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

2 **isolated female rats**

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16

17 **Abstract**

18 Pharmacological inhibition of the enzyme fatty acid amide hydrolase (FAAH), which terminates
19 signalling of the endocannabinoid N-arachidonoylethanolamine (or anandamide, AEA), exerts
20 favourable effects in rodent models of stress-related depression. Yet although depression seems to
21 be more common among women than men and in spite of some evidence of sex differences in
22 treatment efficacy, preclinical development of FAAH inhibitors for the pharmacotherapy of stress-
23 related depression has been predominantly conducted in male animals. Here, adult female rats were
24 exposed to six weeks of social isolation and, starting from the second week, treated with the FAAH
25 inhibitor URB694 (0.3 mg/kg/day, i.p.) or vehicle. Compared to pair-housed females, socially isolated
26 female rats treated with vehicle developed behavioral (mild anhedonia, passive stress coping) and
27 physiological (reduced body weight gain, elevated plasma corticosterone levels) alterations.
28 Moreover, prolonged social isolation provoked a reduction in brain-derived neurotrophic factor
29 (BDNF) and AEA levels within the hippocampus. Together, these changes are indicative of an
30 increased risk of developing a depressive-like state. Conversely, pharmacological inhibition of FAAH
31 activity with URB694 restored both AEA and BDNF levels within the hippocampus of socially isolated
32 rats and prevented the development of behavioral and physiological alterations. These results
33 suggest a potential interplay between AEA-mediated signaling and hippocampal BDNF in the
34 pathogenesis of depression-relevant behaviors and physiological alterations and antidepressant
35 action of FAAH inhibition in socially isolated female rats.

36 **Keywords:** depression; stress; endocannabinoid; BDNF; females

37

38

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.,

67 chronic unpredictable stress and social defeat stress) and presenting a "depressive-like" phenotype
68 (reviewed in Carnevali et al., 2017b). These findings have triggered significant interest in the
69 development of eCB-interacting drugs, including direct-acting receptor ligands and catabolism
70 inhibitors for the pharmacotherapy of depression (Micale et al., 2013). Specifically, within preclinical
71 models, facilitation of AEA signaling through pharmacological inhibition of its degrading enzyme (i.e.,
72 fatty acid amide hydrolase (FAAH)) can enhance monoaminergic transmission, increase cellular
73 plasticity and neurotrophin expression within the hippocampus, dampen HPA axis activity, and evoke
74 antidepressant-like behavioral effects (reviewed in Carnevali et al., 2017b). However, while the
75 literature has been unequivocal in showing that women experience depression at twice the rate of
76 men (e.g., Grigoriadis and Robinson, 2007), very few preclinical studies have been conducted on
77 female experimental animals (Beery, 2018; Kokras and Dalla, 2014). Moreover, despite the
78 existence of sex differences in response to antidepressant treatment (Sloan and Kornstein, 2003),
79 preclinical research on the antidepressant action of FAAH inhibitors has been predominantly
80 conducted in male rodents (Carnevali et al., 2017b; Fowler, 2015). Therefore, there is a clear need
81 to use female animals in preclinical models of stress to either confirm and generalize to females the
82 previously obtained male animal-based findings or underscore potential sex differences in the
83 etiology of depression and/or in the efficacy of new treatments.
84 Based on this background, the purpose of the current study was two-fold. First, we aimed at
85 documenting the development of behavioral (passive stress coping, anhedonia) and biological
86 (reduced hippocampal BDNF levels, HPA axis hyperactivity, body weight loss) alterations in adult
87 female rats exposed to prolonged social isolation, a mild chronic social stressor that has been widely
88 used to model symptoms that are often associated with an increased risk of developing a depressive-
89 like state in rodents (Carnevali et al., 2017a). Second, we tested the hypothesis that pharmacological
90 inhibition of FAAH activity would correct the alterations associated with prolonged social isolation.
91 To this aim, we employed the FAAH inhibitor URB694 (6-hydroxy-[1,1′-biphenyl]-3-yl-
92 cyclohexylcarbamate) which was shown to exhibit higher selectivity and more prolonged and
93 profound access to the brain than the standard inhibitor URB597 (Clapper et al., 2009).
94

