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Anxiety associated with palatable food withdrawal is reversed by the selective FAAH inhibitor PF-3845: A regional analysis of the contribution of endocannabinoid signaling machinery

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Abstract

Objective: Consumption of energy-dense palatable "comfort" food can alleviate stress and negative emotions, while abrupt withdrawal from a palatable diet can worsen these symptoms, causing difficulties with adherence to weight-loss diets. Currently, no pharmacological treatment is effective for obesity-related anxiety, so we investigated the endocannabinoid system (ECS), and specifically the fatty acid amide hydrolase (FAAH), as an interesting emerging target in this context because of its key role in the regulation of both energy homeostasis and emotional behavior.

Methods: Rats were subjected to exposure and subsequent abstinence from a palatable cafeteria diet. During abstinence period, rats were treated with the selective FAAH inhibitor PF-3845 (10 mg/kg; intraperitoneal administration every other day).

Results: Abstinent rats displayed an anxiogenic-like behavior and changes in the proteins of ECS signaling machinery in brain areas involved both in anxiety and food intake regulation. In particular, withdrawal caused a reduction of the expression of cannabinoid receptors in the nucleus accumbens and of enzymes diacylglycerol lipase alpha and monoacylglycerol lipase (MAGL) in the amygdala. Pharmacological inhibition of FAAH exerted an anxiolytic-like effect in abstinent animals and increased both MAGL expression in amygdala and CB2 expression in prefrontal cortex.

Discussion: Overall, our results suggest that emotional disturbances associated with dieting are coupled with region-specific alterations in the cerebral expression of the ECS and that the enhancement of the endocannabinoid signaling by FAAH inhibition

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might represent a novel pharmacological strategy for the treatment of anxiety related to abstinence from palatable food.

Public Significance: The present study focused on evaluating the role of the endocannabinoid system in modulating withdrawal from naturally rewarding activities that have an impact on mood, such as feeding. The variations observed in the emotional behavior of abstinent rats was linked to neuroadaptations of the ECS in specific brain areas.

KEYWORDS
abstinence, anxiety, endocannabinoid system, fatty acid amide hydrolase, obesity, palatable diet, PF-3845

1 | INTRODUCTION

In developed countries, access to palatable food rich in calories (saturated fats and simple sugars) is readily available and has become part of everyday life. Excessive consumption of these ultra-processed, energy-dense foods leads to overweight and obesity (Beslay et al., 2020; Fazzino et al., 2021). It is well-accepted that diets that include an excess of fat and sugars are associated with metabolic dysfunctions and mental health pathologies (Fulton et al., 2022); thus, obesity has been associated with an increased risk of depression and anxiety (Amiri & Behnezhad, 2019; Fulton et al., 2022; Quirk et al., 2013). Moreover, exposure to palatable food provokes anxiety-like behavior in rodents (André et al., 2014; Cottone, Sabino, Roberto, et al., 2009; Cottone, Sabino, Steardo, & Zorrilla, 2009; Iemolo et al., 2012; Sharma et al., 2013).

Obesity and eating disorders can be viewed as chronic, relapsing states with an oscillation between abstinence (dieting) and relapse (Corwin & Grigson, 2009; Johnson & Kenny, 2010; Micioni Di Bonaventura, Di Bonaventura, et al., 2021; Parylak et al., 2011). When individuals with obesity engage in dietary restriction and abstain from highly palatable food, homeostatic eating control circuits may identify food self-deprivation as similar to starvation (Berthoud et al., 2011; Parylak et al., 2011; Volkow et al., 2011). The non-homeostatic brain circuits participating in the control of eating translate such experience into a negative emotional state leading to anxiety that contributes to individuals ceasing to abstain from highly palatable food (Adam & Epel, 2007; Herman et al., 2008; Volkow et al., 2013).

Currently, no effective pharmacological treatment is available to treat obesity-related psychiatric comorbidities, including anxiety. Serotonin-selective reuptake inhibitors (SSRIs, the most widely used class of medication for anxiety disorders) are not appropriate to treat individuals with obesity. Both, weight gain and diabetes type 2 have been reported as side effects of antidepressants, including SSRIs, with important differences in between compounds and patients responses, which make SSRI unreliable therapies for comorbid anxiety/depression (Andersohn et al., 2009; Blumenthal et al., 2014; Uguz et al., 2015).

The endocannabinoid system (ECS) has been considered a valuable target for the identification of novel and effective therapies for the treatment of obesity. ECS and related compounds, including *N*-acylethanolamides (such as anandamide [AEA], palmitoilethanolamide [PEA], and oleoylethanolamide [OEA]), are widely present in brain regions crucial for homeostasis, including the motivation for palatable food consumption and the control of energy metabolism and food intake (DiPatrizio, 2021; Gaetani et al., 2008; Rahman et al., 2021; Romano et al., 2015; Romano et al., 2020). In addition, recent studies have highlighted the role of ECS as a modulator of bioenergetics in the brain and of neuronal and glial mitochondrial respiration (Bénard et al., 2012; Hebert-Chatelain et al., 2016).

Moreover, obesity has been associated with dynamic changes in ECS: individuals with obesity show an increase of the endocannabinoid tone and an overactivation of the ECS (Monteleone et al., 2016; Silvestri & Di Marzo, 2013). Furthermore, clinical and preclinical observations reported that obesity is linked to polymorphisms of genes involved in the ECS (Pucci et al., 2021; Rossi et al., 2018) and that individuals with obesity present altered expression of proteins of ECS both in peripheral tissues (Cocci et al., 2021; Ruiz de Azua & Lutz, 2019) and in the brain (Pucci et al., 2019).

Despite the studies suggesting a role for ECS in the development of obesity, no effective therapy targeting this system has been approved, because of behavioral side effects observed both in preclinical and clinical research (de Ceglia et al., 2021).

