood stream, reaches the adrenal cortex to stimulate corticosterone (CORT) secretion (Ulrich-Lai and Herman, 2009). CORT, in turn, both promotes mobilization of energy stores for rapid, adaptive responses to stress, and exerts a negative feedback to PVN and anterior pituitary to stop the further release of CRH and ACTH (Herman and Cullinan, 1997). Excitation of the HPA axis is driven by selected stress circuits and may vary depending on the type of stressor involved. Physical stress (hemorrhage, hypotension, respiratory distress) activates HPA axis through brainstem catecholaminergic pathways that project directly to CRH expressing neurons of the PVN, while psychological stressors (restraint, forced swimming) activate limbic forebrain structures like amygdala, hippocampus and the prefrontal cortex (Ulrich-Lai and Herman, 2009).

Several lines of evidences support the role of the endocannabinoid system as a modulator of the HPA (Haring et al., 2012; Patel et al., 2004) and of the behavioral responses to stress, including anxiety-related behaviors, mood tone and extinction of fear memories (Cota et al., 2007; Steiner et al., 2008).

The endocannabinoids (ECs) are an endogenous family of retrograde lipid messengers that activate CB1 and CB2 cannabinoid receptors (Piomelli, 2003). They are synthesized on demand through cleavage of membrane phospholipid precursors and are involved in various short-range signaling processes (Piomelli, 2003). The CB1 receptor is the most abundant G-protein-coupled receptor in the central nervous system, and it is expressed at presynaptic level to negatively control neurotransmitter release. Two principal ECs have been broadly characterized so far, namely N-arachidonoyl ethanolamide or anandamide (AEA) and 2- arachidonoylglycerol (2-AG) (Piomelli, 2003). Their actions are terminated by a putative uptake process, followed by degradation by fatty acid amide hydrolase (FAAH) and by monoacylglycerol lipase, respectively. FAAH is widely distributed in the rat brain, mostly in cell bodies juxtaposed to axon terminals that express CB1 receptors suggesting that FAAH activity has an important role in the postsynaptic inactivation of AEA (Piomelli, 2003).

The pharmacological manipulation of AEA tone in the brain has been shown to control several neurobehavioral aspects related to HPA axis activation. Pharmacological inhibition or genetic deletion of FAAH induces analgesia, enhance memory extinction and attenuate anxiety-like behavior *via* an increased activation of CB1 receptors (Bambico et al., 2010; Cassano et al., 2011; Moreira et al., 2008). Selective FAAH inhibitors that significantly increase the brain levels of AEA, but not 2-AG, are currently available. Among these is URB597, which inhibits FAAH activity both *in vitro* (IC_{50}=4.6 nM in rat brain membranes and IC_{50}=0.5 nM in intact neurons) and *in vivo* (ID_{50}=0.15 mg/kg, intraperitoneally (i.p.), in the rat) (Kathuria et al., 2003). By increasing AEA tone and therefore indirectly activating CB1 receptors, URB597 has been shown to elicit marked anxiolytic-like and antidepressant-like responses in different behavioral paradigms, including the elevated zero maze test and the forced swimming test in adult rats, the isolation-induced ultrasonic vocalization test in rat pups, and the tail suspension test in adult mice (Kathuria et al., 2003). Such effects were not accompanied by the broad spectrum of cannabimimetic behavioral actions (Kathuria et al., 2003), and were associated to an increased firing of noradrenergic and serotonergic neurons, respectively, the locus coeruleus (LC) and the dorsal raphe of adult rats, and by an increase of the extracellular levels of serotonin in the hippocampus, an area receiving projections from dorsal raphe (Gobbi et al., 2005). These observations suggested that modulation of endogenous AEA tone, rather than administration of direct CB1 agonists, could represent a novel approach for the treatment of anxiety- and depression-related disorders. Moreover, inhibition of FAAH along with the blockade of transient receptor potential vanilloid type-1 (TRPV1) has been suggested to exert robust anxiolytic effect (John and Currie, 2012). The FAAH inhibition approach could be also useful to modulate the neurobehavioral responses to stress, thus providing a new approach to treat other stress-related neuropsychiatric conditions, such as the posttraumatic stress disorder (Varvel et al., 2007). This hypothesis is supported by the observation that AEA content in the amygdala of rodents subjected to acute restraint stress is significantly decreased, presumably as consequence of increased FAAH activity, and that amygdala AEA levels are indirectly correlated to blood CORT levels (Hill et al., 2009). Based on these considerations, in this study we tested the potential anti-stress effects of systemically administered URB597 to rats subjected to a forced swimming stress. Specifically, we investigated the impact of FAAH inhibition on markers of HPA-axis activation, such as plasma CORT levels, POMC mRNA in the pituitary gland and CRH mRNA in the hypothalamus, as well as on stress-induced neuronal activity in the PVN, the amygdala and the

brainstem, as assessed by *c-fos* (a neuronal activity marker) mRNA induction.

This stress protocol is a modified version of the Porsolt forced swim test which was previously used in our laboratory to demonstrate the antidepressant-like properties of URB597 (Gobbi et al., 2005). Different from our previous study, here animals were not habituated to the test situation, but rather were faced directly to an inescapable 20-min stress. This protocol allowed us to investigate the modulatory effect of URB597 on coping responses to stress never experienced before. It has been clearly demonstrated that varying the environmental conditions and the stressful procedures can lead to different observations (Herman and Cullinan, 1997), thus demonstrating that often the pharmacological actions of a drug cannot be generalized from the specific experimental model used. Most of the available data on the effects of URB597 in acute and repeated stress exposure were generated from animals subjected to restraint stress (Hill et al., 2009; Patel et al., 2004). Therefore using a different experimental protocol of stress gave us also the opportunity to evaluate whether the anti-stress properties of URB597 are independent from the specific experimental context.

Finally, the current literature points at the BLA as a key player in the modulation of the stress response by ECs (Hill et al., 2009) as it is also suggested by the effects of BLA CB1 blockade by the CB1 antagonist AM251 on anxiogenic behavior (Dono and Currie, 2012). Therefore, we investigated also the role played by CB1 receptors in this area in mediating the effects of acute FAAH inhibition.

2. Experimental procedures

2.1. Animals

Male Wistar rats (300–350 g) from Harlan Laboratories, Italy were used in these experiments. Upon arrival, the animals were housed individually in a temperature and humidity controlled room with access to food and water *ad libitum*. The room was maintained at 12:12 h light/dark cycle, with light on at 06:30 h. All procedures met the guidelines from the Italian Ministry of Health, from the United States National Institutes of Health, detailed in the Guide for the Care of Laboratory Animals, and from the European Community directives 86/609/EEC regulating animal research. Animals were accustomed to handling for 7 days before experiments. During the experiments, animals were housed four per cage, euthanized at respective endpoints and brain, pituitary gland and trunk blood were immediately collected. Pituitary glands and brains were snap frozen in 2- methylbutane ($-50\,^{\circ}C$) and stored at $-80\,^{\circ}C$. All efforts were made to minimize animal suffering and to reduce the number of animals used. To this purpose, all the different endpoints considered in this study were measured on the same animals.