95 ## 2. Experimental procedures

96 2.1. Animals and housing conditions

97 Four-month-old female wild-type Groningen rats were used in this study. This rat population,
98 originally derived from the University of Groningen (the Netherlands) and currently bred in our
99 laboratory under standard conditions, shows considerable individual differences in trait-like patterns
100 of behavioral and physiological responses to environmental challenges (Carnevali et al., 2014; de
101 Boer et al., 2017). After weaning, female animals were housed in same-sex sibling pairs and kept in
102 rooms with controlled temperature (22 ± 2 °C) and humidity (50 ± 10 %), under a reversed light-dark
103 cycle (light on from 19:00 to 7:00 h), with food and water ad libitum except when required for the
104 sucrose preference test (see below). A total of 40 pairs were included in the study, but only one
105 female rat from each pair was submitted to the experimental procedures described below.
106 Experiments were performed in accordance with the European Community Council Directive
107 2010/63/UE and approved by the Italian legislation on animal experimentation (D.L. 04/04/2014, n.
108 26, authorization n. 449/2017-PR). All efforts were made to reduce sample size and minimize animal
109 suffering.
110

111 2.2. Experimental design

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

123 (n = 4 SI and n = 4 PH rats), starting with the VEH-treated animals. Experimental animals were
124 tested four times in the sucrose preference test and once in the forced swim test during the dark
125 phase of the daily cycle between 10.00 and 12.00 h. At sacrifice (day 42), trunk blood, adrenal
126 glands, and hippocampus were harvested. Body weight was measured weekly throughout the study.
127 Moreover, the estrous cycle phase of female rats was determined immediately after each behavioral
128 test and before sacrifice using vaginal smear cytology. Vaginal smears were collected by gently
129 introducing a moistened (0.9% NaCl) cotton swab in the rat's vagina. The sample was transferred to
130 a glass slide and examined microscopically following Giemsa staining. The phase of the cycle
131 (metaestrous, diestrous, pro-estrous or estrous) was determined based upon the presence of
132 leukocytes, nucleated epithelial or cornfield epithelial cells (Marcondes et al., 2002).
133
134 2.3. Drug treatment
135 URB694 is a carbamate FAAH inhibitor that irreversibly carbamoylates the nucleophile catalytic
136 serine in FAAH active site (Tarzia et al., 2006). URB694 is a second generation inhibitor with
137 improved metabolic stability and selectivity for FAAH (Clapper et al., 2009). URB694 was freshly
138 dissolved in VEH containing 5% PEG, 5% Tween 80, and 90% saline. VEH (vol:1 ml/kg) or URB694
139 (0.3 mg/kg, i.p.) were injected i.p. between 11.00 and 13.00 h and, on the days of the sucrose
140 solution and forced swim tests, at least 1 h after the completion of the test. URB694 dose was chosen
141 based on our previous studies (Carnevali et al., 2015a; Carnevali et al., 2015b), and a pilot study
142 showing that FAAH activity in the brain of female wild-type Groningen rats was substantially inhibited
143 24 h after administration of this drug dose (Supplemental Figure S1).
144
145 2.4. Sucrose preference test
146 *Ad libitum* 2% sucrose solution was available for 5 days before the beginning of the experimental
147 procedures to allow adaptation to its taste. Food and water were removed from the cage for 16 hours
148 before each sucrose preference test; moreover, one hour before the test, all experimental animals
149 (paired and isolated) were moved into individual cages to ensure accurate fluid intake measurements
150 of paired animals. Water and 2% sucrose solution were placed in premeasured bottles in the

151 individual cage, and fluid intake was monitored for 1 hour. Animals were returned to their respective
152 home cages immediately after the test (Grippo et al., 2007). Sucrose preference tests were
153 conducted in baseline conditions (day -3) and after 11, 25, and 39 days of social isolation (Figure 1).
154 Sucrose solution intake was expressed as the relative percentage of the total liquid intake and was
155 taken as an operational index of anhedonia, defined as reduced sucrose preference relative to
156 control animals and baseline values (Grippo et al., 2007).
157
158 2.5. Forced swim test
159 An adapted version of the forced swim test originally described by Porsolt (Porsolt et al., 1977) was
160 used. On day 35 (Fig. 1), female rats were forced to swim individually for 5 min in a Plexiglas cylinder
161 (height: 40 cm, diameter: 30 cm) filled with water (temperature: 24 ± 2°C; depth: 30 cm). During the
162 test, rats' behavior was video-taped. The overall time spent in immobility (floating and making only
163 those movements necessary to keep the head above water) was scored by a trained experimenter
164 blind to animals' condition and treatment. Immobility during the single session of the forced swim
165 test was used as an index of passive stress coping (Commons et al., 2017).
166
167 2.6. Measurements at sacrifice
168 Twenty-four hours after the last administration of URB694 or VEH (i.e., at 11.00 h; day 42, Figure
169 1), female rats were euthanized by decapitation under isoflurane anesthesia (2% in 100% oxygen).
170 Trunk blood was collected in EDTA-coated tubes (Sarsted AG, Numbrecht, Germany) and plasma
171 was separated by centrifugation (2600 g, 4°C, 10 min). Brains were immediately removed and the
172 hippocampus rapidly dissected and snap-frozen in nitrogen. All samples were stored at -80°C until
173 further analysis, as described below. Adrenal glands were also removed and weighed.
174 *2.6.1. Plasma corticosterone levels*
175 Plasma was deproteinized by addition of two volumes of organic solvent (ice-cold acetonitrile),
176 containing the internal standard dexamethasone (structural analog of corticosterone, 75 nmol/L).
177 After centrifugation (14000 g, 4°C, 10 min), the supernatant was directly injected in the liquid
178 chromatography/tandem mass spectrometry system (HPLC/MS/MS) for quantification of