Indeed, different components of ECS show a unique role in modulating the onset and offset of anxiety induced by stress response (Bedse et al., 2015, 2017; Lisboa et al., 2017; Maldonado et al., 2020). For instance, AEA acts in anxiety onset; on the other hand, a delay in the 2-arachidonoylglycerol (2-AG) signaling dampens anxiety induced by stress exposure. In addition, CB1 agonists modulate anxiety in a dose-dependent manner: low dosages show anxiolytic properties while high dosages seem to be anxiogenic (Petrie et al., 2021). Moreover, other acylethanolamides including PEA and OEA, which share biosynthetic and degradation pathways with AEA, can exert anxiolytic and antidepressant effects (Antón et al., 2017; Borrelli & Izzo, 2009; Petrie et al., 2021).

Previous studies have investigated the role of the ECS in anxiety related to food withdrawal; in particular, it has been suggested that CB1 antagonism (through rimonabant administration) induced an anxiety-like behavior in rats withdrawn from palatable food cycling (Blasio et al., 2013, 2014; Gamble-George et al., 2013), demonstrating that rimonabant treatment was not effective in treating anxiety associated with dieting (Moreira & Crippa, 2009).

In this context, a novel approach to treat anxiety associated with food withdrawal might be the potentiation of endogenous

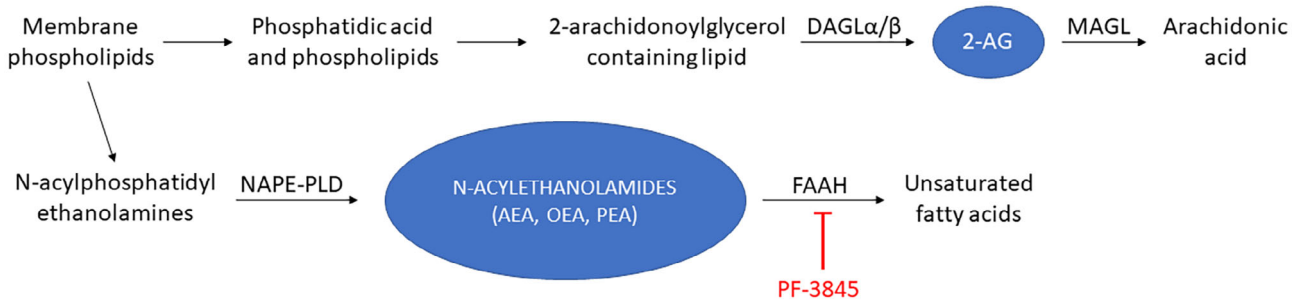

FIGURE 1 Synthetic and degrading pathways of endocannabinoid and endocannabinoid-like molecules. Membrane phospholipids provide the precursors for the synthesis of 2-arachidonoylglycerol (2-AG) and ethanolamides (AEA, anandamide; OEA, oleoylethanolamide; PEA, palmitoilethanolamide). Specifically, 2-arachidonoylglycerol containing lipids are converted in 2-AG by enzyme diacylglycerol lipase (DAGL) α/β. Then, 2-AG is degraded by enzyme monoacylglycerol lipase (MAGL). On the other hand, *N*-acylphosphatidyl ethanolamines are converted by enzyme *N*-acyl phosphatidylethanolamine phospholipase D (NAPE-PLD) in ethanolamides. Ethanolamides are then degraded by fatty acid amide hydrolase (FAAH). PF-3845, the drug used in this study, is an irreversible inhibitor of FAAH.

endocannabinoid and paracannabinoid (*N*-acylethanolamides) signaling, considering that acylethanolamides contribute to the control of both food intake and anxiety.

N-acylethanolamides share crucial metabolic steps; indeed the enzyme fatty acid amide hydrolase (FAAH) is responsible for their degradation (Lu & Mackie, 2016). The pharmacological inhibition of FAAH might be a strategy to increase *N*-acylethanolamide tone (Gaetani et al., 2009) and exert an anxiolytic effect (Bedse et al., 2018).

Based on these premises, in the present study, we investigated the anxiogenic-like behavior of rats exposed to protracted abstinence from cafeteria diet and examined the variations occurring in the expression of proteins related to the ECS in key brain areas involved in both anxiety and food intake control, including hypothalamus (HYPO), amygdala (AMY), nucleus accumbens (ACC), prefrontal cortex (PFC), and periaqueductal gray (PAG). In particular, we focused on proteins involved in the synthesis of *N*-acylethanolamides (*N*-acyl phosphatidylethanolamine phospholipase D, NAPE-PLD) and acylglycerols (diacylglycerol lipase alpha and beta, DAGLα and β), as well as on the enzymes involved in their degradation (FAAH and monoacylglycerol lipase, MAGL). Figure 1 summarizes the relationships between these enzymes and endocannabinoids. We also analyzed the expression of endocannabinoid signaling (CB1 and CB2 receptors).

We hypothesized that the pharmacological treatment with PF-3845, a selective inhibitor of FAAH with elevated oral bioavailability and lower toxicity (Boger et al., 2000), might reduce the anxiety associated with palatable food withdrawal and restore the neurochemical alterations to the ECS.

2 | MATERIALS AND METHODS

2.1 | Cafeteria diet model, behavioral tests, and sacrifice

Adult male Wistar rats (Charles River, Italy; 280–300 g at the beginning of the study) were individually housed under a 12 h light/dark cycle, at constant temperature (20°C–22°C), and humidity (45%–55%) with ad libitum access to water and standard chow (4RF18, Mucedola, Settimo Milanese, Italy) for 2 weeks before the experiment. On day 1 of the experiment, rats were randomly subdivided in two different groups:

- CHOW (ad libitum access to water and standard diet);
- cafeteria (ad libitum access to water, standard diet, and cafeteria diet).

Cafeteria diet was preferred to other diets inducing obesity, because it provided a strong motivational stimulus to voluntarily consume an excessive amount of foods consumed by humans, thanks to the high palatability and variety of selected energy-dense items, reflecting unhealthy human feeding habits that lead to weight gain or obesity (Leigh et al., 2019; Shafat et al., 2009).

Details and procedures of cafeteria diet have been described in our previous studies (Giudetti et al., 2020; Micioni Di Bonaventura, Coman, et al., 2021). The cafeteria diet consisted of a mixture of different high-caloric foods including cookies, chips, cheese, lard, and muffin that were individually weighed before being available to rats. Caloric intake was calculated by weighing each food before and after the meal and by using the nutritional information provided by the manufacturer. Detailed diet content is shown in Supporting Information Material S1.1.

