1 **Antidepressant-like effects of pharmacological inhibition of FAAH activity in socially**

2 **isolated female rats**

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Abstract

Pharmacological inhibition of the enzyme fatty acid amide hydrolase (FAAH), which terminates signalling of the endocannabinoid N-arachidonoylethanolamine (or anandamide, AEA), exerts favourable effects in rodent models of stress-related depression. Yet although depression seems to be more common among women than men and in spite of some evidence of sex differences in treatment efficacy, preclinical development of FAAH inhibitors for the pharmacotherapy of depression has been predominantly conducted in male animals. Here, adult female rats were exposed to six weeks of social isolation and, starting from the second week, treated with the FAAH inhibitor URB694 (0.3 mg/kg/day, i.p.) or vehicle. Compared to pair-housed females, socially isolated female rats treated with vehicle developed behavioral (mild anhedonia, passive stress coping) and biological (reduced body weight gain, elevated plasma corticosterone levels) symptoms related to depression. Moreover, prolonged social isolation provoked a reduction in BDNF content and AEA levels within the hippocampus. Conversely, pharmacological inhibition of FAAH activity with URB694 restored both AEA levels and BDNF content within the hippocampus of socially isolated rats and prevented the development of depressive-like symptoms. These results suggest a potential interplay between AEA-mediated signaling and BDNF at the level of the hippocampus in the pathogenesis of depressive-like symptoms and antidepressant action of FAAH inhibition in socially isolated female rats.

Keywords: depression; stress; endocannabinoid; BDNF; females

1. Introduction

Prolonged or repeated exposure to stressors of psychosocial nature can act as a precipitating factor for the onset of depression (Cohen et al., 2007; Dinan, 2005). One of the most susceptible brain regions to the effects of psychosocial stress is the hippocampus, a component of the limbic system that regulates emotional and cognitive processes related to psychiatric disorders (Belleau et al., 2019; Sheline et al., 2019). The hippocampus is also a major regulator of the hypothalamic-pituitary-adrenal (HPA) axis (Jacobson and Sapolsky, 1991), the neuroendocrine system responsible for the release of glucocorticoid stress hormones (i.e., cortisol in humans, corticosterone in rodents). In patients with depression, hippocampal volume is decreased (Sapolsky, 2000; Sheline, 1996) and the HPA axis is dysregulated (Stetler and Miller, 2011). Depletion of hippocampal neurogenesis has been implicated as one of the substrates that may explain the hippocampal volume loss seen in depression (Duman and Monteggia, 2006; Levone et al., 2015). Specifically, the neurotrophic hypothesis of depression proposes that stress-induced reductions in the expression of brain-derived neurotrophic factor (BDNF), a member of the neurotrophin family regulating synaptic plasticity (Leal et al., 2017; Lu et al., 2014), occur in key limbic structures, including the hippocampus, to contribute to the pathogenesis of depression (Castren et al., 2007; Duman and Monteggia, 2006). Moreover, several lines of clinical and preclinical evidence indicate that conventional antidepressants (e.g., tricyclics, selective serotonin reuptake inhibitors and norepinephrine reuptake inhibitors) may in part exert their effects through BDNF upregulation (Hayley and Anisman, 2013; Pittenger and Duman, 2008; Tardito et al., 2006).

The past two decades have witnessed a driven focus on the identification of novel therapeutic targets for depression, in an attempt to overcome the notable limitations of conventional antidepressant treatments, poor efficacy being perhaps the most critical (Connolly and Thase, 2012). For example, substantial evidence has accumulated implicating a deficit in endocannabinoid (eCB) neurotransmission in the etiology of depression (for a comprehensive review see Gorzalka and Hill, 2011). At the preclinical level, a deficiency in the signaling mediated by the eCB N-arachidonoylethanolamine (or anandamide, AEA) has been noted in the hippocampus, hypothalamus, ventral striatum, and prefrontal cortex of rats exposed to several stressors (i.e.,

chronic unpredictable stress and social defeat stress) and presenting a "depressive-like" phenotype (reviewed in Carnevali et al., 2017b). These findings have triggered significant interest in the development of eCB-interacting drugs, including direct-acting receptor ligands and catabolism inhibitors for the pharmacotherapy of depression (Micale et al., 2013). Specifically, within preclinical models, facilitation of AEA signaling through pharmacological inhibition of its degrading enzyme (i.e., fatty acid amide hydrolase (FAAH)) can enhance monoaminergic transmission, increase cellular plasticity and neurotrophin expression within the hippocampus, dampen HPA axis activity, and evoke antidepressant-like behavioral effects (reviewed in Carnevali et al., 2017b). However, while the literature has been unequivocal in showing that women experience depression at twice the rate of men (e.g., Grigoriadis and Robinson, 2007), very few preclinical studies have been conducted on female experimental animals (Beery, 2018; Kokras and Dalla, 2014). Moreover, despite the existence of sex differences in response to antidepressant treatment (Sloan and Kornstein, 2003), preclinical research on the antidepressant action of FAAH inhibitors has been predominantly conducted in male rodents (Carnevali et al., 2017b; Fowler, 2015). Therefore, there is a clear need to use female animals in preclinical models of stress to either confirm and generalize to females the previously obtained male animal-based findings or underscore potential sex differences in the etiology of depression and/or in the efficacy of new treatments.

Based on this background, the purpose of the current study was two-fold. First, we aimed at documenting the development of key behavioral (passive stress coping, anhedonia) and biological (reduced hippocampal BDNF levels, HPA axis hyperactivity, body weight loss) symptoms of depression in adult female rats exposed to prolonged social isolation, a mild chronic social stressor that has been validated as a rodent model of depression (Carnevali et al., 2017a). Second, we tested the hypothesis that pharmacological inhibition of FAAH activity would exert antidepressant-like effects in socially isolated female rats via upregulation of hippocampal BDNF expression. To this aim, we employed the FAAH inhibitor URB694 (6-hydroxy-[1,1′-biphenyl]-3-yl-cyclohexylcarbamate) which was shown to exhibit higher selectivity and more prolonged and profound access to the brain than the standard inhibitor URB597 (Clapper et al., 2009).

2. Experimental procedures

2.1. Animals and housing conditions

Four-month-old female wild-type Groningen rats were used in this study. This rat population, originally derived from the University of Groningen (the Netherlands) and currently bred in our laboratory under standard conditions, shows considerable individual differences in trait-like patterns of behavioral and physiological responses to environmental challenges (Carnevali et al., 2014; de Boer et al., 2017). After weaning, female animals were housed in same-sex sibling pairs and kept in rooms with controlled temperature (22 ± 2 °C) and humidity (50 ± 10 %), under a reversed light-dark cycle (light on from 19:00 to 7:00 h), with food and water ad libitum except when required for the sucrose preference test (see below). A total of 40 pairs were included in the study, but only one female rat from each pair was submitted to the experimental procedures described below. Experiments were performed in accordance with the European Community Council Directive 2010/63/UE and approved by the Italian legislation on animal experimentation (D.L. 04/04/2014, n. 26, authorization n. 449/2017-PR). All efforts were made to reduce sample size and minimize animal suffering.

2.2. Experimental design

The experimental timeline is depicted in Figure 1. Specific procedures and data analysis are described in the following sections. On day 0, animals were randomly divided in socially isolated (SI) and paired-housed (PH) groups. Female rats from the SI group were separated from their respective sibling and individually housed in a soundproof room for 6 weeks to avoid any sensory (visual, olfactory, and acoustic) contact with their conspecifics. On the contrary, female rats from the PH group were continually housed with their respective sibling and kept in the same room with other pairs. Handling and cage cleaning were matched between the two groups. Starting from the beginning of the third week of the social isolation/pair-housing condition, animals received daily i.p. injection of either the FAAH inhibitor URB694 or vehicle (VEH). Thus, four experimental subgroups emerged: (i) SI + VEH (n = 10), (ii) SI + URB694 (n = 10), (iii) PH + VEH (n =10), and (iv) PH + URB694 (n = 10). Experiments were conducted on separate cohorts of 8 experimental animals each

122 (n = 4 SI and n = 4 PH rats), starting with the VEH-treated animals. Experimental animals were

123 tested four times in the sucrose preference test and once in the forced swim test during the dark

124 phase of the daily cycle between 10.00 and 12.00 h. At sacrifice (day 42), trunk blood, adrenal

125 glands, and hippocampus were harvested. Body weight was measured weekly throughout the study.

126 Moreover, the estrous cycle phase of female rats was determined immediately after each behavioral

127 test and before sacrifice using vaginal smear cytology. Vaginal smears were collected by gently

128 introducing a moistened (0.9% NaCl) cotton swab in the rat's vagina. The sample was transferred to

129 a glass slide and examined microscopically following Giemsa staining. The phase of the cycle

130 (metaestrous, diestrous, pro-estrous or estrous) was determined based upon the presence of

131 leukocytes, nucleated epithelial or cornfield epithelial cells (Marcondes et al., 2002).

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133 2.3. Drug treatment

134 URB694 is a carbamate FAAH inhibitor that irreversibly carbamoylates the nucleophile catalytic

135 serine in FAAH active site (Tarzia et al., 2006). URB694 is a second generation inhibitor with

136 improved metabolic stability and selectivity for FAAH (Clapper et al., 2009). URB694 was freshly

137 dissolved in VEH containing 5% PEG, 5% Tween 80, and 90% saline. VEH (vol:1 ml/kg) or URB694

138 (0.3 mg/kg, i.p.) were injected i.p. between 11.00 and 13.00 h and, on the days of the sucrose

139 solution and forced swim tests, at least 1 h after the completion of the test. URB694 dose was chosen

140 based on our previous studies (Carnevali et al., 2015a; Carnevali et al., 2015b), and a pilot study

141 showing that FAAH activity in the brain of female wild-type Groningen rats was substantially inhibited

142 24 h after administration of this drug dose (Supplemental Figure S1).

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144 2.4. Sucrose preference test

145 *Ad libitum* 2% sucrose solution was available for 5 days before the beginning of the experimental

146 procedures to allow adaptation to its taste. Food and water were removed from the cage for 16 hours

147 before each sucrose preference test; moreover, one hour before the test, all experimental animals

148 (paired and isolated) were moved into individual cages to ensure accurate fluid intake measurements

149 of paired animals. Water and 2% sucrose solution were placed in premeasured bottles in the

individual cage, and fluid intake was monitored for 1 hour. Animals were returned to their respective home cages immediately after the test (Grippo et al., 2007). Sucrose preference tests were conducted in baseline conditions (day -3) and after 11, 25, and 39 days of social isolation (Figure 1). Sucrose solution intake was expressed as the relative percentage of the total liquid intake and was taken as an operational index of anhedonia, defined as reduced sucrose preference relative to control animals and baseline values (Grippo et al., 2007).