2.2. Drugs

URB597 (Sigma, USA) was freshly dissolved in a vehicle (2 ml/kg) made of 5% PEG-400, 5% Tween 80 in saline. AM251 (Tocris bioscience, UK) was dissolved in 5% DMSO, 2% Tween 80 in saline.

2.3. Experiment 1

Thirty minutes after the administration of URB597 or the respective vehicle, rats were either left undisturbed in their home cages (control rats) or were forced to swim for 20 min in a plastic cylindrical water pool (45 cm height $\times$ 30 cm diameter) filled with tap water at $22\pm0.1\,^{\circ}C$ to a depth of 30 cm. URB597 was administered through the intraperitoneal route (i.p.) at 0.1 or 0.3 mg/kg dose. These dosages, as well as 30-minutes interval between URB597 administration and stress exposure, were selected on the basis of our previous observations on the anxiolytic- and antidepressant-like effects in rodents induced by URB597 administration (Kathuria et al., 2003). Moreover, previous studies by other authors used the same time lag between URB597 administration (at dosages comparable to the ones used in our study) and stress exposure (Patel et al., 2004; Roberts et al., 2014).

After completion of the swimming stress procedure, rats were either euthanized (50 min from URB597 or vehicle injection) or carefully dried with a towel and put back into their home cage, to be euthanized 20 minutes later (70 min from URB597 or vehicle injection). Control rats were euthanized at the same time points of stressed rats (Figure 1A). Therefore, experiment 1 included 2 groups of animals (sacrificed at 20 and 40 min, respectively, from the beginning of the stress procedure), each made of 4 subgroups ($n=5$-6/subgroup) as follows: animals injected with vehicle and not exposed to stress (Control); animals injected with vehicle and exposed to the swim stress (Stress); animals injected with URB597 0.1 mg/kg, i.p. and exposed to the swim stress (URB 0.1+Stress); and animals injected with URB597 0.3 mg/kg, i.p. and exposed to stress (URB 0.3+Stress) (Figure 1A).

2.4. Experiment 2

A separate set of animals was anesthetized with equithesin (3.5 ml/kg i.p.) and positioned on a stereotaxic frame. All rats were implanted bilaterally with a stainless steel guide cannula of 23 G aimed at the BLA [stereotaxic coordinates from bregma, AP, -2.8 mm, ML ±4.8 mm, DV -7.5 mm; (Paxinos and Watson, 1998)]. Three steel screws and dental acrylic were used to fix the guide cannulae to the skull. After surgery, animals were allowed to recover for one week, during which 30 G stainless-steel stylets were inserted into the guide cannulae to avoid their blockage. On the day of the experiment, the CB1 receptor antagonist AM251 or its vehicle was administered through the implanted cannulae. Specifically, two injection cannulae of 30 G were connected *via* polyethylene PE10 tubing to two Hamilton microsyringes (50 μl) driven by a microinfusion pump (CMA-400 Microdialysis). The injection cannulae protruded 1.0 mm beyond the guide cannulae implanted in the brain. Microinjection of AM251 (1 ng/side) or corresponding vehicle was performed bilaterally in 500 nl volume per side delivered over 1 min. The injection cannulae were left in position for an additional 1 min before withdrawal, to prevent backflow of the injected liquid.

Experiment 2 included 2 groups of animals as described above with respect to stress application (20 min and 40 min, respectively), each made of 6 subgroups ($n=6$/group) as follows: animals receiving the respective vehicles by intra-BLA and i.p. injection and not exposed to stress (Control); animals injected with vehicle (intra-BLA) and URB597 (0.1 mg/kg, i.p.) and not exposed to stress (URB597); animals injected with AM251 (1 ng/side intra-BLA) and vehicle (i.p.) and not exposed to stress (AM251); animals receiving the respective vehicles by intra-BLA and i.p. injection and exposed to 20-min swim stress (Stress); animals injected with vehicle (intra-BLA) and URB597 (0.1 mg/kg, i.p.) and exposed to the swim stress (URB 0.1+Stress); animals injected with AM251 (1 ng/side intra-BLA) and URB597 (0.1 mg/kg, i.p.) and exposed to stress (AM251 +URB 0.1+Stress) (Figure 4A). The cannulae position was verified postmortem by staining brain slices with cresyl violet using the atlas of Paxinos and Watson (1998) as reference. The histological analysis showed that 86% of cannulae placements were in the desired structure (Figure 4B). Subjects with cannulae outside the area of interest were excluded from the study.