Animals followed the eating schedule from day 1 to 40, until the end of phase 1 of the experiment (see Figure 2; Giudetti et al., 2020). From day 41, cafeteria-fed rats underwent a 28-days abstinence period from cafeteria diet (phase 2); in particular, cafeteria-fed rats had only ad libitum access to standard food and could no longer access the cafeteria diet. At the same time, during phase 2 of the experiment (day 41–68), cafeteria-fed rats were treated every other day with intraperitoneal injections of PF-3845 10 mg/kg (cafeteria PF group) or with vehicle (VEH) consisting in ethanol/tween 80/saline in a proportion 5/5/90 v/v/v (cafeteria VEH group). Similarly, the animals in the CHOW group were treated either with PF-3845 10 mg/kg (CHOW PF group) or with VEH (CHOW VEH group).

We chose to treat the rats every other day since PF-3845 is a covalent FAAH inhibitor with a long-lasting pharmacological action

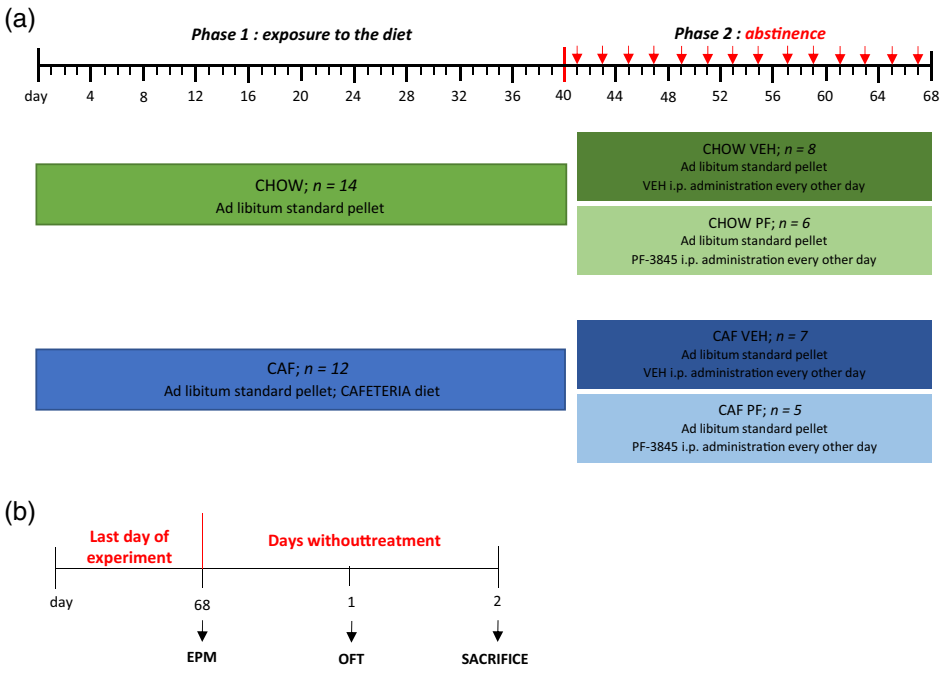

FIGURE 2 (a) Experimental design. Rats were exposed to a cafeteria-style diet (including chips, cheese, lard, muffin, cookies, etc.) for 40 days (CAF). A control group of rats with ad libitum access only to standard chow and water was also included in the study (CHOW). After the first 40 days of cafeteria diet exposure, rats underwent an abstinence period for 28 days, with no longer access to the cafeteria diet but still ad libitum access to standard chow. During the abstinence period, animals were treated either with the FAAH inhibitor PF-3845 (10 mg/kg, i.p.; PF) or its vehicle (VEH) administered every other day. Days of PF-3845 administration are indicated by red arrows. (b) Timeline of behavioral tests (EPM, elevated plus maze; OFT, open field test) and sacrifice.

(Ahn, Johnson, Mileni, et al., 2009), allowing the animals to be treated in alternate days and reducing handling stress. PF-3845 dosage (10 mg/kg) was chosen according to previous studies (Cifani, Avagliano, et al., 2020; Nasirinezhad et al., 2015; Natividad et al., 2017; Rock et al., 2015; Sakin et al., 2015). The experimental design is depicted in Figure 2a.

On day 68, 24 h after the last administration of PF-3845, animals were subjected to elevated plus maze (EPM) paradigm, as described in previous studies (Filaferro et al., 2014; Rodi et al., 2008) and the day after (first day without treatment) animals underwent open field test (OFT), as described in Cifani, Micioni Di Bonaventura, et al. (2020), Micioni Di Bonaventura et al. (2012, 2020). Detailed descriptions of the behavioral tests can be found in Supporting Information Material S1.2. Two days after the last behavioral test, animals were euthanized via CO_2. Brains were extracted and immediately snap-frozen in 2-methyl butane (−50°C) then stored at −80°C until further analyses. The timeline of behavioral tests and sacrifice is shown in Figure 2b. All experiments were performed by following the European directive 2010/63/UE governing animal welfare and with the Italian Ministry of Health guidelines for the care and use of laboratory animals.

2.2 | Brain processing and Western blot analysis

Each brain was sectioned with a cryostat (model HM550; Thermo Fisher Scientific, Kalamazoo, MI) and microdissected into different regions of interest, namely the amygdala (AMY), nucleus accumbens (ACC), prefrontal cortex (PFC), hypothalamus (HYPO), and periacqueductal gray (PAG), as illustrated in Supporting Information Material S1.3 and Figures S1–S4. Sections were collected in microtubes, weighed to a high degree of accuracy, and stored at −80°C until processed. Total proteins from 5 to 15 mg of samples were extracted using 500 μL ice-cold cell lysis buffer for 30 min (López-Gambero et al., 2021). Detailed protocol for Western blot analysis is illustrated in Supporting Information Materials S1.4. Briefly, the antigens probed by western blot analysis were the cannabinoid receptors CB1 and CB2 and the enzymes of synthesis and degradation of endocannabinoids NAPE-PLD, DAGL-α/β, FAAH, and MAGL.