2.5. Forced swim test

An adapted version of the forced swim test originally described by Porsolt (Porsolt et al., 1977) was used. On day 35 (Fig. 1), female rats were forced to swim individually for 5 min in a Plexiglas cylinder (height: 40 cm, diameter: 30 cm) filled with water (temperature: 24 ± 2°C; depth: 30 cm). During the test, rats' behavior was video-taped. The overall time spent in immobility (floating and making only those movements necessary to keep the head above water) was scored by a trained experimenter blind to animals' condition and treatment. Immobility during the single session of the forced swim test was used as an index of passive stress coping (Commons et al., 2017).

2.6. Measurements at sacrifice

Twenty-four hours after the last administration of URB694 or VEH (i.e., at 11.00 h; day 42, Figure 1), female rats were euthanized by decapitation under isoflurane anesthesia (2% in 100% oxygen). Trunk blood was collected in EDTA-coated tubes (Sarsted AG, Numbrecht, Germany) and plasma was separated by centrifugation (2600 g, 4°C, 10 min). Brains were immediately removed and the hippocampus rapidly dissected and snap-frozen in nitrogen. All samples were stored at -80°C until further analysis, as described below. Adrenal glands were also removed and weighed.

2.6.1. Plasma corticosterone levels

Plasma was deproteinized by addition of two volumes of organic solvent (ice-cold acetonitrile), containing the internal standard dexamethasone (structural analog of corticosterone, 75 nmol/L). After centrifugation (14000 g, 4°C, 10 min), the supernatant was directly injected in the liquid chromatography/tandem mass spectrometry system (HPLC/MS/MS) for quantification of

corticosterone levels, in accordance with previously published analytical methods (Plenis et al., 2011).

2.6.2. BDNF hippocampal content

BDNF content in the hippocampus was measured using a commercially available sandwich enzyme-linked immune sorbent assay (ELISA) kit (Quantikine ®ELISA-Total BDNF, R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. A detailed description of the experimental procedure is reported in the Supplemental Material. BDNF tissue content was expressed as ng/g wet weight of tissue.

2.6.3. AEA hippocampal levels

AEA was extracted from 10% w/v hippocampal tissue homogenates employing two volumes of ice-cold acetonitrile containing the deuterated internal standard AEA-d_4 and quantified by HPLC/MS/MS as previously reported (Carnevali et al., 2015a) The analytical standards AEA and AEA-d_4 were purchased from Cayman Chemical (Ann Arbor, MI, USA) as stock solutions in ethanol. AEA levels were expressed as pmol/g wet weight of tissue. A detailed description of the HPLC/MS/MS analytical method and related MS instrumentation is reported in the Supplemental Material.

2.6.4. FAAH activity in the hippocampus

For ex vivo determination of FAAH activity, frozen hippocampi were thawed and homogenized in ice-cold Tris buffer (10 volumes, 50 mM, pH 7.5) containing 0.32 M sucrose. The homogenates were centrifuged (1000 g, 10 min, 4°C) and total protein content was quantified in the supernatant by the bicinchoninic acid (BCA) protein kit (Pierce Biotechnology, Rockford, IL, USA). FAAH activity was measured at 37°C for 30 min in 0.5 mL Tris buffer (50 mM, pH 7.5) containing fatty acid-free bovine serum albumin (BSA) (0.05%, w/v), 50 μg of protein from brain homogenates, 10 µM AEA and [^{3}H]-AEA (10000 disintegrations per minute) as previously described (Clapper et al., 2009). Briefly, the reactions were stopped with 1 mL chloroform:methanol (1:1). After centrifugation (2000 g, 10 min, 4°C), [^{3}H]-ethanolamine was measured in the aqueous phase by liquid scintillation counting. [^{3}H]-AEA (specific activity: 60 Ci/mmol), employed as a substrate for ex vivo FAAH assay, was purchased from American Radiolabeled Chemicals (St. Louis, MI, USA).

2.7. Statistical analysis

All statistical analyses were performed using SPSS v. 25 (IBM software package). Data are presented as mean ± standard error of the mean (SEM). The influence of the estrous cycle phase on behavioral and biochemical measurements was controlled in all statistical analyses. A three-way ANOVA for repeated measures with "condition" (2 levels: isolation, pair-housing) and "treatment" (2 levels: VEH, URB694) as the between subject factors, and "time" as the within subject factor (3 levels: days 11, 25, and 39) was applied on delta changes in sucrose solution preference with respect to baseline. All other data were analyzed with 2 (factor "condition": isolation or pair-housing) x 2 (factor "treatment": URB694 or VEH) factorial design ANOVAs. Follow-up analyses were conducted using Student's "t" tests, with a Bonferroni correction for multiple comparisons. Pearson's r correlations were performed to assess the correlation between plasma corticosterone levels, BDNF hippocampal content and AEA hippocampal levels. Statistical significance was set at $p < 0.05$.

3. Results

3.1. Body weight

There were no significant differences in body weight among groups at the start of the experiment (i.e., when animals were assigned to the different housing conditions) (PH + VEH = 230 ± 2 g; IS + VEH = 237 ± 5 g; PH + URB694 = 231 ± 4 g; IS + URB694 = 226 ± 8 g). However, a significant time x condition interaction emerged on body weight gain calculated as the difference between weight at the end (i.e., immediately before animals were euthanized) and at the start of the experiment (F = 7.1, p = .012). As shown in Figure 2, socially isolated female rats treated with VEH gained significantly less weight compared with their respective pair-housed counterparts (p = .002). This effect of social isolation was prevented by URB694 treatment (SI + URB694 vs SI + VEH, p = .012).

3.2. Sucrose preference test

Total fluid intake did not differ among groups at each assessment point (Supplemental Table S1). Also, there were no significant differences among groups in their baseline preference for the consumption of the sucrose solution (PH + VEH = 85 ± 2 %; IS + VEH = 88 ± 2 %; PH + URB694 = 83 ± 3 %; IS + URB694 = 82 ± 3 %). Of note, the estrous cycle phase had no effect on baseline

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sucrose solution preference (F = 0.3, p = .543). However, factorial ANOVA yielded a significant time x condition interaction (F = 5.1, p = .028) on preference changes during the social isolation period (calculated as the difference between each assessment point and the baseline), with no significant effects of the estrous cycle phase (F = 0.4, p = .497). Specifically, as shown in Figure 3, no group differences were observed on day 11. However, on day 25, socially-isolated female rats treated with VEH showed a significantly larger reduction in the preference for sucrose solution consumption compared with their respective pair-housed counterparts (p = .025). This effect was prevented by URB694 treatment (SI + URB694 vs SI + VEH, p = .003). A similar trend was observed on day 39, although differences did not reach full statistical significance (SI + VEH vs PH + VEH, p = .056; SI + VEH vs SI + URB694, p = .067).

3.3. Forced swim test

Behavior during the forced swim test is illustrated in Figure 4. Factorial ANOVA yielded a significant effect of treatment (F = 4.9, p = .033) and a strong trend for condition x treatment interaction (F = 3.5, p = .071) on immobility time, with no significant effects of estrous cycle phase (F = 0.2, p = .632). Specifically, socially isolated female rats treated with VEH spent significantly more time in immobility compared with their respective pair-housed counterparts (p = .024). This behavioral effect of social isolation was significantly corrected by URB694 treatment (SI + URB694 vs SI + VEH, p = .007).

3.4. Measurements at sacrifice

3.4.1. Plasma corticosterone levels and adrenal weight

Factorial ANOVA yielded a significant condition x treatment interaction (F = 7.1, p = .012) on plasma corticosterone levels at the end of the experimental protocol, with no significant effects of the estrous cycle phase (F = 0.6, p = .430). As depicted in Figure 5, socially isolated female rats treated with VEH had significantly higher plasma corticosterone levels than their respective pair-housed counterparts (p = .016). URB694 treatment prevented the effect of social isolation on plasma corticosterone levels (SI + URB694 vs SI + VEH, p = .003).

There were no significant effects of condition and/or treatment on adrenal weight corrected for body weight at the end of the experiment (PH + VEH = 0.021 ± 0.002 mg/g; IS + VEH = 0.027 ± 0.003 mg/g; PH + URB694 = 0.027 ± 0.002 mg/g; IS + URB694 = 0.026 ± 0.002 mg/g).

3.4.2. BDNF hippocampal content

Factorial ANOVA yielded a significant effect of treatment (F = 7.3, p = .012) and a significant condition x treatment interaction (F = 6.9, p = .014) on BDNF content in the hippocampus at the end of the experimental protocol. As illustrated in Figure 6A, socially isolated female rats treated with VEH showed a significantly lower BDNF hippocampal content compared with their respective pair-housed counterparts (p = .023). This effect of social isolation was prevented by URB694 treatment (SI + URB694 vs SI + VEH, p = .001). Moreover, we found a negative, although not significant, correlation between plasma corticosterone levels and BDNF hippocampal content (Table 1).

3.4.3. AEA hippocampal levels

Factorial ANOVA yielded significant effects of condition (F = 19.7, p < .001) and treatment (F = 27.6, p < .001), and a significant condition x treatment interaction (F = 5.3, p = .028) on AEA hippocampal levels at the end of the experimental protocol. As shown in Figure 6B, socially isolated female rats treated with VEH showed significantly lower AEA hippocampal levels compared with their respective pair housed counterpart (p < .001). As expected, URB694-treated groups showed significantly greater AEA levels than corresponding VEH-treated groups, both in the social isolation (p < .001) and pair-housing (p = .040) condition. Moreover, we found a significant positive correlation between AEA levels and BDNF content within the hippocampus (Table 1), as well as a strong trend for a negative correlation between AEA hippocampal levels and plasma corticosterone levels (Table 1).

3.4.4. FAAH activity

Factorial ANOVA yielded a significant effect of treatment (F = 456.0, p < .001) on FAAH activity in the hippocampus, being, as expected, significantly lower in URB694-treated than VEH-treated rats in both the social isolation (p < .001) and pair-housing (p < .001) condition (Figure 6C).

4. Discussion

The major findings of the current investigation are the following. Compared to pair-housed females, socially isolated female rats developed behavioral (mild anhedonic state, passive stress coping) and biological (reduced body weight gain, elevated plasma corticosterone levels) changes that together are indicative of a depressive-like state, and showed a reduction in BDNF content and AEA levels

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within the hippocampus. Notably, pharmacological inhibition of FAAH activity with URB694 restored AEA and BDNF hippocampal levels, and prevented the development of depressive-like behavioral and biological symptoms following a prolonged period of social isolation.