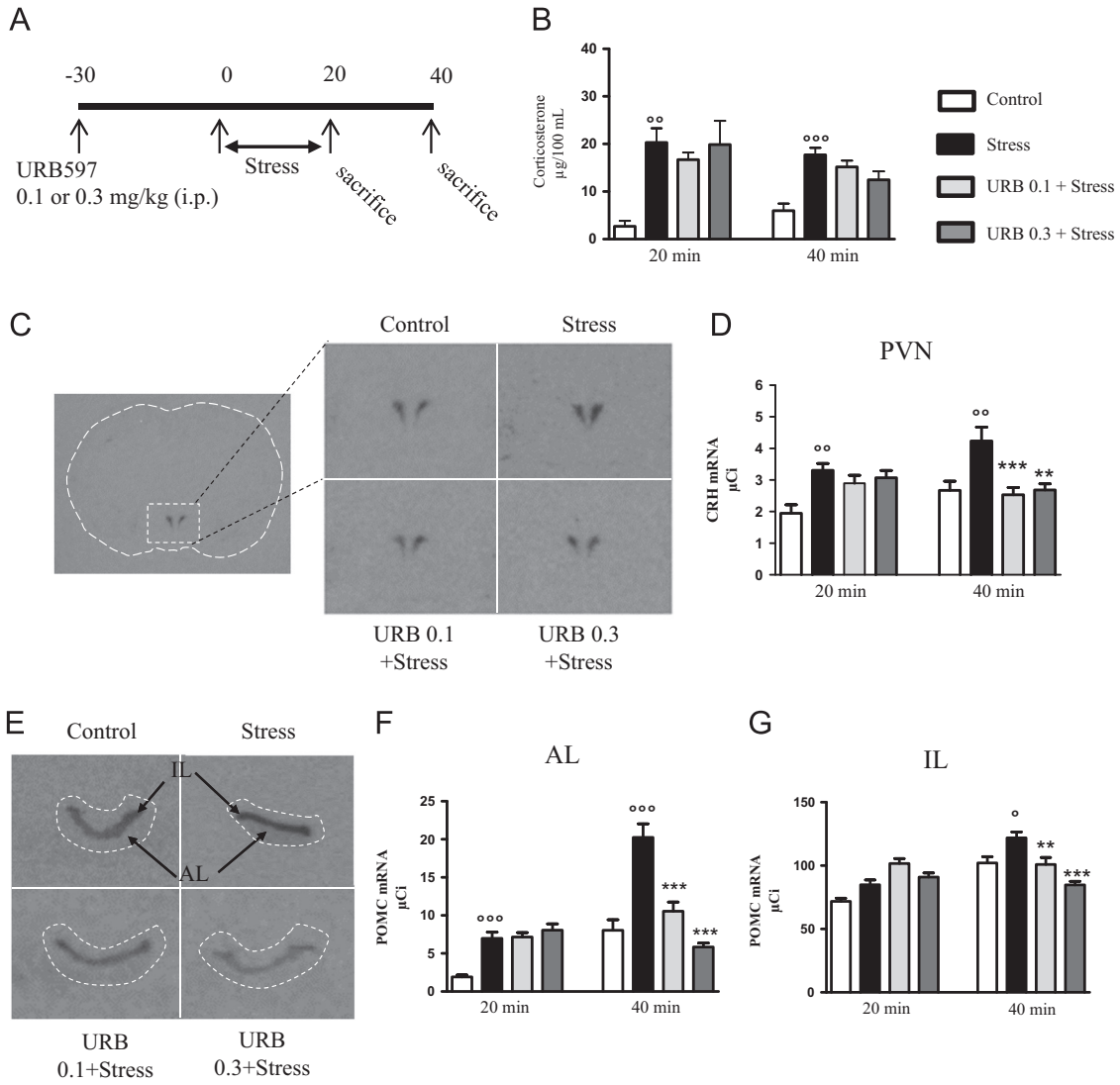

Figure 1 (A) Schematic diagram of protocol used in experiment 1. (B) Effect of acute blockade of fatty acid amide hydrolase (FAAH) by URB597 treatment on plasma corticosterone (CORT) levels. (C) Representative micrographs of CRH mRNA expression in PVN at 40 min taken from scanned autoradiographic film. (D) Effect of URB597 treatment on CRH mRNA levels in PVN. (E) Representative micrographs of POMC mRNA expression in pituitary gland at 40 min taken from scanned autoradiographic film. (F and G) Effects of URB597 treatment on POMC mRNA expression in (F) anterior lobe (AL) and (G) intermediate lobe (IL) of pituitary gland. Data are expressed as average ± SEM (n=5/6 per group). $^{\circ\circ\circ}p<0.001$, $^{\circ\circ}p<0.01$, $^{\circ}p<0.05$ *vs* respective control group; $^{***}p<0.001$ and $^{**}p<0.01$ *vs* respective stress group (Tukey's test for multiple comparisons).

2.5. Corticosterone measurement

Trunk blood was collected in pre-chilled EDTA-tubes. Whole blood was then centrifuged at 2000 × *g* for 30 min to obtain plasma. Plasma samples were aliquoted and frozen at −80 °C. To measure CORT, a competitive enzyme immunoassay (EIA) kit was used (Enzo Life Sciences, Italy). Briefly, 97.5 µl of plasma was added to 2.5 µl of CORT displacement reagent to displace bound CORT present in plasma. The plasma samples were diluted 1:50, and the assay was carried out according to manual instructions.

2.6. *In situ* hybridization

Serial brain coronal sections of 20 µm thickness were cut on a cryostat (−20 °C) and thaw-mounted on RNAse-free positively charged slides. Antisense and sense *c-fos*, CRH and POMC riboprobes were transcribed respectively from rat *c-fos* 667-bp, rat CRH 950-bp, and POMC 599-bp fragments subcloned into pCRII kindly provided by Dr. Jin Fu (University of California, Irvine, USA) in the presence of both, ^{35}S UTP and ^{35}S CTP. Brain sections were then hybridized at 60 °C for 16 h in a buffer containing [^{35}S]cRNA (~45,000 dpm ml^{-1}), 10% dextran sulfate, 50% formamide, 1 × Denhardt's solution, 100 µg ml^{-1} denatured salmon sperm DNA, 0.15 mg ml^{-1} tRNA and 40 mM dithiothreitol. After *c-fos*, CRH and POMC hybridization, brain sections were exposed to Kodak Biomax film (Sigma-Aldrich) for 72, 16, and 16 h, respectively.

2.7. Densitometric analyses

Semi-quantitative analysis was conducted as described in our previous study (Gaetani et al., 2010). Briefly, autoradiography films were scanned (Epson perfection 3200 PHOTO) at high resolution (900 dpi). Brain atlas (Paxinos and Watson, 1998) was used to define

localization of brain areas of interest. Optical densities were converted into radioactivity concentrations by densitometric analysis of ^{14}C-microscale standards (American Radiolabeled Chemicals) to create a calibration curve with a linear coefficient $r^2>0.9$ for each film. Measurements were obtained from at least 4 consecutive tissue sections containing the desired brain structure.

2.8. Statistical analysis

The results are expressed as mean values ± SEM. The effects of URB597 on stress-induced CORT secretion, CRH, POMC, and *c-fos* mRNA expression were analyzed by one way analysis of variance (ANOVA) (Hill et al., 2009; Patel et al., 2004). All post hoc comparisons were made using Tukey's test for multiple comparisons. The threshold for statistical significance was always set at $p<0.05$.

3. Results

3.1. Experiment 1

3.1.1. Forced swim induces CORT secretion

Plasma CORT levels were measured to confirm that 20 min of swimming stress activated the HPA axis and to evaluate the effect of URB597 on this parameter.

Stress exposure significantly increased plasma CORT levels in rats at both 20 ($p<0.01$) and 40 min ($p<0.001$). Moreover, one-way ANOVA revealed no significant effect of URB597 treatment on plasma CORT levels of stressed-animals at either time points (Figure 1B).

3.1.2. FAAH inhibition attenuates stress-induced CRH mRNA expression in PVN

The hybridization signal for CRH mRNA was evident in the PVN (Figure 1C) and significantly increased at 20 and 40 min ($p<0.01$) after stress exposure. Moreover, the statistical analysis of average optical densities revealed a significant effect of URB597 on stress-induced CRH mRNA levels at 40 min ($F_{3,106}=6.322$, $p=0.0006$), but not at 20 min. *Post hoc* analysis revealed that both doses of URB597 significantly attenuated stress-induced expression of CRH mRNA compared to vehicle-treated stressed rats at 40 min ($p<0.001$ and $p<0.01$, for URB 0.1 and URB 0.3, respectively) (Figure 1D). In CeA, neither stress nor URB597 treatment significantly altered CRH mRNA levels (data not shown).

3.1.3. FAAH inhibition attenuated stress-induced POMC mRNA expression in pituitary gland

Overall POMC expression was higher in the intermediate than the anterior lobe of the pituitary gland (Figure 1E-G). Twenty min of stress significantly increased POMC mRNA expression in both anterior ($p<0.001$ at 20 and 40 min) and intermediate lobes ($p<0.05$ at 40 min) of the pituitary gland. URB597 treatment significantly affected stress-induced POMC mRNA expression in the anterior ($F_{3,\ 563}=27.71$, $p<0.0001$) and intermediate lobe ($F_{3,\ 448}=11.89$, $p<0.0001$) of the pituitary gland at 40 min, but not at 20 min. *Post hoc* analysis revealed that both doses of URB597 significantly decreased stress-induced POMC levels at 40 min in anterior ($p<0.001$) and intermediate lobe of pituitary gland ($p<0.01$ and $p<0.001$ for URB 0.1 and URB 0.3, respectively).