2.3 | Statistical analysis

All data are expressed as mean ± *SEM*. Data obtained from the daily monitoring of the body weight gain and food intake in phase one (exposure to the diet) were analyzed by a two-way ANOVA for repeated measures, setting "diet" (CHOW, cafeteria) and "time" as fixed variables; Bonferroni's test was used to correct post hoc analyses for multiple comparisons. Similarly, data from phase two (abstinence from cafeteria diet) were analyzed with a three-way ANOVA for repeated measures by setting "diet" (CHOW, cafeteria), "treatment" (VEH, PF), and "time" as fixed variables; Bonferroni's test was used to correct post hoc analyses for multiple comparisons.

Data from behavioral tests and Western blot analyses were analyzed by two-way ANOVA (factors: group, treatment). Subsequent multiple comparisons among groups were carried out by using Tukey post hoc HSD. Following the suggestion of Chen et al. (2018), post hoc analysis was performed even if the ANOVA was not significant. Besides, analysis of correlations were run to relate behavioral parameters with caloric consumption and the expression of the ECS in the different areas. These analyses were run as two-tailed Pearson correlations. Statistical significance was set at $p < .05$. The software used for statistics and graphics were IBM SPSS Statistics 22 and GraphPad Prism 8.

3 | RESULTS

3.1 | Body weight and food intake

During *phase 1*, rats exposed to the cafeteria diet showed increased body weight. From day 10 on, there was a significant difference between the weight of cafeteria-fed group compared with the CHOW-fed one, as shown in Figure 3a. Significant body weight gain variation between the two groups was related to significant variations in food intake: the analysis of daily caloric intake showed that animals exposed to the cafeteria diet consumed significantly more calories compared with the CHOW-fed group beginning on day 1 (Figure 3b). Results of two-way ANOVA for repeated measures analysis for body weight and food intake are shown in the Supporting Information Material section S2.1.

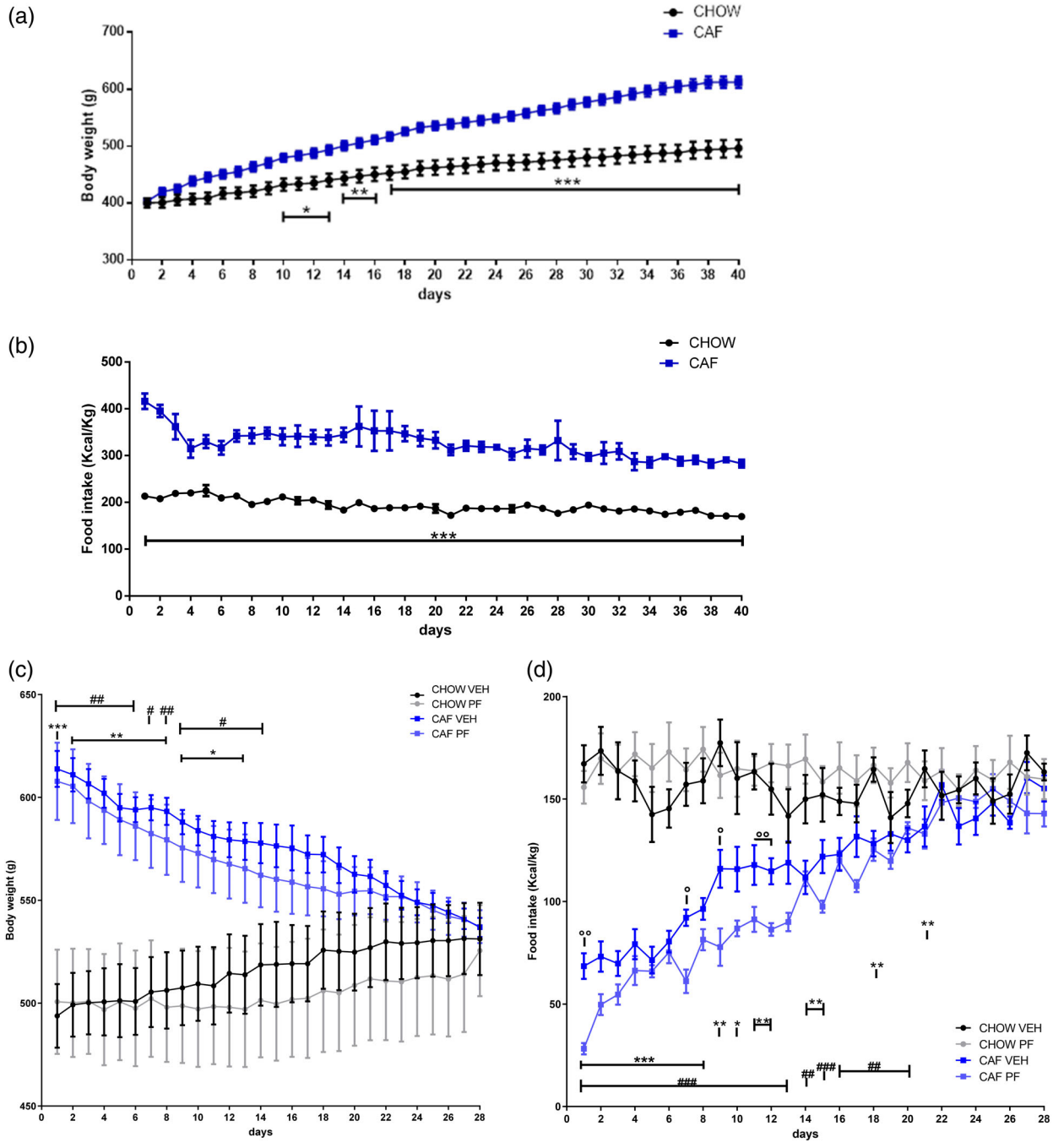

FIGURE 3 Body weight (a,c) and food intake (BnnnnD) of rats respectively during phase 1 and phase 2 of the experiment. Two-way ANOVA for repeated measures, Bonferroni post hoc analysis. $^{*}p < .05$, $^{**}p < .01$, $^{***}p < .001$ between CHOW VEH and cafeteria VEH animals; $^{\#}p < .05$, $^{\#\#}p < .01$, $^{\#\#\#}p < .001$ between CHOW PF and cafeteria PF animals; $^{\circ}p < .05$, $^{\circ\circ}p < .01$, $^{\circ\circ\circ}p < .001$ between cafeteria VEH and cafeteria PF animals.