179 corticosterone levels, in accordance with previously published analytical methods (Plenis et al.,
180 2011). A detailed description of the HPLC/MS/MS analytical method and related MS instrumentation
181 is reported in the Supplemental Material.

182 *2.6.2. BDNF hippocampal content*

183 BDNF content in the hippocampus was measured using a commercially available sandwich enzyme-
184 linked immune sorbent assay (ELISA) kit (Quantikine ®ELISA-Total BDNF, R&D Systems,
185 Minneapolis, MN, USA) according to the manufacturer's instructions. A detailed description of the
186 experimental procedure is reported in the Supplemental Material. BDNF tissue content was
187 expressed as a percentage of the control group (PH+VEH rats).

188 *2.6.3. AEA hippocampal levels*

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

195 *2.6.4. FAAH activity in the hippocampus*

196 For ex vivo determination of FAAH activity, frozen hippocampi were thawed and homogenized in ice-
197 cold Tris buffer (10 volumes, 50 mM, pH 7.5) containing 0.32 M sucrose. The homogenates were
198 centrifuged (1000 g, 10 min, 4°C) and total protein content was quantified in the supernatant by the
199 bicinchoninic acid (BCA) protein kit (Pierce Biotechnology, Rockford, IL, USA). FAAH activity was
200 measured at 37°C for 30 min in 0.5 mL Tris buffer (50 mM, pH 7.5) containing fatty acid-free bovine
201 serum albumin (BSA) (0.05%, w/v), 50 μg of protein from brain homogenates, 10 μM AEA and $[^3H]$-
202 AEA (10000 disintegrations per minute) as previously described (Clapper et al., 2009). Briefly, the
203 reactions were stopped with 1 mL chloroform:methanol (1:1). After centrifugation (2000 g, 10 min,
204 4°C), $[^3H]$-ethanolamine was measured in the aqueous phase by liquid scintillation counting. $[^3H]$-
205 AEA (specific activity: 60 Ci/mmol), employed as a substrate for ex vivo FAAH assay, was purchased
206 from American Radiolabeled Chemicals (St. Louis, MI, USA).

207 2.7. Statistical analysis

208 All statistical analyses were performed using SPSS v. 25 (IBM software package). Data are 209 presented as mean ± standard error of the mean (SEM). The influence of the estrous cycle phase 210 on behavioral and biochemical measurements was controlled in all statistical analyses. A three-way 211 ANOVA for repeated measures with "condition" (2 levels: isolation, pair-housing) and "treatment" (2 212 levels: VEH, URB694) as the between subject factors, and "time" as the within subject factor (3 213 levels: days 11, 25, and 39) was applied on delta changes in sucrose solution preference with respect 214 to baseline. All other data were analyzed with 2 (factor "condition": isolation or pair-housing) x 2 215 (factor "treatment": URB694 or VEH) factorial design ANOVAs. Follow-up analyses were conducted 216 using Student's "t" tests, with a Bonferroni correction for multiple comparisons. Pearson's r 217 correlations were performed to assess the correlation between plasma corticosterone levels, BDNF 218 hippocampal content and AEA hippocampal levels. Statistical significance was set at $p < 0.05$.

219

220 ## 3. Results

221 3.1. Body weight

222 There were no significant differences in body weight among groups at the start of the experiment 223 (i.e., when animals were assigned to the different housing conditions) (PH + VEH = 230 ± 2 g; IS + 224 VEH = 237 ± 5 g; PH + URB694 = 231 ± 4 g; IS + URB694 = 226 ± 8 g). However, a significant time 225 x condition interaction emerged on body weight gain calculated as the difference between weight at 226 the end (i.e., immediately before animals were euthanized) and at the start of the experiment ($F =$ 227 7.1, $p = .012$). As shown in Figure 2, socially isolated female rats treated with VEH gained 228 significantly less weight compared with their respective pair-housed counterparts ($p = .002$). This 229 effect of social isolation was prevented by URB694 treatment (SI + URB694 vs SI + VEH, $p = .012$).