4.1. Depressive-like syndrome in socially isolated female rats

Psychiatric disorders in humans have been linked prevalently with social stress and/or reduced social interaction (Bjorkqvist, 2001; Heinrich and Gullone, 2006). Within preclinical models, the social defeat paradigm has been shown to have a substantial impact on depression-relevant behavioral and physiological parameters in adult male rats, while solitary housing is particularly effective in precipitating depressive-like symptoms in previously group-housed female rats (Beery and Kaufer, 2015; Carnevali et al., 2017a). Accordingly, in the current study we found that six weeks of social isolation in adult female rats produce several changes that mimic the symptoms of human depression (American Psychiatry Association, 2013). Of note, the social isolation protocol adopted in this study included both solitary housing and long-term deprivation of sensory stimuli originating from the surrounding social environment. Therefore, it is likely that the described effects are due to a combination of both. Specifically, female rats showed a reduction in body weight gain, signs of a mild anhedonic-like state (i.e., reduced preference for the consumption of a sucrose solution), passive coping (i.e., increased immobility in the forced swim test), and elevated plasma corticosterone levels. Deficits in body weight gain in isolated rats may be explained by reduced food intake, as previously demonstrated in individually housed mice and rats (Izadi et al., 2018; Sun et al., 2014), particularly around light-dark phase transitions (Sun et al., 2014). Interestingly, reductions in heat production and in the respiratory exchange ratio were also found during light-dark transitions in individually housed mice (Sun et al., 2014), suggesting that metabolic functions may have been affected also in our socially isolated rats. Moreover, the mild reduction in the preference for the consumption of a palatable solution observed only after 25 days of social isolation resembles the time course of changes reported in female Wistar rats exposed to chronic mild stress (Grippo et al., 2005) and in socially isolated female prairie voles (Grippo et al., 2007). However, we acknowledge that the interpretation of this result is limited by the difference, albeit not statistically significant, between the two stressed groups on day 11 (i.e., before the start of the pharmacological treatment).

Notably, the estrous cycle phase did not seem to have any effect on any of the behavioral and biological variables assessed in the current study, although our analysis is limited by the small sample size given that four different stages were considered. Nevertheless, this is in line with empirical research across multiple rodent species demonstrating that estrous cyclicity is not a major source of variability in females or, at least, is not greater than intrinsic variability in males (Beery, 2018; Finnell et al., 2018; Kokras et al., 2015).

Animal and human studies have provided support for the role of stress in the pathogenesis of depression via alterations in BDNF-mediated signaling (Hashimoto, 2010; Stepanichev et al., 2014), a neurotrophin that primarily regulates synaptic plasticity (Leal et al., 2017; Lu et al., 2014). In line with these findings, we found that BDNF content was reduced in the hippocampus of socially isolated female rats with depressive-like symptoms. Remarkably, such downregulation of hippocampal BDNF was paralleled by a decrease in AEA hippocampal levels. Converging lines of evidence support the possibility that AEA signaling at the cannabinoid receptor 1 (CB1R) may be an important mediator of neuroplastic phenomena within the hippocampus (Aguado et al., 2005; Hashimotodani et al., 2007; Hill et al., 2010; Scarante et al., 2017; Burstein et al., 2018). Particularly relevant for the current results are findings of decreased BDNF levels in the hippocampus of CB1R knockout mice (Aso et al., 2008). Thus, we hypothesize that a deficiency in AEA-mediated signaling at the CB1R might be implicated in the downregulation of BDNF hippocampal content observed in socially isolated female rats. Moreover, the positive correlation found here between AEA levels and BDNF content further supports a role for the eCB system in adult hippocampal neurogenesis (Scarante et al., 2017). Notably, while one study reported a similar decrease in AEA levels in the hippocampus of chronically stressed male rats (Hill et al., 2008), other studies showed no changes in AEA hippocampal levels upon chronic stress exposure (Bortolato et al., 2007; Carnevali et al., 2015a; Hill et al., 2005). Of note, our data suggest that reduced AEA levels in the hippocampus of socially isolated rats were not due to an upregulation of FAAH enzymatic activity. This is in line with previous studies showing that FAAH activity is not affected by chronic stress exposure in rats (Bortolato et al., 2007; Hill et al., 2008), suggesting that the stress-induced decline in the hippocampal pool of AEA might be due to diminished biosynthetic mechanisms. Empirical evidence indicates the eCB system may be a

biochemical effector of glucocorticoids in the brain (Hill and McEwen, 2010). Notably, the hippocampus itself is particularly sensitive to the action of glucocorticoid stress hormones due the rich concentration of receptor sites for glucocorticoids (De Kloet et al., 1998). The negative, although only marginally significant, correlation found between plasma corticosterone levels and AEA hippocampal levels prompts further investigation into the specific mechanisms underlying the effects of stress exposure on AEA metabolism and their causal relationship with BDNF hippocampal downregulation. Interestingly, sex-specific mechanisms of eCB-mediated synaptic modulation within the hippocampus have been proposed to partly explain sex disparities in prevalence of depression (Huang and Woolley, 2012; Tabatadze et al., 2015). Decreased levels of BDNF may contribute to the atrophy of the hippocampus that has been observed in patients with depression (Sheline, 1996; Sheline et al., 2019). Recently, Belleau and colleagues (Belleau et al., 2019) proposed a model according to which chronic life stress can trigger the initial development of hippocampal volume reduction. However, this reduction would be neither necessary nor sufficient to produce a major depressive episode (Belleau et al., 2019). On the other hand, stress also initiates a set of neurotoxic processes (HPA axis dysregulation, inflammation, and neurotransmitter disturbances) that interact and may drive the development of a more chronic type of depression marked by further hippocampal volume reduction (Belleau et al., 2019). Although hippocampal volume was not assessed in the current study, we speculate that AEA–BDNF interactions might be implicated in the development of depressive symptoms and hippocampal volume decline under chronic life stress. Future longitudinal studies in rodent models of social stress-induced depression may be informative in this regard.

4.2. Antidepressant action of the FAAH inhibitor URB694

In an attempt to replicate findings of our previous study demonstrating antidepressant-like effects of the FAAH inhibitor URB694 in chronically stressed male rats (Carnevali et al., 2015a), pharmacological treatment with URB694 started after two weeks of social isolation (i.e., we anticipated that depressive-like behaviors would already have begun to manifest by then). However, contrary to our expectations, we failed to conclusively demonstrate the onset of an anhedonic-like state before the start of the treatment. Thus, the fact that URB694-treated females did not show depressive-like behavioral and biological symptoms after a prolonged period of social isolation

suggests, more cautiously, that inhibition of FAAH activity represents an effective preventive measure in this animal model. These results are in line with a growing body of evidence demonstrating that pharmacological inhibition of FAAH activity produces an antidepressant response in chronically stressed male rodents (Carnevali et al., 2017b). Interestingly, FAAH inhibitors have been shown to increase hippocampal neurogenesis in adult rats (Goncalves et al., 2008; Hill et al., 2006; Marchalant et al., 2009) and prevent stress-induced BDNF downregulation in the brain (Burstein et al., 2018), supposedly via facilitation of CB1R-mediated activation of the extracellular signal-regulated kinase signaling pathway (Derkinderen et al., 2003; Rubino et al., 2006). Therefore, given that CB1Rs are highly abundant in the rodent (and human) hippocampus (Mackie, 2005), we hypothesize that the antidepressant-like action of the FAAH inhibitor URB694 in socially isolated female rats may be partly mediated by a preservation of hippocampal BDNF content via enhancement of AEA signaling at the CB1R. However, the antidepressant-like effects of URB694 may also be interpreted in light of experimental evidence showing that AEA-signaling enhancement at the CB1R facilitates adaptive stress coping behaviors (Haller et al., 2013) and attenuates the neuroendocrine response to psychological stressors (Gorzalka et al., 2008). Moreover, given that FAAH inhibitors also increase the levels of other fatty acid amines with activity at peroxisome proliferator activated receptor-α (N-oleoylethanolamine (OEA) and N-palmitoylethanolamine (PEA)), the possibility of other non-cannabinoid receptor-mediated mechanisms cannot be completely ruled out. For example, a growing body of preclinical evidence suggests that PEA could have antidepressant-like activity (De Gregorio et al., 2019). On the other hand, increases in the endogenous levels of OEA may reduce food intake by regulating systems that control hunger and satiety in the brain (Romano et al., 2015). However, these compounds might also prolong and enhance AEA biological activity by competing with AEA for FAAH-mediated degradation (Petrosino et al., 2009). Of note, the current drug regimen had no effects on control animals, suggesting that the FAAH inhibitor did not affect normal biological processes and behavioral responses.

4.3. Conclusion

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401   The results of this study suggest a potential interplay between AEA-mediated signaling and BDNF

402   at the level of the hippocampus in the pathogenesis of depressive-like symptoms in socially isolated

403   female rats, and document the ability of the FAAH inhibitor URB694 to correct the alterations

404   associated with prolonged social isolation, a naturalistic rodent model of depression with high face,

405   construct, and predictive validity (Carnevali et al., 2017a). These findings complement accumulating

406   evidence on the antidepressant-like effects of FAAH inhibitors in male rodents exposed to chronic

407   stress (reviewed in Carnevali et al., 2017b). Recently, sex differences in hippocampal response to

408   pharmacological inhibition of FAAH activity have been reported in rats after acute intense stress

409   (Zer-Aviv and Akirav, 2016). This suggests that preclinical development of FAAH inhibitors for the

410   pharmacotherapy of depression should aim at comparing the underlying neurobiological

411   mechanisms between males and females. We must acknowledge that, at present, clinical research

412   on FAAH inhibitors has been slowed down by the serious adverse effects caused by the FAAH

413   inhibitor BIA 10–2474 for the treatment of pain (von Schaper, 2016), which displayed both intrinsic

414   toxic effects at high doses and off-targets effects (van Esbroeck et al., 2017). Investigations

415   conducted by a Temporary Specialist Scientific Committee concluded that the toxicity of BIA 10-

416   2474 is unlikely due to FAAH inhibition (Temporary Specialist Scientific Committee, 2016). A

417   communication from the U.S. Food and Drug Administration also reported that the unique toxicity of

418   this drug does not extend to other FAAH inhibitors (Food and Drug Administration, 2016), which are

419   well tolerated by patients enrolled in clinical trials, and remarkably lack of the common adverse

420   events elicited by exogenous cannabinoid-like compounds, including impairment in cognition, motor

421   coordination, and psychoses (Mallet et al., 2016). The disorders for which these agents are being

422   tested are mostly neuropsychiatric, such as pain conditions, depression, anxiety disorders, and

423   phobias (Mallet et al., 2016). Nevertheless, the current results in female rats and previous research

424   in male rodents using the carbamate FAAH inhibitors URB597 (e.g. Bortolato et al., 2007) and

425   URB694 (Carnevali et al., 2015a) warrant more translational studies to examine the mood-

426   modulating properties of this class of FAAH inhibitors and their underlying mechanism of action for

427   the pharmacotherapy of depression in both sexes (Gururajan et al., 2019).