During *phase 2*, rats experiencing withdrawal from the cafeteria diet significantly reduced their body weight within the first 14 days of phase 2, as shown in Figure 3c. In fact, both cafeteria VEH and cafeteria PF rats significantly reduced their body weight, when compared with CHOW-fed rats administered the same treatment. Similarly, a significant reduction of food intake was observed in both cafeteria-fed VEH and cafeteria-fed PF rats until day 21 of phase 2, when compared with CHOW-fed rats administered the same treatment, as shown in Figure 3d.

At the end of the experiment, a significant difference was not observed in either body weight or food intake of animals, as shown in Figure 3c. PF-3845 reduced significantly the food intake of cafeteria-fed PF rats only in days 1, 7, 9, 11, and 12 of phase 2, when compared with cafeteria-fed VEH rats, as shown in Figure 3d. The details of the statistical analysis are shown in Supporting Information Material section S2.1. Supporting Information material also contains analysis of body weight gain expressed as change respect to the first day of the treatment (Supporting Information results S2.2 and Figure S5).

3.2 | Behavioral tests

Overall, rats subjected to diet exposure and withdrawal (cafeteria-fed VEH) displayed anxiogenic-like behavior. In fact, when compared with CHOW-fed VEH, cafeteria-fed VEH animals showed a significant reduction of both central distance traveled and zone entries in OFT; a significant increase in the time spent in closed arms and a significant decrease of the time spent in open arms in EPM, as depicted in Figure 4b–e.

FAAH inhibition exerted an anxiolytic-like effect in abstinent rats: cafeteria-fed PF animals, when compared with VEH-treated ones, showed a significant increase of both central distance traveled and zone entries in OFT, a significant decrease in the time spent in closed arms and a significant increase of the time spent in open arms in EPM. Results of two-way ANOVA are shown in Supporting Information Materials S2.3.

Moreover, we found a correlation between total food intake during the experiment (expressed in kcal/kg) and both time spent in open arms ($r = -.66$; $p < .01$) and zone entries ($r = -.51$; $p < .05$). This finding is of pivotal importance, since it demonstrates that anxiogenic-like behavior is directly related to caloric consumption.

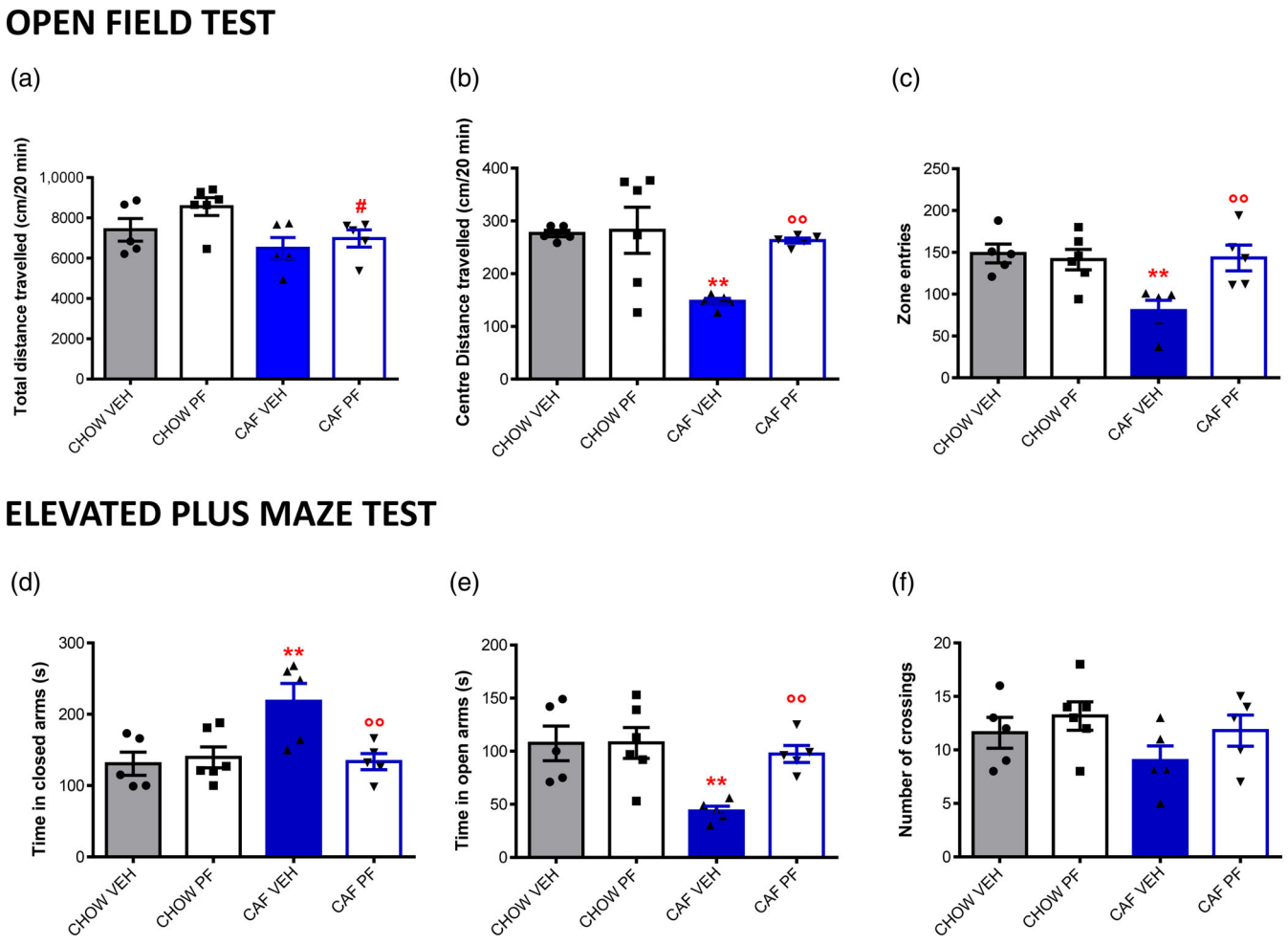

FIGURE 4 Behavioral analysis of anxiety-like behaviors. Total distance traveled (a), center distance traveled (b) and zone entries (c) were measured during open field test. In the elevated plus maze, time spent in closed (d) and open arms and number of total crossings (F) were evaluated. Data were analyzed with two-way ANOVA, Tukey post hoc results are shown: $^{**}p < .01$ vs. CHOW VEH animals; $^{\circ\circ}p < .01$ vs. cafeteria VEH animals $^{\#}p < .05$ vs. cafeteria PF.