230 3.2. Sucrose preference test

231 Total fluid intake did not differ among groups at each assessment point (Supplemental Table S1). 232 Also, there were no significant differences among groups in their baseline preference for the 233 consumption of the sucrose solution (PH + VEH = 85 ± 2 %; IS + VEH = 88 ± 2 %; PH + URB694 = 234 83 ± 3 %; IS + URB694 = 82 ± 3 %). Of note, the estrous cycle phase had no effect on baseline

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

245 3.3. Forced swim test

246 Behavior during the forced swim test is illustrated in Figure 4. Factorial ANOVA yielded a significant
247 effect of treatment (F = 4.9, p = .033) and a strong trend for condition x treatment interaction (F =
248 3.5, p = .071) on immobility time, with no significant effects of estrous cycle phase (F = 0.2, p = .632).
249 Specifically, socially isolated female rats treated with VEH spent significantly more time in immobility
250 compared with their respective pair-housed counterparts (p = .024). This behavioral effect of social
251 isolation was significantly corrected by URB694 treatment (SI + URB694 vs SI + VEH, p = .007).

252 3.4. Measurements at sacrifice

253 *3.4.1. Plasma corticosterone levels and adrenal weight*

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

260 There were no significant effects of condition and/or treatment on adrenal weight corrected for body
261 weight at the end of the experiment (PH + VEH = 0.021 ± 0.002 mg/g; IS + VEH = 0.027 ± 0.003
262 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 physiological (reduced body weight gain, elevated plasma corticosterone levels) changes, and showed a reduction in BDNF and AEA levels within the hippocampus. Together, these changes are

291 indicative of an increased risk of developing a depressive-like state. Notably, pharmacological
292 inhibition of FAAH activity with URB694 restored AEA and BDNF hippocampal levels, and prevented
293 the development of behavioral and physiological alterations following prolonged social isolation.

294 4.1. Depressive-like changes in socially isolated female rats

295 Psychiatric disorders in humans have been linked prevalently with social stress and/or reduced
296 social interaction (Bjorkqvist, 2001; Heinrich and Gullone, 2006). Within preclinical models, the social
297 defeat paradigm has been shown to have a substantial impact on depression-relevant behavioral
298 and physiological parameters in adult male rats, while solitary housing is particularly effective in
299 precipitating depressive-like symptoms in previously group-housed female rats (Beery and Kaufer,
300 2015; Carnevali et al., 2017a). Of note, the social isolation protocol adopted in this study included
301 both solitary housing and long-term deprivation of sensory stimuli originating from the surrounding
302 social environment. Therefore, it is likely that the described effects are due to a combination of both.
303 Specifically, female rats showed a reduction in body weight gain, signs of a mild anhedonic-like state
304 (i.e., reduced preference for the consumption of a sucrose solution), passive coping (i.e., increased
305 immobility in the forced swim test), and elevated plasma corticosterone levels. Deficits in body weight
306 gain in isolated rats may be explained by reduced food intake, as previously demonstrated in
307 individually housed mice and rats (Izadi et al., 2018; Sun et al., 2014), particularly around light-dark
308 phase transitions (Sun et al., 2014). Interestingly, reductions in heat production and in the respiratory
309 exchange ratio were also found during light-dark transitions in individually housed mice (Sun et al.,
310 2014), suggesting that metabolic functions may have been affected also in our socially isolated rats.
311 Moreover, the mild reduction in the preference for the consumption of a palatable solution observed
312 only after 25 days of social isolation resembles the time course of changes reported in female Wistar
313 rats exposed to chronic mild stress (Grippo et al., 2005) and in socially isolated female prairie voles
314 (Grippo et al., 2007). However, we acknowledge that the interpretation of this result is limited by the
315 difference, albeit not statistically significant, between the two stressed groups on day 11 (i.e., before
316 the start of the pharmacological treatment). Notably, the estrous cycle phase did not seem to have
317 any effect on any of the behavioral and biological variables assessed in the current study, although
318 our analysis is limited by the small sample size given that four different stages were considered.