428   **Table 1** Correlation matrix between plasma corticosterone levels, brain-derived neurotrophic factor

429   (BDNF) hippocampal content, and anandamide (AEA) hippocampal levels at the end of the

430   experimental protocol.

		Corticosterone	BDNF	AEA
Corticosterone	r	-		
	p			
BDNF	r	-.32	-	
	p	.082		
AEA	r	-.31	.44	-
	p	.068	.015	

431

432

Figure legends

Figure 1. Timeline of experimental procedures.

Figure 2. Body weight gain of paired-housed (PH) and socially isolated (SI) female rats treated with vehicle (VEH) or URB694, calculated as the difference between weight at the end (immediately before animals were euthanized) and at the start (when animals were assigned to the different housing conditions) of the experiment (n = 10 per group). * = significantly different from corresponding PH + VEH group; # = significantly different from corresponding SI + VEH group (p values are reported in the text).

Figure 3. Changes in sucrose solution preference in paired-housed (PH) and socially isolated (SI) female rats treated with vehicle (VEH) or URB694, calculated as the difference between each assessment point during the social isolation period and the baseline (n = 10 per group). * = significantly different from corresponding PH + VEH group; # = significantly different from corresponding SI + VEH group (p values are reported in the text).

Figure 4. Time spent in immobility during the forced swim test by paired-housed (PH) and socially isolated (SI) female rats treated with vehicle (VEH) or URB694 (n = 10 per group). * = significantly different from corresponding PH + VEH group; # = significantly different from corresponding SI + VEH group (p values are reported in the text).

Figure 5. Plasma corticosterone levels at the end of the experimental protocol in paired-housed (PH) and socially isolated (SI) female rats treated with vehicle (VEH) or URB694 (n = 10 per group). * = significantly different from corresponding PH + VEH group; # = significantly different from corresponding SI + VEH group (p values are reported in the text).

Figure 6. Tissue content of brain-derived neurotrophic factor (BDNF; panel A), anandamide levels (panel B), and fatty acid amide hydrolase (FAAH) activity (panel C) in the hippocampus of paired-housed (PH) and socially isolated (SI) female rats treated with vehicle (VEH) or URB694 (n = 10

18

462   per group). * = significantly different from corresponding PH + VEH group; # = significantly different

463   from corresponding VEH group (p values are reported in the text).

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1   **Antidepressant-like effects of pharmacological inhibition of FAAH activity in socially**

2   **isolated female rats**

3

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Abstract

Pharmacological inhibition of the enzyme fatty acid amide hydrolase (FAAH), which terminates signalling of the endocannabinoid N-arachidonoylethanolamine (or anandamide, AEA), exerts favourable effects in rodent models of stress-related depression. Yet although depression seems to be more common among women than men and in spite of some evidence of sex differences in treatment efficacy, preclinical development of FAAH inhibitors for the pharmacotherapy of depression has been predominantly conducted in male animals. Here, adult female rats were exposed to six weeks of social isolation and, starting from the second week, treated with the FAAH inhibitor URB694 (0.3 mg/kg/day, i.p.) or vehicle. Compared to pair-housed females, socially isolated female rats treated with vehicle developed behavioral (mild anhedonia, passive stress coping) and biological (reduced body weight gain, elevated plasma corticosterone levels) symptoms related to depression. Moreover, prolonged social isolation provoked a reduction in BDNF content and AEA levels within the hippocampus. Conversely, pharmacological inhibition of FAAH activity with URB694 restored both AEA levels and BDNF content within the hippocampus of socially isolated rats and prevented the development of depressive-like symptoms. These results suggest a potential interplay between AEA-mediated signaling and BDNF at the level of the hippocampus in the pathogenesis of depressive-like symptoms and antidepressant action of FAAH inhibition in socially isolated female rats.

Keywords: depression; stress; endocannabinoid; BDNF; females

1. Introduction

Prolonged or repeated exposure to stressors of psychosocial nature can act as a precipitating factor for the onset of depression (Cohen et al., 2007; Dinan, 2005). One of the most susceptible brain regions to the effects of psychosocial stress is the hippocampus, a component of the limbic system that regulates emotional and cognitive processes related to psychiatric disorders (Belleau et al., 2019; Sheline et al., 2019). The hippocampus is also a major regulator of the hypothalamic-pituitary-adrenal (HPA) axis (Jacobson and Sapolsky, 1991), the neuroendocrine system responsible for the release of glucocorticoid stress hormones (i.e., cortisol in humans, corticosterone in rodents). In patients with depression, hippocampal volume is decreased (Sapolsky, 2000; Sheline, 1996) and the HPA axis is dysregulated (Stetler and Miller, 2011). Depletion of hippocampal neurogenesis has been implicated as one of the substrates that may explain the hippocampal volume loss seen in depression (Duman and Monteggia, 2006; Levone et al., 2015). Specifically, the neurotrophic hypothesis of depression proposes that stress-induced reductions in the expression of brain-derived neurotrophic factor (BDNF), a member of the neurotrophin family regulating synaptic plasticity (Leal et al., 2017; Lu et al., 2014), occur in key limbic structures, including the hippocampus, to contribute to the pathogenesis of depression (Castren et al., 2007; Duman and Monteggia, 2006). Moreover, several lines of clinical and preclinical evidence indicate that conventional antidepressants (e.g., tricyclics, selective serotonin reuptake inhibitors and norepinephrine reuptake inhibitors) may in part exert their effects through BDNF upregulation (Hayley and Anisman, 2013; Pittenger and Duman, 2008; Tardito et al., 2006).

The past two decades have witnessed a driven focus on the identification of novel therapeutic targets for depression, in an attempt to overcome the notable limitations of conventional antidepressant treatments, poor efficacy being perhaps the most critical (Connolly and Thase, 2012). For example, substantial evidence has accumulated implicating a deficit in endocannabinoid (eCB) neurotransmission in the etiology of depression (for a comprehensive review see Gorzalka and Hill, 2011). At the preclinical level, a deficiency in the signaling mediated by the eCB N-arachidonoylethanolamine (or anandamide, AEA) has been noted in the hippocampus, hypothalamus, ventral striatum, and prefrontal cortex of rats exposed to several stressors (i.e.,

chronic unpredictable stress and social defeat stress) and presenting a "depressive-like" phenotype (reviewed in Carnevali et al., 2017b). These findings have triggered significant interest in the development of eCB-interacting drugs, including direct-acting receptor ligands and catabolism inhibitors for the pharmacotherapy of depression (Micale et al., 2013). Specifically, within preclinical models, facilitation of AEA signaling through pharmacological inhibition of its degrading enzyme (i.e., fatty acid amide hydrolase (FAAH)) can enhance monoaminergic transmission, increase cellular plasticity and neurotrophin expression within the hippocampus, dampen HPA axis activity, and evoke antidepressant-like behavioral effects (reviewed in Carnevali et al., 2017b). However, while the literature has been unequivocal in showing that women experience depression at twice the rate of men (e.g., Grigoriadis and Robinson, 2007), very few preclinical studies have been conducted on female experimental animals (Beery, 2018; Kokras and Dalla, 2014). Moreover, despite the existence of sex differences in response to antidepressant treatment (Sloan and Kornstein, 2003), preclinical research on the antidepressant action of FAAH inhibitors has been predominantly conducted in male rodents (Carnevali et al., 2017b; Fowler, 2015). Therefore, there is a clear need to use female animals in preclinical models of stress to either confirm and generalize to females the previously obtained male animal-based findings or underscore potential sex differences in the etiology of depression and/or in the efficacy of new treatments.

Based on this background, the purpose of the current study was two-fold. First, we aimed at documenting the development of key behavioral (passive stress coping, anhedonia) and biological (reduced hippocampal BDNF levels, HPA axis hyperactivity, body weight loss) symptoms of depression in adult female rats exposed to prolonged social isolation, a mild chronic social stressor that has been validated as a rodent model of depression (Carnevali et al., 2017a). Second, we tested the hypothesis that pharmacological inhibition of FAAH activity would exert antidepressant-like effects in socially isolated female rats via upregulation of hippocampal BDNF expression. To this aim, we employed the FAAH inhibitor URB694 (6-hydroxy-[1,1′-biphenyl]-3-yl-cyclohexylcarbamate) which was shown to exhibit higher selectivity and more prolonged and profound access to the brain than the standard inhibitor URB597 (Clapper et al., 2009).

2. Experimental procedures

2.1. Animals and housing conditions

Four-month-old female wild-type Groningen rats were used in this study. This rat population, originally derived from the University of Groningen (the Netherlands) and currently bred in our laboratory under standard conditions, shows considerable individual differences in trait-like patterns of behavioral and physiological responses to environmental challenges (Carnevali et al., 2014; de Boer et al., 2017). After weaning, female animals were housed in same-sex sibling pairs and kept in rooms with controlled temperature (22 ± 2 °C) and humidity (50 ± 10 %), under a reversed light-dark cycle (light on from 19:00 to 7:00 h), with food and water ad libitum except when required for the sucrose preference test (see below). A total of 40 pairs were included in the study, but only one female rat from each pair was submitted to the experimental procedures described below. Experiments were performed in accordance with the European Community Council Directive 2010/63/UE and approved by the Italian legislation on animal experimentation (D.L. 04/04/2014, n. 26, authorization n. 449/2017-PR). All efforts were made to reduce sample size and minimize animal suffering.

2.2. Experimental design

The experimental timeline is depicted in Figure 1. Specific procedures and data analysis are described in the following sections. On day 0, animals were randomly divided in socially isolated (SI) and paired-housed (PH) groups. Female rats from the SI group were separated from their respective sibling and individually housed in a soundproof room for 6 weeks to avoid any sensory (visual, olfactory, and acoustic) contact with their conspecifics. On the contrary, female rats from the PH group were continually housed with their respective sibling and kept in the same room with other pairs. Handling and cage cleaning were matched between the two groups. Starting from the beginning of the third week of the social isolation/pair-housing condition, animals received daily i.p. injection of either the FAAH inhibitor URB694 or vehicle (VEH). Thus, four experimental subgroups emerged: (i) SI + VEH (n = 10), (ii) SI + URB694 (n = 10), (iii) PH + VEH (n =10), and (iv) PH + URB694 (n = 10). Experiments were conducted on separate cohorts of 8 experimental animals each

122   (n = 4 SI and n = 4 PH rats), starting with the VEH-treated animals. Experimental animals were

123   tested four times in the sucrose preference test and once in the forced swim test during the dark

124   phase of the daily cycle between 10.00 and 12.00 h. At sacrifice (day 42), trunk blood, adrenal

125   glands, and hippocampus were harvested. Body weight was measured weekly throughout the study.