It is important to underscore that behavioral tests were also conducted on day 40, at the end of phase 1, but no significant behavioral variation was observed at that time point related to dietary exposure (*data not shown*); based on these results, we chose to perform further analyses only in animals subjected to diet exposure and subsequent abstinence.

3.3 | Western blot analysis

3.3.1 | Cannabinoid receptors

Results of Western blot analysis of CB1 and CB2 receptors are shown in Figure 5a–e. Results were normalized to γ-adaptin.

In AMY (Figure 5a), two-way ANOVA analysis of the results of CB1 expression revealed a significant interaction. PF-3845 administration significantly dampened CB1 expression in CHOW-fed PF group, when compared with the CHOW-fed VEH group. This effect was not observed in cafeteria-fed PF animals, which exhibited a significant increase in CB1 expression, when compared with the CHOW-fed PF group. Otherwise, no significant variation was observed in CB2 expression in this region.

On the other hand, diet exposure and abstinence affected both CB1 and CB2 receptors expression in ACC (Figure 5b). In particular, statistical analysis showed a significant effect of diet on CB2 expression. Specifically, cafeteria-fed VEH rats displayed a significant decrease in the expression of both receptors, when compared with

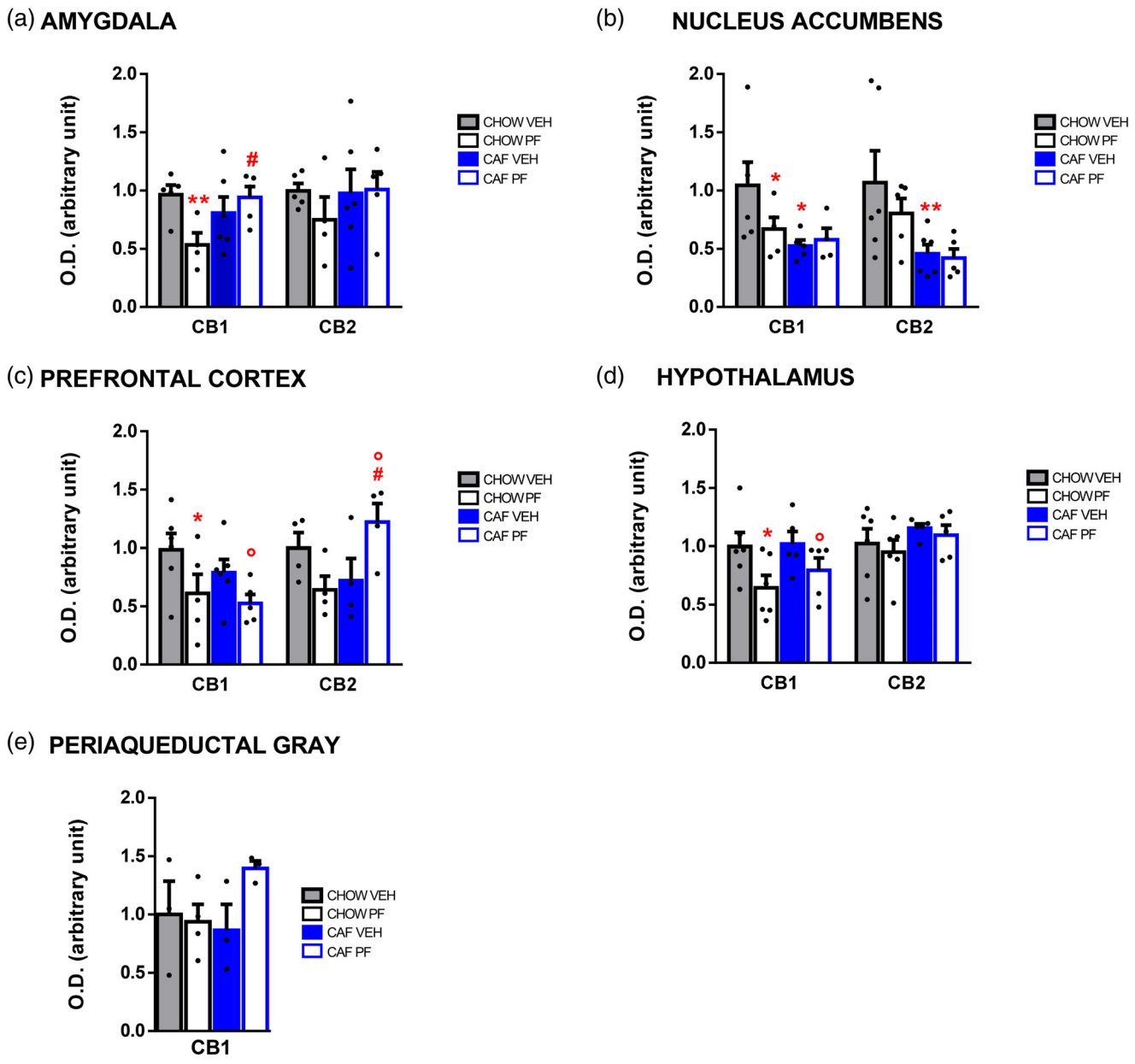

FIGURE 5 Protein expression of CB1 and CB2 receptor in amygdala (a), nucleus accumbens (b), prefrontal cortex (c), hypothalamus (d) and periaqueductal gray (e) measured by Western blot analysis and normalized to γ-adaptin. Two-way ANOVA statistical analysis was performed, Tukey post hoc results are shown in the figure. $^{*}p < .05$, $^{**}p < .01$ vs. CHOW VEH; $^{\circ}p < .05$ vs. cafeteria VEH; $^{\#}p < .05$ vs. CHOW PF. No staining for CB2 in periaqueductal gray was detected.

not abstinent rats (CHOW-fed VEH). Pharmacological treatment did not affect this difference.