3.1.4. FAAH inhibition attenuates stress-induced *c-fos* mRNA expression

Twenty min of swimming stress significantly increased *c-fos* mRNA expression in different brain regions involved in the HPA axis activation, including BLA ($p<0.01$ at 20 min and $p<0.001$ at 40 min), CeA ($p<0.05$ at 40 min), MeA ($p<0.001$ at 20 min and 40 min) and PVN ($p<0.001$ at 20 min and 40 min) (Figure 2A-F).

In BLA, URB597 treatment had a significant effect on stress-induced *c-fos* mRNA expression at 40 min ($F_{3,\ 119}=10.92$, $p<0.0001$), but not at 20 min. *Post hoc* analysis revealed that both doses of URB597 treatment significantly dampened the stress-induced *c-fos* mRNA elevation ($p<0.01$ and $p<0.001$) at 40 min (Figure 2C). URB597 treatment also affected stress-induced *c-fos* mRNA expression in CeA at 20 min ($F_{3,\ 120}=14.44$, $p<0.0001$) and 40 min ($F_{3,\ 118}=5.422$, $p=0.0016$). *Post hoc* analysis revealed that at 20 min 0.1 mg/kg dose of URB597 significantly increased *c-fos* mRNA levels ($p<0.001$) compared to vehicle-treated stressed rats (Figure 2D). Conversely, at 40 min only the highest dose of URB597 significantly attenuated ($p<0.05$) stress-induced *c-fos* mRNA expression (Figure 2D).

In MeA, URB597 treatment showed a significant effect on stress-induced *c-fos* mRNA expression at both time points (20 min, $F_{3,\ 104}=53.12$, $p<0.0001$; 40 min, $F_{3,\ 118}=33.34$, $p<0.0001$). *Post hoc* analysis revealed that the highest dose of URB597 attenuated stress-induced *c-fos* mRNA expression at both time points ($p<0.001$) (Figure 2E).

In PVN, URB597 treatment affected *c-fos* mRNA expression at 20 min ($F_{3,\ 53}=34.90$, $p<0.0001$) and 40 min ($F_{3,\ 45}=29.29$, $p<0.0001$). *Post hoc* analysis showed a significant effect of either doses of URB597 at 20 min ($p<0.001$); *c-fos* remained attenuated also at 40 min but only in animals treated with the highest dose ($p<0.01$) (Figure 2F).

As shown in Figure 3, 20 min of swimming stress induced *c-fos* mRNA expression in two hindbrain regions, the LC and the nucleus of solitary tract (NST). In both areas, URB597 treatment significantly increased the stress-induced *c-fos* mRNA expression (LC, $F_{3,\ 98}=13.52$, $p<0.0001$; NTS, $F_{3,\ 85}=16.91$, $p<0.0001$) at 20 min but not at 40 min (Figure 3C). In particular, in LC URB597 effect reached statistical significance only at the lowest dose ($p<0.01$) (Figure 3B and C left panel), while in NTS both doses of URB597 ($p<0.001$ and $p<0.01$, for URB 0.1 and URB 0.3, respectively) were effective (Figure 3B and C, right panel).

3.2. Experiment 2

3.2.1. Pharmacological blockade of CB1 receptors in BLA reverses URB597 effect on the stress-induced increase of *c-fos* mRNA

To explore whether the activation of CB1 receptors expressed in BLA was involved in the dampening effects of URB597 on *c-fos* levels in PVN and BLA, the CB1 antagonist AM251 (1 ng/500 nl/side) or its corresponding vehicle was injected bilaterally into the BLA (experiment 2) at the same time of URB597 systemic administration (Figure 4A and B). In this experiment the effects of either pharmacological treatment alone in non-stressed rats were also assessed. The statistical analyses of the results showed that in non-stressed rats URB597 treatment caused an early (20 min) increase of