126   Moreover, the estrous cycle phase of female rats was determined immediately after each behavioral

127   test and before sacrifice using vaginal smear cytology. Vaginal smears were collected by gently

128   introducing a moistened (0.9% NaCl) cotton swab in the rat's vagina. The sample was transferred to

129   a glass slide and examined microscopically following Giemsa staining. The phase of the cycle

130   (metaestrous, diestrous, pro-estrous or estrous) was determined based upon the presence of

131   leukocytes, nucleated epithelial or cornfield epithelial cells (Marcondes et al., 2002).

132

133   2.3. Drug treatment

134   URB694 is a carbamate FAAH inhibitor that irreversibly carbamoylates the nucleophile catalytic

135   serine in FAAH active site (Tarzia et al., 2006). URB694 is a second generation inhibitor with

136   improved metabolic stability and selectivity for FAAH (Clapper et al., 2009). URB694 was freshly

137   dissolved in VEH containing 5% PEG, 5% Tween 80, and 90% saline. VEH (vol:1 ml/kg) or URB694

138   (0.3 mg/kg, i.p.) were injected i.p. between 11.00 and 13.00 h and, on the days of the sucrose

139   solution and forced swim tests, at least 1 h after the completion of the test. URB694 dose was chosen

140   based on our previous studies (Carnevali et al., 2015a; Carnevali et al., 2015b), and a pilot study

141   showing that FAAH activity in the brain of female wild-type Groningen rats was substantially inhibited

142   24 h after administration of this drug dose (Supplemental Figure S1).

143

144   2.4. Sucrose preference test

145   *Ad libitum* 2% sucrose solution was available for 5 days before the beginning of the experimental

146   procedures to allow adaptation to its taste. Food and water were removed from the cage for 16 hours

147   before each sucrose preference test; moreover, one hour before the test, all experimental animals

148   (paired and isolated) were moved into individual cages to ensure accurate fluid intake measurements

149   of paired animals. Water and 2% sucrose solution were placed in premeasured bottles in the

individual cage, and fluid intake was monitored for 1 hour. Animals were returned to their respective home cages immediately after the test (Grippo et al., 2007). Sucrose preference tests were conducted in baseline conditions (day -3) and after 11, 25, and 39 days of social isolation (Figure 1). Sucrose solution intake was expressed as the relative percentage of the total liquid intake and was taken as an operational index of anhedonia, defined as reduced sucrose preference relative to control animals and baseline values (Grippo et al., 2007).

2.5. Forced swim test

An adapted version of the forced swim test originally described by Porsolt (Porsolt et al., 1977) was used. On day 35 (Fig. 1), female rats were forced to swim individually for 5 min in a Plexiglas cylinder (height: 40 cm, diameter: 30 cm) filled with water (temperature: 24 ± 2°C; depth: 30 cm). During the test, rats' behavior was video-taped. The overall time spent in immobility (floating and making only those movements necessary to keep the head above water) was scored by a trained experimenter blind to animals' condition and treatment. Immobility during the single session of the forced swim test was used as an index of passive stress coping (Commons et al., 2017).

2.6. Measurements at sacrifice

Twenty-four hours after the last administration of URB694 or VEH (i.e., at 11.00 h; day 42, Figure 1), female rats were euthanized by decapitation under isoflurane anesthesia (2% in 100% oxygen). Trunk blood was collected in EDTA-coated tubes (Sarsted AG, Numbrecht, Germany) and plasma was separated by centrifugation (2600 g, 4°C, 10 min). Brains were immediately removed and the hippocampus rapidly dissected and snap-frozen in nitrogen. All samples were stored at -80°C until further analysis, as described below. Adrenal glands were also removed and weighed.

2.6.1. Plasma corticosterone levels

Plasma was deproteinized by addition of two volumes of organic solvent (ice-cold acetonitrile), containing the internal standard dexamethasone (structural analog of corticosterone, 75 nmol/L). After centrifugation (14000 g, 4°C, 10 min), the supernatant was directly injected in the liquid chromatography/tandem mass spectrometry system (HPLC/MS/MS) for quantification of

178 corticosterone levels, in accordance with previously published analytical methods (Plenis et al.,

179 2011).

2.6.2. BDNF hippocampal content

181 BDNF content in the hippocampus was measured using a commercially available sandwich enzyme-

182 linked immune sorbent assay (ELISA) kit (Quantikine ®ELISA-Total BDNF, R&D Systems,

183 Minneapolis, MN, USA) according to the manufacturer's instructions. A detailed description of the

184 experimental procedure is reported in the Supplemental Material. BDNF tissue content was

185 expressed as ng/g wet weight of tissue.

2.6.3. AEA hippocampal levels

187 AEA was extracted from 10% w/v hippocampal tissue homogenates employing two volumes of ice-

188 cold acetonitrile containing the deuterated internal standard AEA-d_4 and quantified by HPLC/MS/MS

189 as previously reported (Carnevali et al., 2015a) The analytical standards AEA and AEA-d_4 were

190 purchased from Cayman Chemical (Ann Arbor, MI, USA) as stock solutions in ethanol. AEA levels

191 were expressed as pmol/g wet weight of tissue. A detailed description of the HPLC/MS/MS analytical

192 method and related MS instrumentation is reported in the Supplemental Material.

2.6.4. FAAH activity in the hippocampus

194 For ex vivo determination of FAAH activity, frozen hippocampi were thawed and homogenized in ice-

195 cold Tris buffer (10 volumes, 50 mM, pH 7.5) containing 0.32 M sucrose. The homogenates were

196 centrifuged (1000 g, 10 min, 4°C) and total protein content was quantified in the supernatant by the

197 bicinchoninic acid (BCA) protein kit (Pierce Biotechnology, Rockford, IL, USA). FAAH activity was

198 measured at 37°C for 30 min in 0.5 mL Tris buffer (50 mM, pH 7.5) containing fatty acid-free bovine

199 serum albumin (BSA) (0.05%, w/v), 50 μg of protein from brain homogenates, 10 µM AEA and [^{3}H]-

200 AEA (10000 disintegrations per minute) as previously described (Clapper et al., 2009). Briefly, the

201 reactions were stopped with 1 mL chloroform:methanol (1:1). After centrifugation (2000 g, 10 min,

202 4°C), [^{3}H]-ethanolamine was measured in the aqueous phase by liquid scintillation counting. [^{3}H]-

203 AEA (specific activity: 60 Ci/mmol), employed as a substrate for ex vivo FAAH assay, was purchased

204 from American Radiolabeled Chemicals (St. Louis, MI, USA).

205

206 2.7. Statistical analysis

207 All statistical analyses were performed using SPSS v. 25 (IBM software package). Data are

208 presented as mean ± standard error of the mean (SEM). The influence of the estrous cycle phase

209 on behavioral and biochemical measurements was controlled in all statistical analyses. A three-way

210 ANOVA for repeated measures with "condition" (2 levels: isolation, pair-housing) and "treatment" (2

211 levels: VEH, URB694) as the between subject factors, and "time" as the within subject factor (3

212 levels: days 11, 25, and 39) was applied on delta changes in sucrose solution preference with respect

213 to baseline. All other data were analyzed with 2 (factor "condition": isolation or pair-housing) x 2

214 (factor "treatment": URB694 or VEH) factorial design ANOVAs. Follow-up analyses were conducted

215 using Student's "t" tests, with a Bonferroni correction for multiple comparisons. Pearson's r

216 correlations were performed to assess the correlation between plasma corticosterone levels, BDNF

217 hippocampal content and AEA hippocampal levels. Statistical significance was set at p < 0.05.

218

219 **3. Results**

220 3.1. Body weight

221 There were no significant differences in body weight among groups at the start of the experiment

222 (i.e., when animals were assigned to the different housing conditions) (PH + VEH = 230 ± 2 g; IS +

223 VEH = 237 ± 5 g; PH + URB694 = 231 ± 4 g; IS + URB694 = 226 ± 8 g). However, a significant time

224 x condition interaction emerged on body weight gain calculated as the difference between weight at

225 the end (i.e., immediately before animals were euthanized) and at the start of the experiment (F =

226 7.1, p = .012). As shown in Figure 2, socially isolated female rats treated with VEH gained

227 significantly less weight compared with their respective pair-housed counterparts (p = .002). This

228 effect of social isolation was prevented by URB694 treatment (SI + URB694 vs SI + VEH, p = .012).

229 3.2. Sucrose preference test

230 Total fluid intake did not differ among groups at each assessment point (Supplemental Table S1).

231 Also, there were no significant differences among groups in their baseline preference for the

232 consumption of the sucrose solution (PH + VEH = 85 ± 2 %; IS + VEH = 88 ± 2 %; PH + URB694 =

233 83 ± 3 %; IS + URB694 = 82 ± 3 %). Of note, the estrous cycle phase had no effect on baseline

sucrose solution preference (F = 0.3, p = .543). However, factorial ANOVA yielded a significant time x condition interaction (F = 5.1, p = .028) on preference changes during the social isolation period (calculated as the difference between each assessment point and the baseline), with no significant effects of the estrous cycle phase (F = 0.4, p = .497). Specifically, as shown in Figure 3, no group differences were observed on day 11. However, on day 25, socially-isolated female rats treated with VEH showed a significantly larger reduction in the preference for sucrose solution consumption compared with their respective pair-housed counterparts (p = .025). This effect was prevented by URB694 treatment (SI + URB694 vs SI + VEH, p = .003). A similar trend was observed on day 39, although differences did not reach full statistical significance (SI + VEH vs PH + VEH, p = .056; SI + VEH vs SI + URB694, p = .067).

3.3. Forced swim test

Behavior during the forced swim test is illustrated in Figure 4. Factorial ANOVA yielded a significant effect of treatment (F = 4.9, p = .033) and a strong trend for condition x treatment interaction (F = 3.5, p = .071) on immobility time, with no significant effects of estrous cycle phase (F = 0.2, p = .632). Specifically, socially isolated female rats treated with VEH spent significantly more time in immobility compared with their respective pair-housed counterparts (p = .024). This behavioral effect of social isolation was significantly corrected by URB694 treatment (SI + URB694 vs SI + VEH, p = .007).