319 Nevertheless, this is in line with empirical research across multiple rodent species demonstrating
320 that estrous cyclicity is not a major source of variability in females or, at least, is not greater than
321 intrinsic variability in males (Beery, 2018; Finnell et al., 2018; Kokras et al., 2015).
322 Animal and human studies have provided support for the role of stress in the pathogenesis of
323 depression via alterations in BDNF-mediated signaling (Hashimoto, 2010; Stepanichev et al., 2014),
324 a neurotrophin that primarily regulates synaptic plasticity (Leal et al., 2017; Lu et al., 2014). In line
325 with these findings, we found that BDNF content was reduced in the hippocampus of socially isolated
326 female rats with depressive-like symptoms. Remarkably, such downregulation of hippocampal BDNF
327 was paralleled by a decrease in AEA hippocampal levels. Converging lines of evidence support the
328 possibility that AEA signaling at the cannabinoid receptor 1 (CB1R) may be an important mediator
329 of neuroplastic phenomena within the hippocampus (Aguado et al., 2005; Hashimotodani et al.,
330 2007; Hill et al., 2010; Scarante et al., 2017; Burstein et al., 2018). Particularly relevant for the current
331 results are findings of decreased BDNF levels in the hippocampus of CB1R knockout mice (Aso et
332 al., 2008). Thus, we hypothesize that a deficiency in AEA-mediated signaling at the CB1R might be
333 implicated in the downregulation of BDNF hippocampal content observed in socially isolated female
334 rats. Moreover, the positive correlation found here between AEA levels and BDNF content further
335 supports a role for the eCB system in adult hippocampal neurogenesis (Scarante et al., 2017).
336 Notably, while one study reported a similar decrease in AEA levels in the hippocampus of chronically
337 stressed male rats (Hill et al., 2008), other studies showed no changes in AEA hippocampal levels
338 upon chronic stress exposure (Bortolato et al., 2007; Carnevali et al., 2015a; Hill et al., 2005). Of
339 note, our data suggest that reduced AEA levels in the hippocampus of socially isolated rats were not
340 due to an upregulation of FAAH enzymatic activity. This is in line with previous studies showing that
341 FAAH activity is not affected by chronic stress exposure in rats (Bortolato et al., 2007; Hill et al.,
342 2008), suggesting that the stress-induced decline in the hippocampal pool of AEA might be due to
343 diminished biosynthetic mechanisms. Empirical evidence indicates the eCB system may be a
344 biochemical effector of glucocorticoids in the brain (Hill and McEwen, 2010). Notably, the
345 hippocampus itself is particularly sensitive to the action of glucocorticoid stress hormones due the
346 rich concentration of receptor sites for glucocorticoids (De Kloet et al., 1998). The negative, although

347 only marginally significant, correlation found between plasma corticosterone levels and AEA
348 hippocampal levels prompts further investigation into the specific mechanisms underlying the effects
349 of stress exposure on AEA metabolism and their causal relationship with BDNF hippocampal
350 downregulation. Interestingly, sex-specific mechanisms of eCB-mediated synaptic modulation within
351 the hippocampus have been proposed to partly explain sex disparities in prevalence of depression
352 (Huang and Woolley, 2012; Tabatadze et al., 2015). Decreased levels of BDNF may contribute to
353 the atrophy of the hippocampus that has been observed in patients with depression (Sheline, 1996;
354 Sheline et al., 2019). Recently, Belleau and colleagues (Belleau et al., 2019) proposed a model
355 according to which chronic life stress can trigger the initial development of hippocampal volume
356 reduction. However, this reduction would be neither necessary nor sufficient to produce a major
357 depressive episode (Belleau et al., 2019). On the other hand, stress also initiates a set of neurotoxic
358 processes (HPA axis dysregulation, inflammation, and neurotransmitter disturbances) that interact
359 and may drive the development of a more chronic type of depression marked by further hippocampal
360 volume reduction (Belleau et al., 2019). Although hippocampal volume was not assessed in the
361 current study, we speculate that AEA–BDNF interactions might be implicated in the development of
362 depressive symptoms and hippocampal volume decline under chronic life stress. Future longitudinal
363 studies in rodent models of social stress may be informative in this regard.

364 4.2. Antidepressant-like effects of the FAAH inhibitor URB694

365 In an attempt to replicate findings of our previous study demonstrating antidepressant-like effects of
366 the FAAH inhibitor URB694 in chronically stressed male rats (Carnevali et al., 2015a),
367 pharmacological treatment with URB694 started after two weeks of social isolation (i.e., we
368 anticipated that depressive-like behaviors would already have begun to manifest by then). However,
369 contrary to our expectations, we failed to conclusively demonstrate the onset of an anhedonic-like
370 state before the start of the treatment. Thus, the fact that URB694-treated females did not show
371 depressive-like behavioral and biological symptoms after a prolonged period of social isolation
372 suggests, more cautiously, that inhibition of FAAH activity represents an effective preventive
373 measure in this animal model. These results are in line with a growing body of evidence
374 demonstrating that pharmacological inhibition of FAAH activity produces an antidepressant response