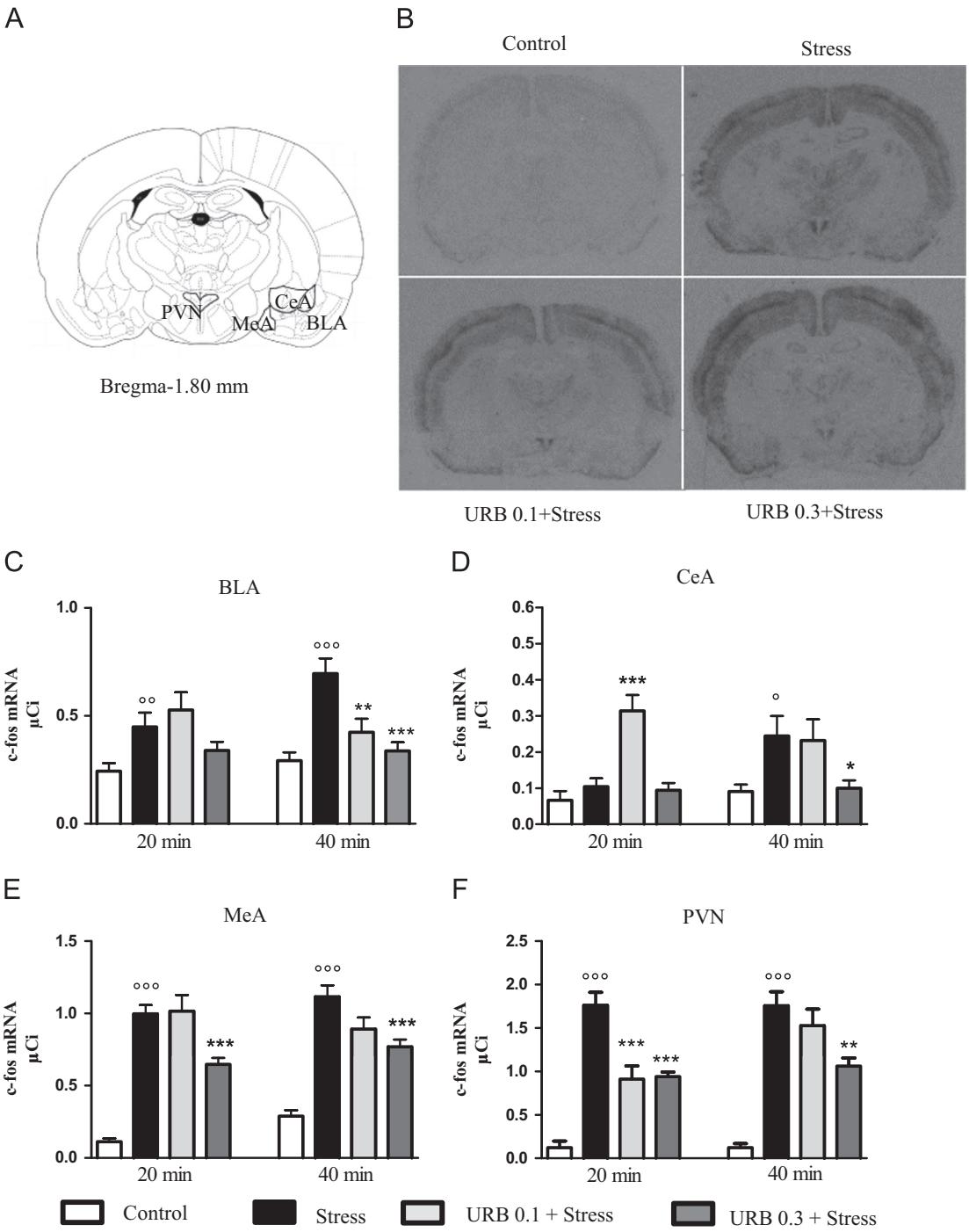

Figure 2 Effect of URB597 treatment on forced swimming induced *c-fos* expression in the brain. (A) Brain diagram adapted from Paxinos and Watson (1998) showing the anatomical location of PVN, BLA, CeA and MeA; (B) Representative micrographs of rat brain coronal sections scanned from autoradiographic film showing *c-fos* mRNA expression in the brain at 20 min. (C–F) Effects of URB597 treatment on forced swim induced *c-fos* expression in BLA (C), CeA (D), MeA (E) and PVN (F). Data are expressed as average ± SEM (n=5/6 per group). °°°$p<0.001$, °°$p<0.01$, °$p<0.05$ *vs* respective control group; ***$p<0.001$, **$p<0.01$, and *$p<0.05$ *vs* respective stress group (Tukey's test for multiple comparisons).

c-fos mRNA in BLA ($p<0.05$, Figure 4C) and MeA ($p<0.01$, Figure 4E), as compared to control rats treated with vehicle. A similar early increase was also observed in BLA ($p<0.01$), CeA ($p<0.05$), MeA ($p<0.01$) but not in PVN, of rats treated with AM251 and not subjected to stress (Figure 4C–F). In these animals, we observed the same effects of URB597 treatment, as previously seen in experiment 1, in BLA, MeA and PVN at both 20 and 40 min (Figure 4C, E and F). Minor differences in *c-fos* mRNA expression pattern were observed at the earliest time point in CeA as compared to experiment 1. In particular, at 20 min, the CeA of stressed rats of experiment 2 showed higher *c-fos* mRNA levels than control rats (Figure 4D), whereas

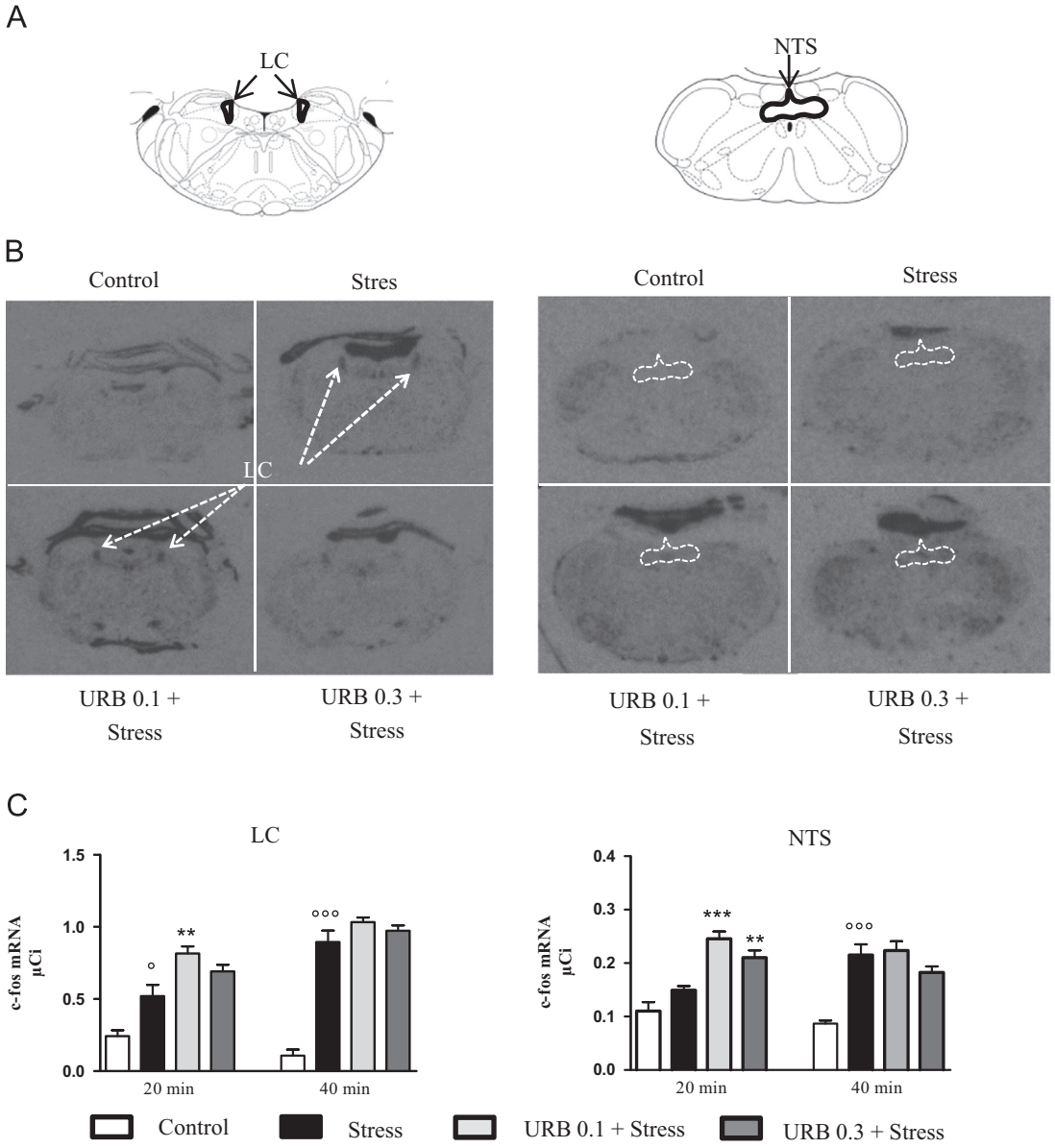

Figure 3 Effects of URB597 treatment and forced swimming stress on neuronal activation of LC and NTS. (A) Brain diagram adapted from Paxinos and Watson (1998) showing the anatomical location of LC (left panel) and NTS (right panel). (B) Representative micrographs of rat brain coronal sections scanned from autoradiographic film showing *c-fos* mRNA expression in the LC (left panel) and in the NTS (right panel) at 20 min. (C) Effects of URB597 treatment on *c-fos* mRNA expression levels in LC (left panel) and NTS (right panel). Data are expressed as average ± SEM ($n=5/6$ per group). $^{\circ\circ\circ}p<0.001$, $^{\circ}p<0.05$ *vs* respective control group; $^{***}p<0.001$ and $^{**}p<0.01$ *vs* respective stress group (Tukey's test for multiple comparisons).

this difference was not observed in experiment 1 (Figure 2D). This small difference might be due to cannulation. Also, the significant increase of *c-fos* in the CeA of rats treated with 0.1 mg/kg of URB597 at 20 min was not evident in experiment 2 at the same time point (Figure 4D). The significant difference might have been covered due to increased *c-fos* levels in stress group rats of experiment 2.