Expression of CB1 receptor was significantly affected by treatment in both PFC (Figure 5c) and HYPO (Figure 5d). In fact, the PF-3845 administration significantly reduced the expression of CB1 receptors in both CHOW-fed PF and cafeteria-fed PF groups, when compared with VEH-treated animals. Moreover, a significant interaction was found for CB2 expression in PFC; the cafeteria-fed PF group showed a significant increase in the expression of CB2 receptors, when compared with both cafeteria-fed VEH and CHOW-fed PF animals. No significant effect was noted in HYPO for CB2 expression.

No significant variation in CB1 expression was noted in PAG (Figure 5e). No appropriate staining was obtained for CB2 expression in this region.

In summary, analysis of cannabinoid receptor expression showed a significant decrease of both CB1 and CB2 receptor in ACC of cafeteria-fed VEH-treated rats (when compared with the control). Most importantly, PF-3845 administration caused a significant decrease of CB1 expression in PFC and HYPO both in CHOW-fed and cafeteria-fed abstinent rats. PF-3845 treatment also increased CB2 expression in the PFC of abstinent animals.

A positive correlation between the zone entries and the expression of CB2 in PFC was found ($r = .55$; $p < .05$). Considering that, we might speculate that anxiolytic-like effect of PF-3845 in abstinent animals is related to increased CB2 expression in the PFC of these animals.

Results obtained from two-way ANOVA analyses conducted for each parameter are reported in Supporting Information Material S2.4; Tukey's post hoc results are shown in Figure 5. Representative images of Western blot analyses are shown in Figures S6 and S7.

3.3.2 | Enzymes for synthesis and degradation of endocannabinoids

Results of Western blot analyses of enzymes responsible for synthesis and for degradation of endocannabinoids are shown in Figure 6a–e. Results were normalized to γ-adaptin.

Western blot analyses revealed that exposure and subsequent abstinence from cafeteria diet induces several impairments to endocannabinoid signaling in the brain regions analyzed; such alterations are only partially reverted by the pharmacological treatment as shown in Figure 6.

In the AMY (Figure 6a), statistical analysis of the expression of enzymes responsible for synthesis and for degradation of endocannabinoids revealed a significant interaction for DAGLα and MAGL. Rats subjected to diet exposure and abstinence (cafeteria-fed VEH group) showed a significant decrease in the expression of NAPE-PLD, DAGLα, and MAGL in AMY, when compared with CHOW-fed VEH group, thus suggesting a hypoactivation of the ECS in cafeteria-fed VEH animals in this brain region. When compared with cafeteria-fed VEH animals, cafeteria-fed PF rats manifested a significant increase in the expression of MAGL and a trend in an increase in the expression of NAPE-PLD and DAGLα.

On the other hand, in the ACC (Figure 6b), two-way ANOVA evidenced a significant effect of diet in DAGLα and FAAH expression, and a significant effect of treatment on FAAH expression. Diet exposure and abstinence caused a significant decrease in the expression of DAGLα in both cafeteria-fed VEH and cafeteria-fed PF animals, when compared with CHOW-fed ones. In the same region, pharmacological treatment caused an increase of FAAH expression in CHOW-fed PF animals, when compared with CHOW-fed VEH. Cafeteria-fed PF animals showed a significant decrease in FAAH O.D., when compared with CHOW-fed PF.

In the PFC (Figure 6c), Western blot analysis did not show significant variations in the expression of the ECS. In this region statistical analysis showed a significant effect of the diet in DAGLβ expression; in fact cafeteria-fed VEH animals displayed a significant decrease in the expression of DAGLβ, when compared with CHOW-fed VEH ones.

Similarly, in the HYPO (Figure 6d), cafeteria-fed VEH rats showed a significant reduction of the expression of DAGLβ, when compared with CHOW-fed VEH ones (significant effect of diet). Moreover, a trend in the increase in the expression of DAGLα was observed in cafeteria-fed VEH rats, when compared with CHOW-fed VEH ones. Treatment with PF-3845 significantly reversed this trend. In fact, cafeteria-fed PF animals showed a significant reduction of the expression of DAGLα, when compared with cafeteria-fed VEH ones. Additionally, in this area PF-3845 administration per se caused a significant decrease in the expression of NAPE-PLD and DAGLβ in CHOW-fed PF animals, when compared with vehicle-treated ones (significant effect of the treatment).

No significant variations in the expression of proteins relating to the ECS were observed in PAG (Figure 6e), due to diet abstinence and pharmacological treatment, except for a significant increase in FAAH expression in CHOW-fed PF group, when compared with CHOW-fed VEH (significant effect of the treatment). No appropriate staining was observed for DAGLβ and MAGL.

In summary, it is important to underline the findings obtained in the AMY, where cafeteria-fed VEH rats show a significant decrease of NAPE-PLD, DAGLa, and MAGL enzymes expression when compared with the CHOW-fed group. In this area, PF-3845 administration in abstinent rats significantly increased the expression of MAGL, compared with vehicle-treated rats. At the same time, PF-3845 treatment significantly reduced DAGLα expression in HYPO of abstinent rats compared with not-treated ones.

Results obtained from two-way ANOVA analyses conducted for each parameter are reported in Supporting Information Material S2.5 (Figure 6). Representative images of Western blot analyses are shown in Figures S8–S12. Supporting Information materials contain data showing the ratio between enzymes of synthesis and degradation of endocannabinoids for each area (Figure S13) and detailed images of Western blot analysis for each membrane (Figures S14–S18).