375 in chronically stressed male rodents (Carnevali et al., 2017b). Interestingly, FAAH inhibitors have
376 been shown to increase hippocampal neurogenesis in adult rats (Goncalves et al., 2008; Hill et al.,
377 2006; Marchalant et al., 2009) and prevent stress-induced BDNF downregulation in the brain
378 (Burstein et al., 2018), supposedly via facilitation of CB1R-mediated activation of the extracellular
379 signal-regulated kinase signaling pathway (Derkinderen et al., 2003; Rubino et al., 2006). Therefore,
380 given that CB1Rs are highly abundant in the rodent (and human) hippocampus (Mackie, 2005), we
381 hypothesize that the antidepressant-like action of the FAAH inhibitor URB694 in socially isolated
382 female rats may be partly mediated by a preservation of hippocampal BDNF content via
383 enhancement of AEA signaling at the CB1R. However, the antidepressant-like effects of URB694
384 may also be interpreted in light of experimental evidence showing that AEA-signaling enhancement
385 at the CB1R facilitates adaptive stress coping behaviors (Haller et al., 2013) and attenuates the
386 neuroendocrine response to psychological stressors (Gorzalka et al., 2008). Moreover, given that
387 FAAH inhibitors also increase the levels of other fatty acid amines with activity at peroxisome
388 proliferator activated receptor-α (N-oleoylethanolamine (OEA) and N-palmitoylethanolamine (PEA)),
389 the possibility of other non-cannabinoid receptor-mediated mechanisms cannot be completely ruled
390 out. For example, a growing body of preclinical evidence suggests that PEA could have
391 antidepressant-like activity (De Gregorio et al., 2019). On the other hand, increases in the
392 endogenous levels of OEA may reduce food intake by regulating systems that control hunger and
393 satiety in the brain (Romano et al., 2015). However, these compounds might also prolong and
394 enhance AEA biological activity by competing with AEA for FAAH-mediated degradation (Petrosino
395 et al., 2009). Of note, the current drug regimen had no effects on control animals, suggesting that
396 the FAAH inhibitor did not affect normal biological processes and behavioral responses.
397

398 ### 4.3. Conclusion

399 The results of this study suggest a potential interplay between AEA-mediated signaling and BDNF
400 at the level of the hippocampus in the development of depression-relevant behaviors and
401 physiological changes in female rats exposed to prolonged social isolation. Moreover, the current
402 results document the ability of the FAAH inhibitor URB694 to correct the alterations associated with

403 prolonged social isolation. One should note, however, that the current results are merely suggestive,
404 and their translational implications for depression should be interpreted within the context of their
405 limitations. First, the behavioral and biological changes described here after social isolation are often
406 associated with an increased risk of developing major depression, but are not clinical symptoms of
407 major depression per se. Second, we adopted a rodent model of prolonged social isolation, which
408 should not be intended as a diagnostic model, but rather as a model of risk and vulnerability factor
409 of stress-related depression. Moreover, we must acknowledge that, at present, clinical research on
410 FAAH inhibitors has been slowed down by the serious adverse effects caused by the FAAH inhibitor
411 BIA 10–2474 for the treatment of pain (von Schaper, 2016), which displayed both intrinsic toxic
412 effects at high doses and off-targets effects (van Esbroeck et al., 2017). Investigations conducted by
413 a Temporary Specialist Scientific Committee concluded that the toxicity of BIA 10-2474 is unlikely
414 due to FAAH inhibition (Temporary Specialist Scientific Committee, 2016). A communication from
415 the U.S. Food and Drug Administration also reported that the unique toxicity of this drug does not
416 extend to other FAAH inhibitors (Food and Drug Administration, 2016), which are well tolerated by
417 patients enrolled in clinical trials, and remarkably lack of the common adverse events elicited by
418 exogenous cannabinoid-like compounds, including impairment in cognition, motor coordination, and
419 psychoses (Mallet et al., 2016). The disorders for which these agents are being tested are mostly
420 neuropsychiatric, such as pain conditions, depression, anxiety disorders, and phobias (Mallet et al.,
421 2016). Nevertheless, the current results in female rats and previous research in male rodents using
422 the carbamate FAAH inhibitors URB597 (e.g. Bortolato et al., 2007) and URB694 (Carnevali et al.,
423 2015a) warrant more translational studies to examine the mood-modulating properties of this class
424 of FAAH inhibitors (Gururajan et al., 2019). Recently, sex differences in hippocampal response to
425 pharmacological inhibition of FAAH activity have been reported in rats after acute intense stress
426 (Zer-Aviv and Akirav, 2016). This suggests that preclinical development of FAAH inhibitors for the
427 pharmacotherapy of stress-related depression should aim at comparing the underlying
428 neurobiological mechanisms between males and females.