In BLA, there was a significant effect of URB597 administration on stress-induced *c-fos* mRNA expression ($F_{5,300}=14.91$, $p<0.0001$), as observed in experiment 1 at 40 min. *Post hoc* analysis revealed that 0.1 mg/kg of URB597 significantly attenuated stress-induced *c-fos* mRNA ($p<0.01$) (Figure 4C). Animals that received an intra-BLA infusion of AM251 with a systemic injection of URB597 exhibited no differences in stress-induced *c-fos* mRNA levels compared to vehicle-treated stressed rats ($p>0.05$) at 40 min (Figure 4C). A similar significant effect of URB597 on stress-induced *c-fos* mRNA expression was observed in the PVN at the earliest time point (20 min) ($F_{5,206}=68.37$, $p<0.0001$). *Post hoc* analysis revealed that in animals treated with URB597 stress-induced *c-fos* mRNA expression was significantly lower compared to vehicle-treated stressed rats ($p<0.001$) (Figure 4F). However, AM251 administration was not able to completely prevent the effects of URB597 in this brain area, as suggested by the observation that *c-fos* mRNA levels in rats receiving both drugs remained

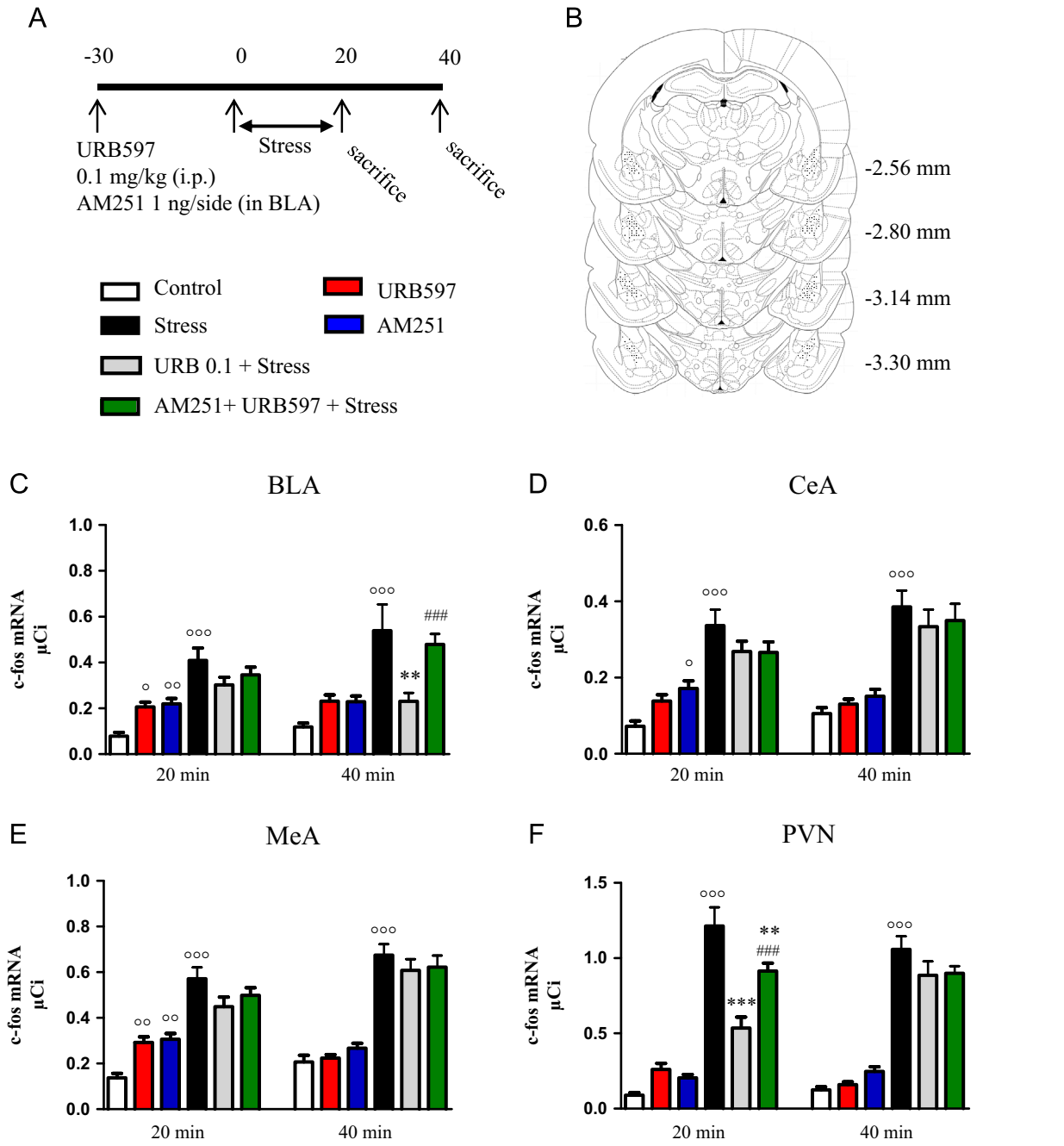

Figure 4 (A) Schematic diagram of protocol used in experiment 2. (B) Brain diagrams adapted from Paxinos and Watson (1998) showing cannulae tip positions for all rats receiving AM251 or vehicle infusion into the BLA. (C–F) Effects of CB1 blockade by AM251 administered into the BLA on URB597-induced effects on *c-fos* mRNA in BLA (C), CeA (D), MeA (E) and PVN (F). Data are expressed as average ± SEM ($n=6$ per group). $^{\circ\circ\circ}p<0.001$, $^{\circ\circ}p<0.01$ and $^{\circ}p<0.05$ *vs* respective control group; $^{***}p<0.001$ and $^{**}p<0.01$ *vs* respective stressed group; $^{\#\#\#}p<0.001$ *vs* respective URB 0.1+Stress group (Tukey's test for multiple comparisons).

statistically different from those observed in vehicle-treated stressed rats ($p<0.01$) (Figure 4F). CB1 antagonism in BLA did not affect URB597 effects in CeA and MeA (Figure 4D and E).

3.2.2. Pharmacological blockade of CB1 receptors in BLA blocked URB597 effect on the stress-induced increase of CRH mRNA

Besides confirming the results obtained from experiment 1, showing that URB597 (0.1 mg/kg, i.p.) could decrease stress-induced CRH mRNA (at 40 min), the results obtained from experiment 2 also showed that animals receiving a concomitant intra-BLA infusion of AM251 with the systemic injection of URB597 exhibited no differences in stress-induced CRH mRNA levels compared to vehicle-treated stressed rats ($p>0.05$) at 40 min (Figure 5), thus demonstrating that the blockade of BLA CB1 receptors could prevent the effects induced by URB597 systemic administration. No significant changes in CRH mRNA expression in PVN were observed in non stressed rats treated with either URB597 alone or AM251 alone.