3.4. Measurements at sacrifice

3.4.1. Plasma corticosterone levels and adrenal weight

Factorial ANOVA yielded a significant condition x treatment interaction (F = 7.1, p = .012) on plasma corticosterone levels at the end of the experimental protocol, with no significant effects of the estrous cycle phase (F = 0.6, p = .430). As depicted in Figure 5, socially isolated female rats treated with VEH had significantly higher plasma corticosterone levels than their respective pair-housed counterparts (p = .016). URB694 treatment prevented the effect of social isolation on plasma corticosterone levels (SI + URB694 vs SI + VEH, p = .003).

There were no significant effects of condition and/or treatment on adrenal weight corrected for body weight at the end of the experiment (PH + VEH = 0.021 ± 0.002 mg/g; IS + VEH = 0.027 ± 0.003 mg/g; PH + URB694 = 0.027 ± 0.002 mg/g; IS + URB694 = 0.026 ± 0.002 mg/g).

3.4.2. BDNF hippocampal content

Factorial ANOVA yielded a significant effect of treatment (F = 7.3, $p = .012$) and a significant condition x treatment interaction (F = 6.9, $p = .014$) on BDNF content in the hippocampus at the end of the experimental protocol. As illustrated in Figure 6A, socially isolated female rats treated with VEH showed a significantly lower BDNF hippocampal content compared with their respective pair-housed counterparts ($p = .023$). This effect of social isolation was prevented by URB694 treatment (SI + URB694 vs SI + VEH, $p = .001$). Moreover, we found a negative, although not significant, correlation between plasma corticosterone levels and BDNF hippocampal content (Table 1).

3.4.3. AEA hippocampal levels

Factorial ANOVA yielded significant effects of condition (F = 19.7, $p < .001$) and treatment (F = 27.6, $p < .001$), and a significant condition x treatment interaction (F = 5.3, $p = .028$) on AEA hippocampal levels at the end of the experimental protocol. As shown in Figure 6B, socially isolated female rats treated with VEH showed significantly lower AEA hippocampal levels compared with their respective pair housed counterpart ($p < .001$). As expected, URB694-treated groups showed significantly greater AEA levels than corresponding VEH-treated groups, both in the social isolation ($p < .001$) and pair-housing ($p = .040$) condition. Moreover, we found a significant positive correlation between AEA levels and BDNF content within the hippocampus (Table 1), as well as a strong trend for a negative correlation between AEA hippocampal levels and plasma corticosterone levels (Table 1).

3.4.4. FAAH activity

Factorial ANOVA yielded a significant effect of treatment (F = 456.0, $p < .001$) on FAAH activity in the hippocampus, being, as expected, significantly lower in URB694-treated than VEH-treated rats in both the social isolation ($p < .001$) and pair-housing ($p < .001$) condition (Figure 6C).

4. Discussion

The major findings of the current investigation are the following. Compared to pair-housed females, socially isolated female rats developed behavioral (mild anhedonic state, passive stress coping) and biological (reduced body weight gain, elevated plasma corticosterone levels) changes that together are indicative of a depressive-like state, and showed a reduction in BDNF content and AEA levels

11

within the hippocampus. Notably, pharmacological inhibition of FAAH activity with URB694 restored AEA and BDNF hippocampal levels, and prevented the development of depressive-like behavioral and biological symptoms following a prolonged period of social isolation.

4.1. Depressive-like syndrome in socially isolated female rats

Psychiatric disorders in humans have been linked prevalently with social stress and/or reduced social interaction (Bjorkqvist, 2001; Heinrich and Gullone, 2006). Within preclinical models, the social defeat paradigm has been shown to have a substantial impact on depression-relevant behavioral and physiological parameters in adult male rats, while solitary housing is particularly effective in precipitating depressive-like symptoms in previously group-housed female rats (Beery and Kaufer, 2015; Carnevali et al., 2017a). Accordingly, in the current study we found that six weeks of social isolation in adult female rats produce several changes that mimic the symptoms of human depression (American Psychiatry Association, 2013). Of note, the social isolation protocol adopted in this study included both solitary housing and long-term deprivation of sensory stimuli originating from the surrounding social environment. Therefore, it is likely that the described effects are due to a combination of both. Specifically, female rats showed a reduction in body weight gain, signs of a mild anhedonic-like state (i.e., reduced preference for the consumption of a sucrose solution), passive coping (i.e., increased immobility in the forced swim test), and elevated plasma corticosterone levels. Deficits in body weight gain in isolated rats may be explained by reduced food intake, as previously demonstrated in individually housed mice and rats (Izadi et al., 2018; Sun et al., 2014), particularly around light-dark phase transitions (Sun et al., 2014). Interestingly, reductions in heat production and in the respiratory exchange ratio were also found during light-dark transitions in individually housed mice (Sun et al., 2014), suggesting that metabolic functions may have been affected also in our socially isolated rats. Moreover, the mild reduction in the preference for the consumption of a palatable solution observed only after 25 days of social isolation resembles the time course of changes reported in female Wistar rats exposed to chronic mild stress (Grippo et al., 2005) and in socially isolated female prairie voles (Grippo et al., 2007). However, we acknowledge that the interpretation of this result is limited by the difference, albeit not statistically significant, between the two stressed groups on day 11 (i.e., before the start of the pharmacological treatment).

Notably, the estrous cycle phase did not seem to have any effect on any of the behavioral and biological variables assessed in the current study, although our analysis is limited by the small sample size given that four different stages were considered. Nevertheless, this is in line with empirical research across multiple rodent species demonstrating that estrous cyclicity is not a major source of variability in females or, at least, is not greater than intrinsic variability in males (Beery, 2018; Finnell et al., 2018; Kokras et al., 2015).

Animal and human studies have provided support for the role of stress in the pathogenesis of depression via alterations in BDNF-mediated signaling (Hashimoto, 2010; Stepanichev et al., 2014), a neurotrophin that primarily regulates synaptic plasticity (Leal et al., 2017; Lu et al., 2014). In line with these findings, we found that BDNF content was reduced in the hippocampus of socially isolated female rats with depressive-like symptoms. Remarkably, such downregulation of hippocampal BDNF was paralleled by a decrease in AEA hippocampal levels. Converging lines of evidence support the possibility that AEA signaling at the cannabinoid receptor 1 (CB1R) may be an important mediator of neuroplastic phenomena within the hippocampus (Aguado et al., 2005; Hashimotodani et al., 2007; Hill et al., 2010; Scarante et al., 2017; Burstein et al., 2018). Particularly relevant for the current results are findings of decreased BDNF levels in the hippocampus of CB1R knockout mice (Aso et al., 2008). Thus, we hypothesize that a deficiency in AEA-mediated signaling at the CB1R might be implicated in the downregulation of BDNF hippocampal content observed in socially isolated female rats. Moreover, the positive correlation found here between AEA levels and BDNF content further supports a role for the eCB system in adult hippocampal neurogenesis (Scarante et al., 2017). Notably, while one study reported a similar decrease in AEA levels in the hippocampus of chronically stressed male rats (Hill et al., 2008), other studies showed no changes in AEA hippocampal levels upon chronic stress exposure (Bortolato et al., 2007; Carnevali et al., 2015a; Hill et al., 2005). Of note, our data suggest that reduced AEA levels in the hippocampus of socially isolated rats were not due to an upregulation of FAAH enzymatic activity. This is in line with previous studies showing that FAAH activity is not affected by chronic stress exposure in rats (Bortolato et al., 2007; Hill et al., 2008), suggesting that the stress-induced decline in the hippocampal pool of AEA might be due to diminished biosynthetic mechanisms. Empirical evidence indicates the eCB system may be a

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biochemical effector of glucocorticoids in the brain (Hill and McEwen, 2010). Notably, the hippocampus itself is particularly sensitive to the action of glucocorticoid stress hormones due the rich concentration of receptor sites for glucocorticoids (De Kloet et al., 1998). The negative, although only marginally significant, correlation found between plasma corticosterone levels and AEA hippocampal levels prompts further investigation into the specific mechanisms underlying the effects of stress exposure on AEA metabolism and their causal relationship with BDNF hippocampal downregulation. Interestingly, sex-specific mechanisms of eCB-mediated synaptic modulation within the hippocampus have been proposed to partly explain sex disparities in prevalence of depression (Huang and Woolley, 2012; Tabatadze et al., 2015). Decreased levels of BDNF may contribute to the atrophy of the hippocampus that has been observed in patients with depression (Sheline, 1996; Sheline et al., 2019). Recently, Belleau and colleagues (Belleau et al., 2019) proposed a model according to which chronic life stress can trigger the initial development of hippocampal volume reduction. However, this reduction would be neither necessary nor sufficient to produce a major depressive episode (Belleau et al., 2019). On the other hand, stress also initiates a set of neurotoxic processes (HPA axis dysregulation, inflammation, and neurotransmitter disturbances) that interact and may drive the development of a more chronic type of depression marked by further hippocampal volume reduction (Belleau et al., 2019). Although hippocampal volume was not assessed in the current study, we speculate that AEA–BDNF interactions might be implicated in the development of depressive symptoms and hippocampal volume decline under chronic life stress. Future longitudinal studies in rodent models of social stress-induced depression may be informative in this regard.

4.2. Antidepressant action of the FAAH inhibitor URB694

In an attempt to replicate findings of our previous study demonstrating antidepressant-like effects of the FAAH inhibitor URB694 in chronically stressed male rats (Carnevali et al., 2015a), pharmacological treatment with URB694 started after two weeks of social isolation (i.e., we anticipated that depressive-like behaviors would already have begun to manifest by then). However, contrary to our expectations, we failed to conclusively demonstrate the onset of an anhedonic-like state before the start of the treatment. Thus, the fact that URB694-treated females did not show depressive-like behavioral and biological symptoms after a prolonged period of social isolation

suggests, more cautiously, that inhibition of FAAH activity represents an effective preventive measure in this animal model. These results are in line with a growing body of evidence demonstrating that pharmacological inhibition of FAAH activity produces an antidepressant response in chronically stressed male rodents (Carnevali et al., 2017b). Interestingly, FAAH inhibitors have been shown to increase hippocampal neurogenesis in adult rats (Goncalves et al., 2008; Hill et al., 2006; Marchalant et al., 2009) and prevent stress-induced BDNF downregulation in the brain (Burstein et al., 2018), supposedly via facilitation of CB1R-mediated activation of the extracellular signal-regulated kinase signaling pathway (Derkinderen et al., 2003; Rubino et al., 2006). Therefore, given that CB1Rs are highly abundant in the rodent (and human) hippocampus (Mackie, 2005), we hypothesize that the antidepressant-like action of the FAAH inhibitor URB694 in socially isolated female rats may be partly mediated by a preservation of hippocampal BDNF content via enhancement of AEA signaling at the CB1R. However, the antidepressant-like effects of URB694 may also be interpreted in light of experimental evidence showing that AEA-signaling enhancement at the CB1R facilitates adaptive stress coping behaviors (Haller et al., 2013) and attenuates the neuroendocrine response to psychological stressors (Gorzalka et al., 2008). Moreover, given that FAAH inhibitors also increase the levels of other fatty acid amines with activity at peroxisome proliferator activated receptor-α (N-oleoylethanolamine (OEA) and N-palmitoylethanolamine (PEA)), the possibility of other non-cannabinoid receptor-mediated mechanisms cannot be completely ruled out. For example, a growing body of preclinical evidence suggests that PEA could have antidepressant-like activity (De Gregorio et al., 2019). On the other hand, increases in the endogenous levels of OEA may reduce food intake by regulating systems that control hunger and satiety in the brain (Romano et al., 2015). However, these compounds might also prolong and enhance AEA biological activity by competing with AEA for FAAH-mediated degradation (Petrosino et al., 2009). Of note, the current drug regimen had no effects on control animals, suggesting that the FAAH inhibitor did not affect normal biological processes and behavioral responses.