429 **Table 1** Correlation matrix between plasma corticosterone levels, brain-derived neurotrophic factor
430 (BDNF) hippocampal content, and anandamide (AEA) hippocampal levels at the end of the
431 experimental protocol.

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

432

433

434 **Figure legends**

435 **Figure 1.** Timeline of experimental procedures.

436

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

443

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

449

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

454

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

459

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

463 Data are expressed mean±SEM. BDNF values are expressed as a percentage of the control group

464 (PH+VEH rats). * = significantly different from corresponding PH + VEH group; # = significantly

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

466

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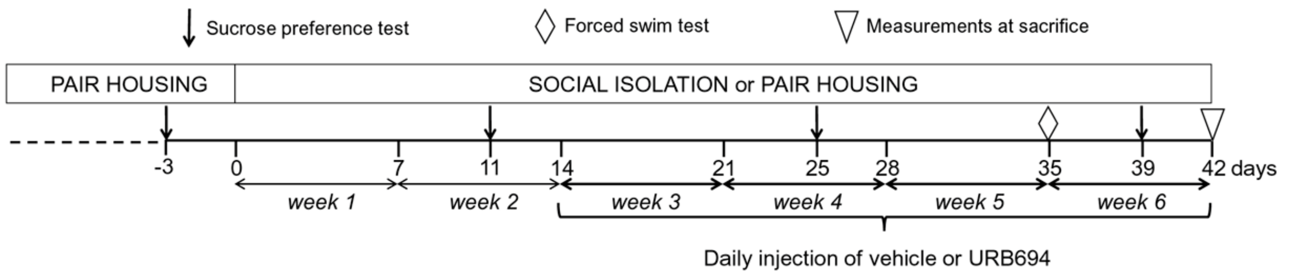

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669 FIGURE 1

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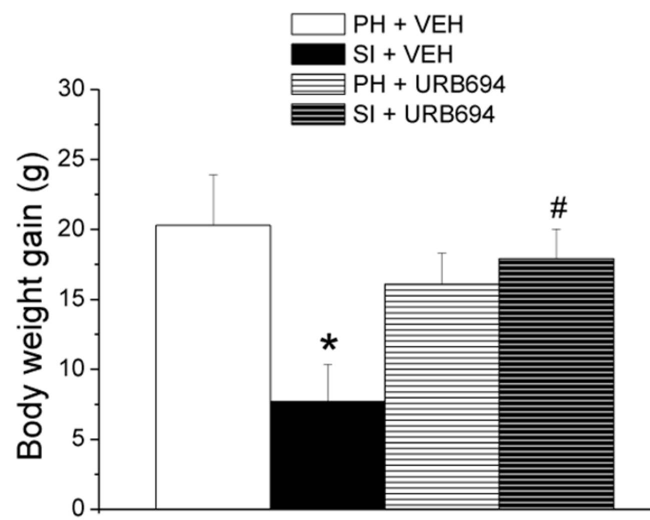

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673 FIGURE 2

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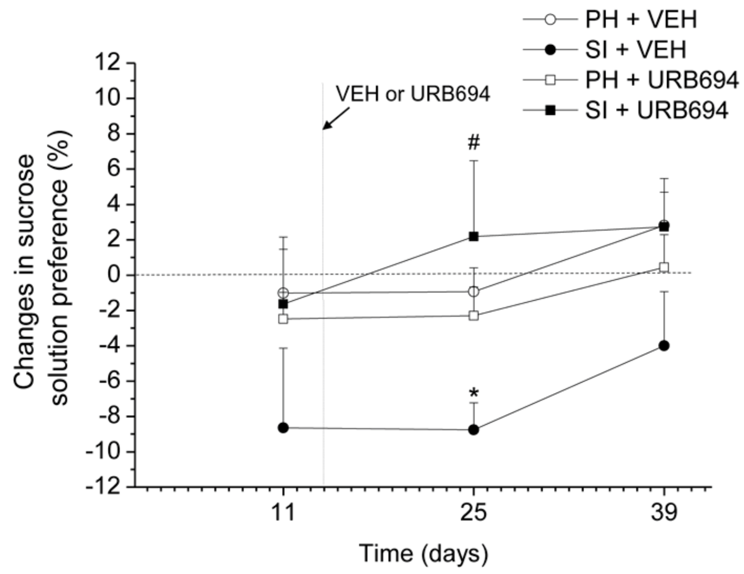