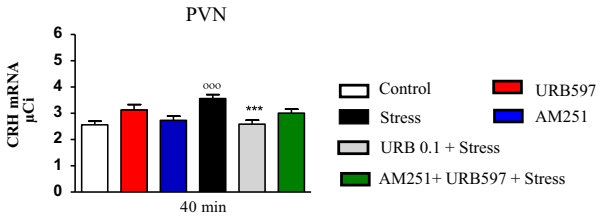

Figure 5 Effects of CB1 blockade by AM251 administered into BLA on URB597-induced effects on CRH mRNA in PVN. Data are expressed as average ± SEM ($n=6$ per group). °°°$p<0.001$ *vs* respective control group; ***$p<0.001$ *vs* respective stressed group (Tukey's test for multiple comparisons).

4. Discussion

In this study we showed that systemic administration of the FAAH inhibitor URB597 dampened the hypothalamic and pituitary responses to forced-swimming stress through a mechanism that, at least in part, involves the activation of CB1 receptors in the BLA. More specifically, URB597 treatment decreased 1) stress-induced CRH mRNA expression in PVN; 2) stress-induced POMC mRNA expression, dose dependently, in the pituitary gland; and 3) stress-induced *c-fos* mRNA in amygdala and PVN. Conversely, URB597 increased *c-fos* mRNA in LC and NTS. Local application of the CB1 receptor antagonist AM251 within the BLA partially prevented URB597 effects in the PVN and completely blocked its effect in the BLA.

All the effects observed were not paralleled by significant changes in circulating CORT levels, which were however sensitive to the stress procedure, as previously reported in the literature (Steiner and Wotjak, 2008). This result is in agreement with several previous findings showing that acute systemic administration of URB597 (Figure 1B) does not affect stress-induced CORT levels in plasma (Hill et al., 2010b; Kerr et al., 2012; Roberts et al., 2014; Steiner and Wotjak, 2008). However, to our knowledge, there is only one paper reporting a decrease of CORT secretion in mice subjected to restraint-stress (Patel et al., 2004).

The discrepancy with more recent studies was in part justified by some authors as a consequence of the different time points for blood sampling (Kerr et al., 2012). We hypothesize that it might be due to different types of stress used in the experiments. In support to this hypothesis and in accordance with more recent findings showing no effects of FAAH inhibitors on stress-induced CORT levels, same authors of the former study recently demonstrated with a detailed time-point experiment, that URB597 administration (0.1 mg/kg i.p.) to mice subjected to a 30-min restraint stress (30 min after treatment) does not cause any decrease of CORT plasma levels at any time (from 0 to 90 min after the end of the stress exposure) (Roberts et al., 2014). The authors argue that the opposite findings with respect to their previous work might be due to differences in the stress protocol (such as the rigidity of the plastic tube used for the restraint and the impact of this detail on body temperature), thus highlighting how even small differences in the stress methods might differentially impact AEA-mediated neuromodulatory pathways that affect the HPA axis response to stress.

This hypothesis remained poorly explored and it is at the basis of the rationale of our study aimed to shed new light on this issue.

Our results also confirm a previous report showing that 10 min of forced swimming increases CRH heteronuclear RNA (hnRNA) in PVN, which returns to basal levels within 2 h (Jiang et al., 2004). As expected, the increase of CRH mRNA in PVN was accompanied by increased POMC mRNA levels in the pituitary gland, as already well documented by previous studies (Jiang et al., 2004). Although it has been shown that CRH mRNA levels increase in CeA after 1–1.5 h of acute exposure to psychological stress (Makino et al., 1999), we did not detect any change at either time point analyzed, probably because of the shorter time frame considered. Acute physical and psychological stress can increase POMC mRNA expression in the anterior and intermediate lobe of the rat pituitary (Garcia-Garcia et al., 1997; Harbuz and Lightman, 1989). In our study, we found that URB597 treatment decreased CRH mRNA in the PVN and POMC mRNA in the pituitary gland, suggesting an attenuation of the hypothalamic and pituitary response to stress.

Substantial levels of CB1 (Wenger et al., 1999) and FAAH (Thomas et al., 1997) have been described in the anterior and intermediate lobes of the pituitary gland, although CB1-like immunoreactivity seems to be hardly present on corticotrophs (Wenger et al., 1999). This observation suggests that the effect of URB597 on POMC levels in the pituitary gland does not involve the local activation of CB1 receptors, but may result from reduced CRH secretion from PVN neurons. In agreement with this hypothesis, Barna et al. (2004) showed that *in vitro* ACTH secretion by the anterior pituitary gland is not affected by either CB1 gene disruption nor the incubation with a cannabinoid agonist.

Overall, our results clearly show that the acute administration of URB597 attenuates the hypothalamic and pituitary responses to stress. To gain some insight on the possible mechanisms that mediate such effects, we investigated the involvement of key brain structures by analyzing the pattern of *c-fos* mRNA expression.

Using *in-situ* hybridization followed by densitometric analyses, we showed that 20 min of forced swimming stress dramatically increased *c-fos* mRNA expression in PVN, MeA, BLA and LC and produced a moderate expression in CeA and NTS (Cullinan et al., 1995). Our results are also in line with previous reports showing that psychological stress (forced swimming, restraint and noise) elicits *c-fos* expression strongly in MeA as compared to CeA, while physical stress (hemorrhage and immune challenge) strongly induces *c-fos* in CeA (Dayas et al., 2001). Previous tracing studies have demonstrated that amygdaloidal nuclei project to PVN both directly (Dayas et al., 1999; Sawchenko and Swanson, 1983; Silverman et al., 1981; Tribollet and Dreifuss, 1981) and indirectly, *via* the posterior bed nucleus of stria terminalis (BNST) (Choi et al., 2007), DMH (Herman et al., 2003) and the periventricular nucleus of hypothalamus (Canteras et al., 1995). Moreover, both NTS (A2 region) and LC (A6 region) directly and indirectly projects to PVN, which can be activated by both of them under stress conditions (Cunningham and Sawchenko, 1988). In our study, the pattern of *c-fos* induction in stressed rats treated with vehicle is in agreement with all these previous observations made with retro-tracing approaches.