4.3. Conclusion

The results of this study suggest a potential interplay between AEA-mediated signaling and BDNF at the level of the hippocampus in the pathogenesis of depressive-like symptoms in socially isolated female rats, and document the ability of the FAAH inhibitor URB694 to correct the alterations associated with prolonged social isolation, a naturalistic rodent model of depression with high face, construct, and predictive validity (Carnevali et al., 2017a). These findings complement accumulating evidence on the antidepressant-like effects of FAAH inhibitors in male rodents exposed to chronic stress (reviewed in Carnevali et al., 2017b). Recently, sex differences in hippocampal response to pharmacological inhibition of FAAH activity have been reported in rats after acute intense stress (Zer-Aviv and Akirav, 2016). This suggests that preclinical development of FAAH inhibitors for the pharmacotherapy of depression should aim at comparing the underlying neurobiological mechanisms between males and females. We must acknowledge that, at present, clinical research on FAAH inhibitors has been slowed down by the serious adverse effects caused by the FAAH inhibitor BIA 10–2474 for the treatment of pain (von Schaper, 2016), which displayed both intrinsic toxic effects at high doses and off-targets effects (van Esbroeck et al., 2017). Investigations conducted by a Temporary Specialist Scientific Committee concluded that the toxicity of BIA 10-2474 is unlikely due to FAAH inhibition (Temporary Specialist Scientific Committee, 2016). A communication from the U.S. Food and Drug Administration also reported that the unique toxicity of this drug does not extend to other FAAH inhibitors (Food and Drug Administration, 2016), which are well tolerated by patients enrolled in clinical trials, and remarkably lack of the common adverse events elicited by exogenous cannabinoid-like compounds, including impairment in cognition, motor coordination, and psychoses (Mallet et al., 2016). The disorders for which these agents are being tested are mostly neuropsychiatric, such as pain conditions, depression, anxiety disorders, and phobias (Mallet et al., 2016). Nevertheless, the current results in female rats and previous research in male rodents using the carbamate FAAH inhibitors URB597 (e.g. Bortolato et al., 2007) and URB694 (Carnevali et al., 2015a) warrant more translational studies to examine the mood-modulating properties of this class of FAAH inhibitors and their underlying mechanism of action for the pharmacotherapy of depression in both sexes (Gururajan et al., 2019).

428 **Table 1** Correlation matrix between plasma corticosterone levels, brain-derived neurotrophic factor

429 (BDNF) hippocampal content, and anandamide (AEA) hippocampal levels at the end of the

430 experimental protocol.

		Corticosterone	BDNF	AEA
Corticosterone	r	-		
	p			
BDNF	r	-.32	-	
	p	.082		
AEA	r	-.31	.44	-
	p	.068	.015	

431

432

Figure legends

Figure 1. Timeline of experimental procedures.

Figure 2. Body weight gain of paired-housed (PH) and socially isolated (SI) female rats treated with

vehicle (VEH) or URB694, calculated as the difference between weight at the end (immediately

before animals were euthanized) and at the start (when animals were assigned to the different

housing conditions) of the experiment (n = 10 per group). * = significantly different from

corresponding PH + VEH group; # = significantly different from corresponding SI + VEH group (p

values are reported in the text).

Figure 3. Changes in sucrose solution preference in paired-housed (PH) and socially isolated (SI)

female rats treated with vehicle (VEH) or URB694, calculated as the difference between each

assessment point during the social isolation period and the baseline (n = 10 per group). * =

significantly different from corresponding PH + VEH group; # = significantly different from

corresponding SI + VEH group (p values are reported in the text).

Figure 4. Time spent in immobility during the forced swim test by paired-housed (PH) and socially

isolated (SI) female rats treated with vehicle (VEH) or URB694 (n = 10 per group). * = significantly

different from corresponding PH + VEH group; # = significantly different from corresponding SI +

VEH group (p values are reported in the text).

Figure 5. Plasma corticosterone levels at the end of the experimental protocol in paired-housed (PH)

and socially isolated (SI) female rats treated with vehicle (VEH) or URB694 (n = 10 per group). * =

significantly different from corresponding PH + VEH group; # = significantly different from

corresponding SI + VEH group (p values are reported in the text).

Figure 6. Tissue content of brain-derived neurotrophic factor (BDNF; panel A), anandamide levels

(panel B), and fatty acid amide hydrolase (FAAH) activity (panel C) in the hippocampus of paired-

housed (PH) and socially isolated (SI) female rats treated with vehicle (VEH) or URB694 (n = 10

462   per group). * = significantly different from corresponding PH + VEH group; # = significantly different

463   from corresponding VEH group (p values are reported in the text).