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676 FIGURE 3

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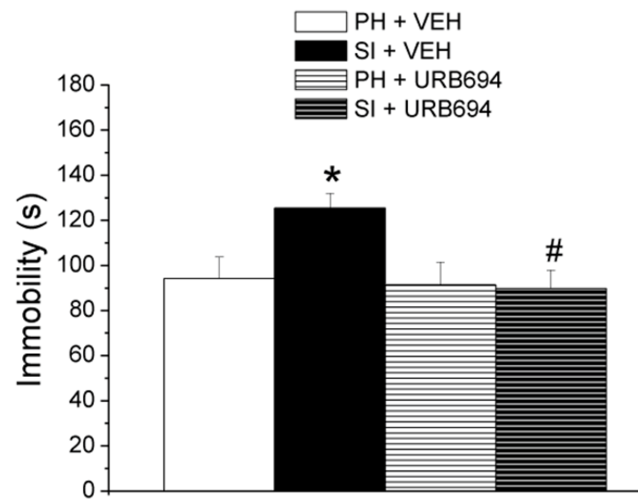

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680 FIGURE 4

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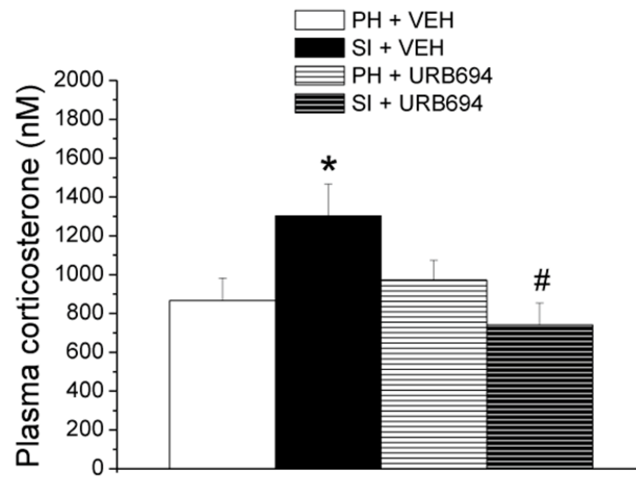
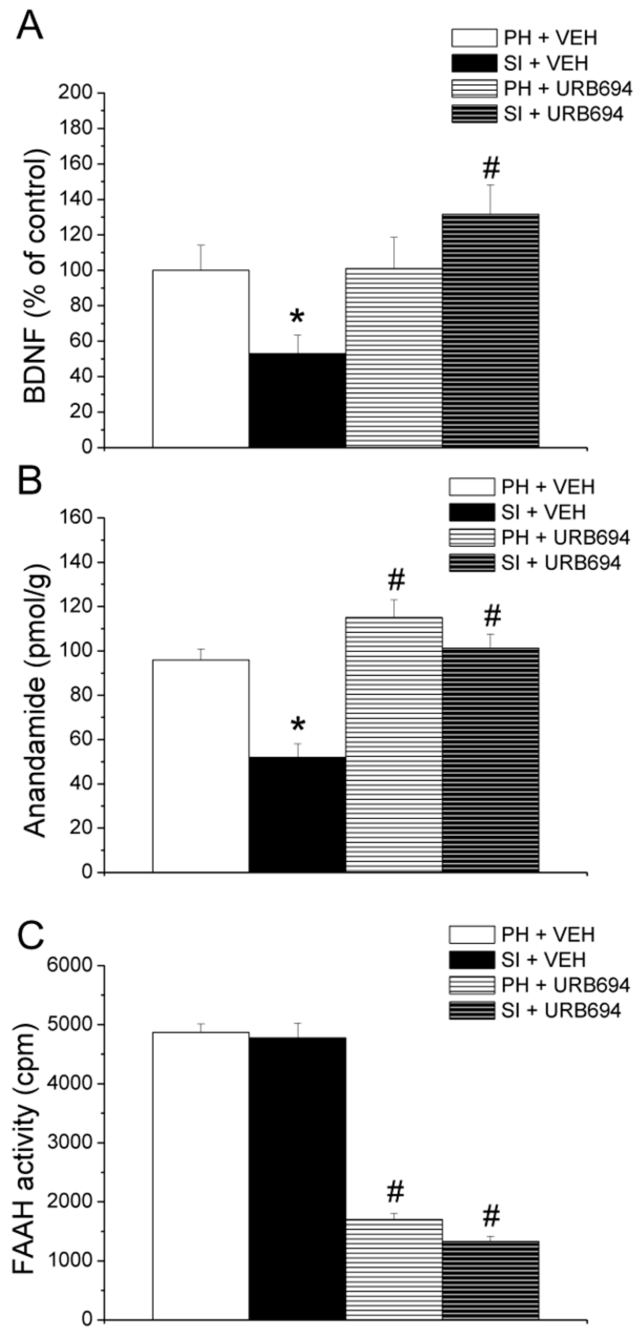


683 FIGURE 5



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685 FIGURE 6