There is strong evidence that ECs act as "gatekeeper" of the HPA axis and negatively modulate its stress-induced activation (Gorzalka and Hill, 2009). Indeed, studies on pharmacological blockade of CB1 by SR141716 administration (Patel et al., 2004) and on CB1 knockout mice showed enhanced circulating levels of ACTH (Barna et al., 2004), CORT (Cota et al., 2007; Steiner et al., 2008), as well as anxiety-like behavioral responses (Haller et al., 2002; Maccarrone et al., 2002; Martin et al., 2002). Our results are in agreement with these observations. Moreover, it has been shown that systemic administration of glucocorticoids increases tissue contents of AEA in amygdala, hippocampus and hypothalamus (Hill et al., 2010a). CB1 receptors and FAAH are highly expressed in BLA and moderately expressed in CeA. In BLA, CB1 receptors are abundant on cholecystokinin (CCK)-positive GABAergic interneurons and moderately expressed on glutamatergic terminals (Bodor et al., 2005; Katona et al., 2001; Kawamura et al., 2006; Marsicano and Lutz, 1999). There is also an evidence that CB1 receptors are present in PVN, LC and NTS, and that FAAH is expressed in PVN and NTS at low levels (Haring et al., 2012). These observations suggest that URB597 may increase AEA levels (Kathuria et al., 2003) in these brain regions (mainly in BLA, PFC and PVN) and thereby potentiate AEA effects at CB1 receptors. In keeping with these findings, we showed that administration of the CB1 antagonist AM251 into the BLA blocked the dampening effect of URB597 on stress-induced neuronal activation in PVN and BLA, confirming the involvement of CB1 receptors expressed in the BLA (Hill et al., 2009). However, AEA also shows low affinity for TRPV1 receptors (Ross, 2003), therefore, the role of TRVP1 receptors cannot be ruled out. Activation of TRPV1 receptors exerts opposite effects with respect to the activation of CB1 receptors in the neural control of anxiety (Rubino et al., 2008). It has been previously demonstrated that simultaneous blockade of FAAH and TRVP1 within the BLA is required to exert anxiolytic effects in rats (John and Currie, 2012). In line with this observation, intra-BLA administration of a low dose of URB597 (0.1 μg), but not a higher dose (1 μg), was able to attenuate stress-induced CORT levels, suggesting that the higher dose of URB597 might have caused a hyper activation of TRPV1 receptors (Hill et al., 2009).

AM251 administration did not completely block URB597 effects in PVN. In fact, while it did prevent URB597-induced decrease of CRH mRNA levels in stressed rats (at 40 min), it slightly dampened, but not prevented, the decrease of c-fos mRNA observed at 20 min. This observation suggests that the effects of URB597 in this brain area are only partially mediated by CB1 activation in the BLA and that other areas might be involved. One possibility is that PVN activation is attenuated by the stimulation of CB1 receptor in the PFC and/or hippocampus, (Hill et al., 2006, 2011; Hu et al., 2011). In fact, it has been proposed that activation of CB1 receptors within the mPFC decreases GABA release, which could increase the outflow of the glutamatergic neurons to inhibitory GABAergic neurons of BNST or peri-PVN, that, in turn, project to the PVN. Therefore, the activation of either these inhibitory brain subnuclei could dampen the neuronal activation of PVN (Hill et al., 2011). Moreover, we cannot rule out that local inhibition of FAAH in PVN might be also involved. In fact, it has been shown that ECs are involved in a fast feedback mechanism of HPA axis that is driven by CORT acting within the PVN. In particular, it was demonstrated that the activation of glucocorticoids receptors expressed on CRH-positive neurons of the PVN by circulating CORT stimulates ECs synthesis, which, in turn, activate presynaptic CB1 receptors expressed on glutamatergic terminals projecting to the PVN (Di et al., 2003). Simulation of CB1 results in the suppression of glutamate release in the PVN leading to decreased activation of this nucleus and, therefore, decreased activation of the HPA axis (Di et al., 2003). In agreement with this observation, Malcher-Lopes et al. (2006) showed that local application of glucocorticoids increased AEA and 2-AG levels in PVN slices. Based on these considerations, we speculate that URB597 might cause an accumulation of AEA in PVN, which might, in turn, contribute to the suppression of HPA axis activation. According to this hypothesis the blockade of CB1 receptors in the BLA should not affect this mechanism.

Our results showing that URB597 increases neuronal activation of LC are consistent with previous findings that URB597 increases NMDA induced firing activity of noradrenergic neurons in the LC (Gobbi et al., 2005; Mendiguren and Pineda, 2004). CB1 activation suppresses the inhibition of noradrenergic cells produced by stimulation of the nucleus prepositus hypoglossi, the main GABAergic input to the LC (Muntoni et al., 2006), thus providing a mechanism by which cannabinoids may increase spontaneous firing of noradrenergic neurons in this area. Previous studies demonstrated that LC is activated by acute stress and after activation it increases NA outflow in stress related limbic forebrain regions, which mainly include hippocampus, PFC and amygdala. Amygdala uniquely has facilitating effects over the stress response in contrast to hippocampus and PFC, which have inhibitory effects (Herman and Cullinan, 1997). NTS can also stimulate NA outflow within the BLA, either directly or through the activation of LC-NA neurons (McGaugh, 2004). Previously reported electrophysiological studies demonstrated that URB597 could induce a slow increase in LC-NA-neurons in anesthetized rats (Gobbi et al., 2005). However, microdialysis experiments reported in the same paper have shown that URB597 treatment did not increase NA outflow in PFC. To our knowledge, no data is currently available on the possible effects of URB597 treatment on NA outflow in other projection areas, such as the BLA, as well as on the effects on NA neurons of the LC under stress conditions. Based on these considerations we cannot exclude that in our experiments LC and NTS might increase amygdala activation in URB597-treated stressed animals. However, since our results show that *c-fos* induction in the BLA is reduced and this reduction is coupled to a decreased stress-induced activation of PVN, other compensatory mechanisms might be involved so that LC and NTS activation does not correspond to BLA activation in our experimental context. One possibility is that the local increase of AEA tone within the BLA could overcompensate the facilitating role of LC and NTS, thus dampening the stress response at PVN. This compensatory mechanism could be more evident after the administration of the highest dose of URB597 (0.3 mg/kg), as suggested by the loss of the stress dampening effect observed at later time point (40 min) in the PVN of stressed rats treated with the lowest dose of URB597.

Activation of the LC and NTS by URB597-treatment in stressed rats might be also the possible reason for not observing significantly reduced CORT levels. In support to this hypothetical explanation, previous observations were reported on the ability of URB597 treatment to decrease CORT levels in animals exposed to restraint stress only if directly injected into the BLA (0.1 μg) (Hill et al., 2009) but not if systemically administered in a single injection (0.1 or 0.3 mg/kg) (Hill et al., 2010b; Roberts et al., 2014). This might suggest that systemic administration of URB597 could activate some brain regions such as LC and NTS, which could reduce the dampening effect of URB597 on stress-induced CORT secretion. All these aspects deserve further investigations and were beyond the aim of our study.

Overall our findings supports the notion that enhancement of endogenous anadamide signaling by inhibiting FAAH represents a novel pharmacological target for the management of stress and anxiety-related disorders. Although the precise mechanism by which cannabinoid receptors control neuroendocrine functions related to stress response remains to be fully clarified, our findings suggest an important role of CB1 receptors in the BLA.

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Contributors

Gaurav Bedse, Roberto Colangeli, Silvana Gaetani and Tommaso Cassano designed the study, and wrote protocol. Gaurav Bedse, Angelo M. Lavecchia and Adele Romano managed experimental part. Gaurav Bedse, Roberto Colangeli and Carlo Cifani managed the literature searches and analyses. Fabio Altieri managed all riboprobes required for this study.

Tommaso Cassano and Silvana Gaetani undertook the statistical analysis. Gaurav Bedse wrote the first draft of manuscript. All authors contributed to and have approved the final manuscript.

Conflict of interest

The authors have no actual or potential conflicts of interest including any financial, personal or other relationships with other people or organizations within three years of beginning the work submitted that could inappropriately influence (bias) our work.

No author's institution has contracts relating to this research through which it or any other organization may stand to gain financially now or in the future.

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