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Figure 1
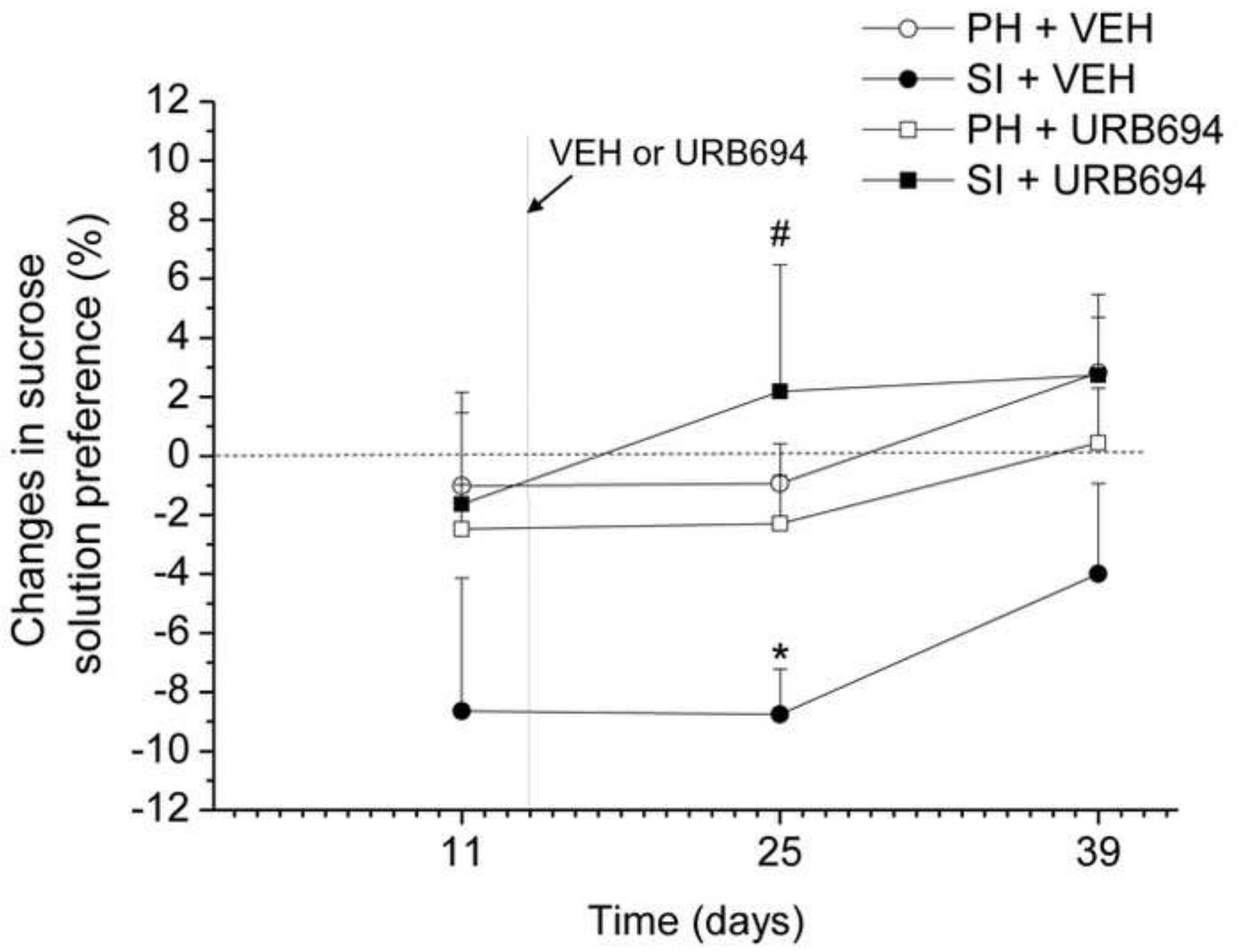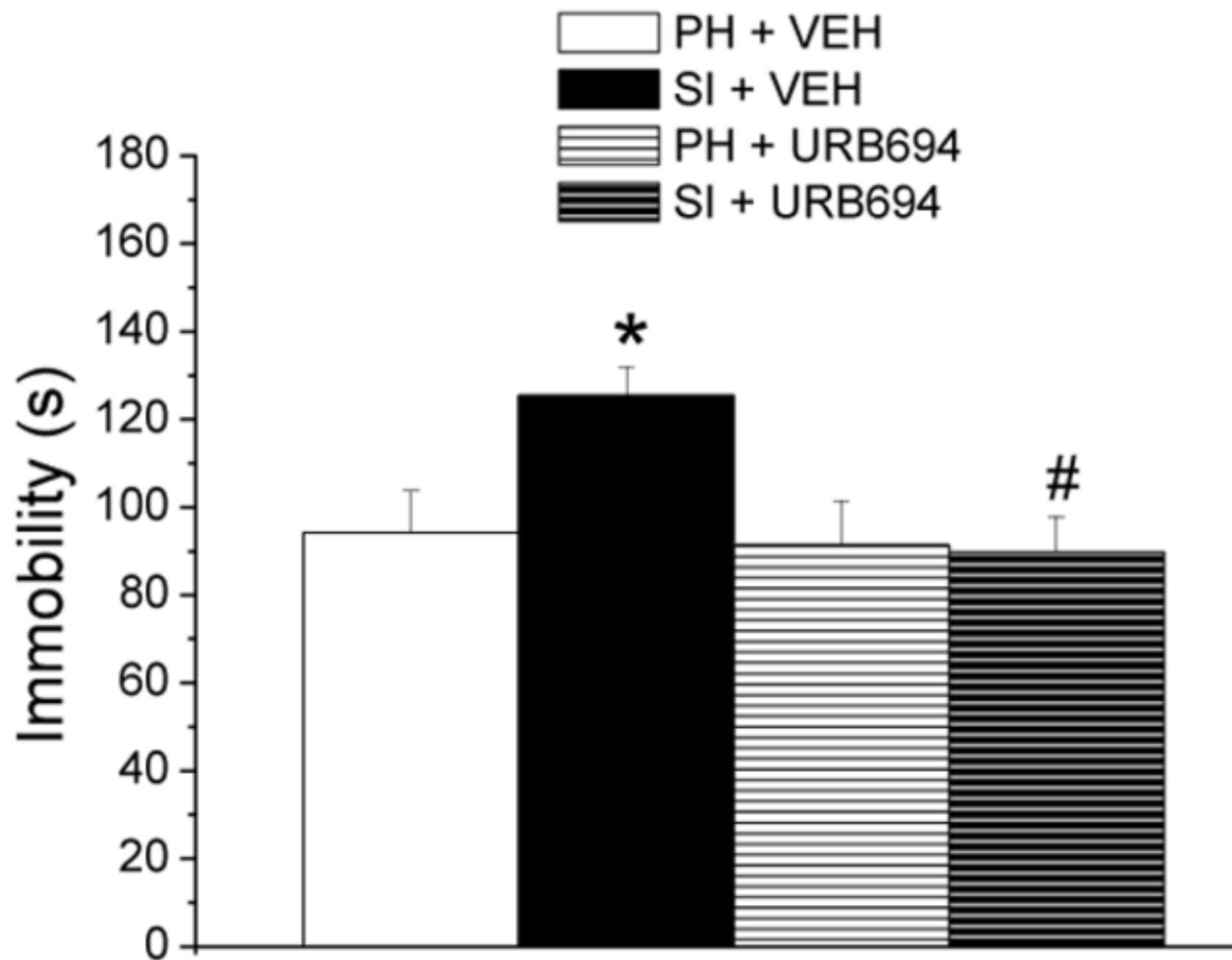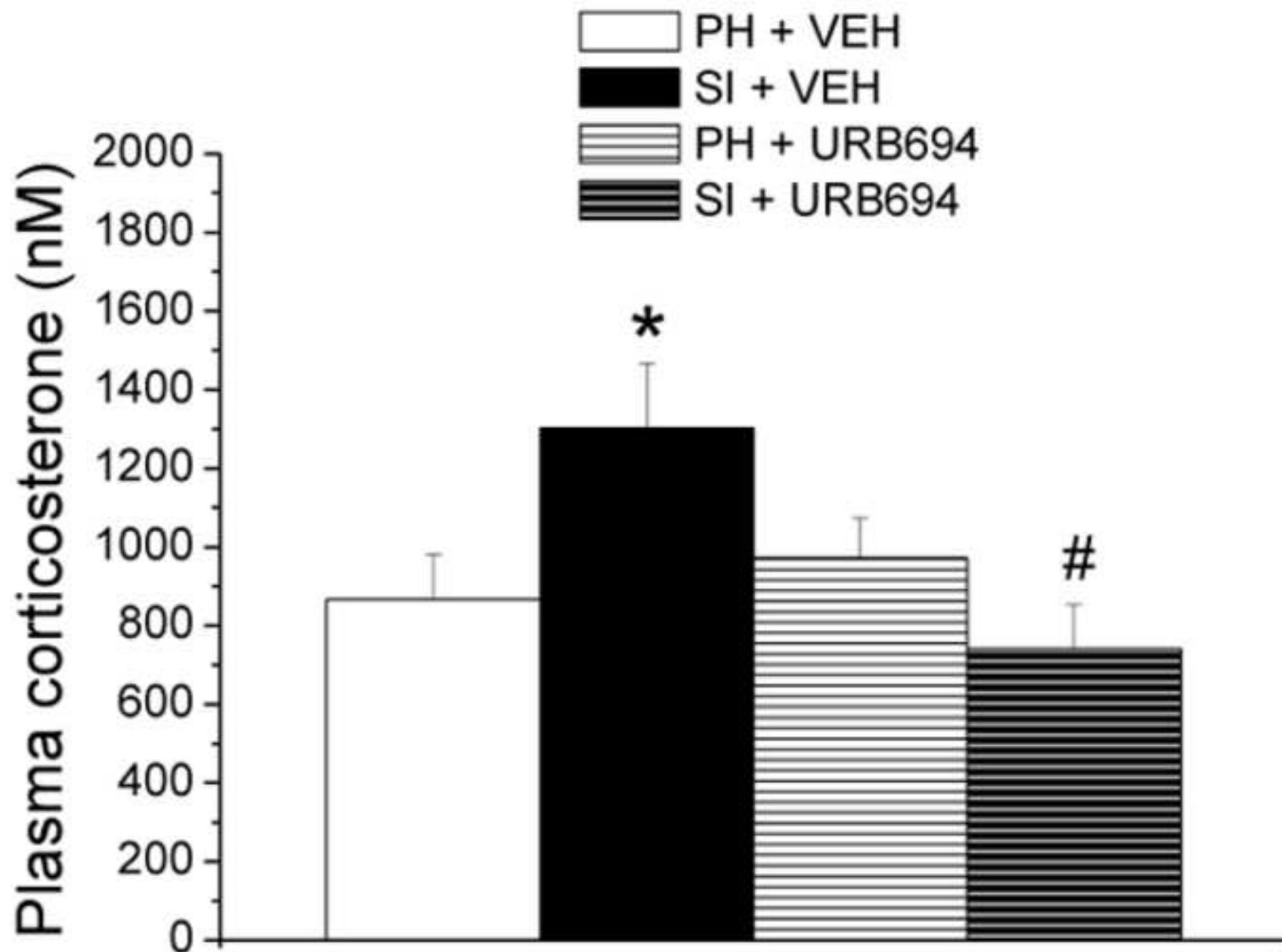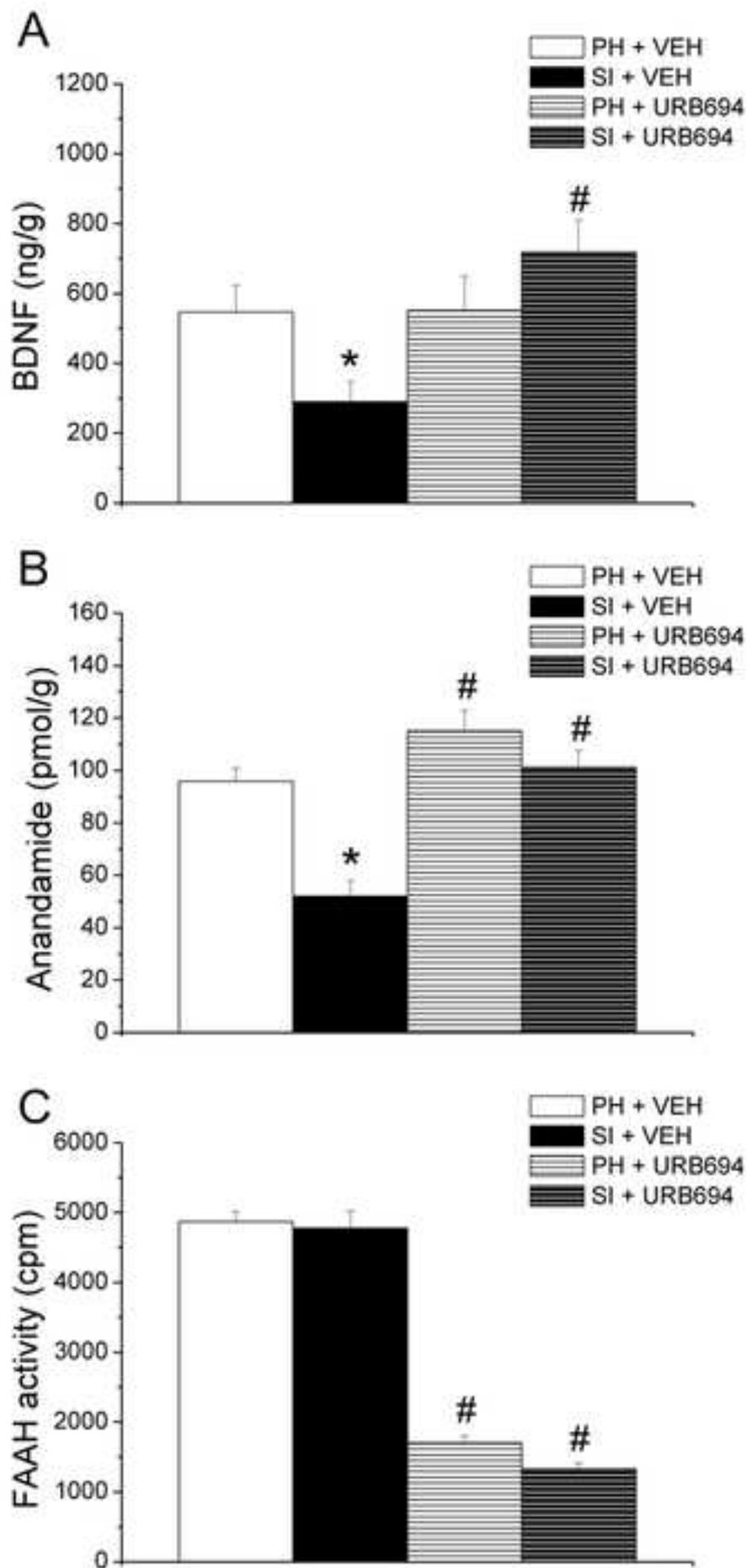
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Role of the Funding Source

Contributors

Authors LC and RS performed the experiments and analysed the data. Authors FV and FF analyzed the data. Authors LC, RS and AS designed the study. Author GS synthesized URB694. Author LC wrote the first draft of the manuscript. Authors RS, FV, SR, MM and AS revised the article critically for important intellectual content. All authors interpreted the results and contributed to and have approved the final manuscript.

Conflict of Interest

All authors declare that they have no conflicts of interest.

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