Further, correlation analysis showed a positive correlation between time in the open arms and the expression of both DAGLa in

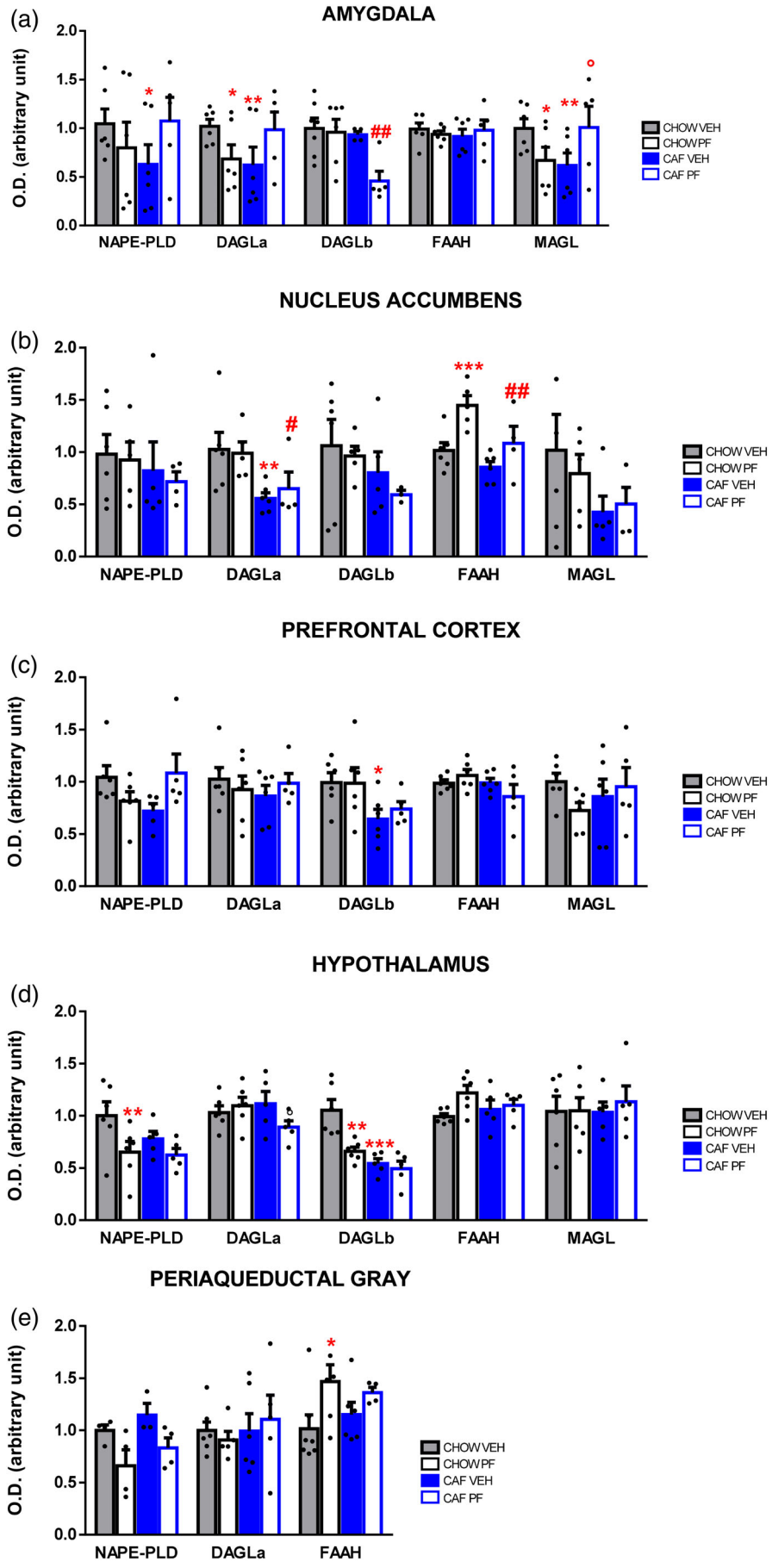

FIGURE 6 Protein expression of enzymes partaking to the endocannabinoid system in amygdala (a), nucleus accumbens (b), prefrontal cortex (c), hypothalamus (d), periaqueductal gray (e) measured by Western blot analysis and normalized to γ-adaptin. Two-way ANOVA statistical analysis was performed, Tukey post hoc results are shown in the figure. $^{*}p < .05$, $^{**}p < .01$, $^{***}p < .001$ vs. CHOW VEH; $^{\circ}p < .05$ vs. cafeteria VEH; $^{\#}p < .05$, $^{\#\#}p < .01$ vs. CHOW PF. DAGL-a/b, diacylglycerol lipase a/b; FAAH, fatty acid amide hydrolase; MAGL, monoacylglycerol lipase; NAPE-PLD, *N*-acyl phosphatidylethanolamine phospholipase D.

ACC ($r = .59$; $p < .05$) and DAGLb in PFC ($r = .69$; $p < .01$) indicating that the reduction of the expression of these enzymes in abstinent animals can be related with their anxiogenic-like behavior.

4 | DISCUSSION

The results of this study demonstrate that consumption of highly palatable food and subsequent abstinence can cause long-term anxiety-like behavior in experimental rats and that the severity of behavioral variations can be related to increased caloric intake. This anxiety-like behavior is associated to permanent changes in the expression of specific proteins of the ECS, mainly affecting the synthesis of endocannabinoids in brain areas related to anxiety and food consumption, such as the PFC, ACC, AMY, PAG, and HYPO.

Several observations suggest that palatable food consumption causes neuroadaptations in both modulatory or excitatory transmissions (Adermark et al., 2021; Arcego et al., 2020; Brown et al., 2017; de Sa Nogueira et al., 2021; Moore et al., 2020; Sharma, 2021). These neuroadaptations may be a basis for food addiction and for a relapse in the consumption of palatable food following a period of restriction (Cottone et al., 2008). Anxiety and negative feelings associated with diet restriction may drive patients to abandon their diet (D'Addario et al., 2014; Herman et al., 2008).

An innovative feature of this study is that it is focused on evaluating the potential role of the ECS in modulating withdrawal from naturally rewarding activities that have an impact on mood, such as feeding. The variations observed in the emotional behavior of abstinent rats can be linked to neuroadaptations of ECS in specific brain areas, as the nature of these changes differed across brain regions and conditions (withdrawal from palatable food or treatment). However, it is important to highlight the limitation of Western blot technique that cannot distinguish in which cellular type the observed changes are produced and that the gross dissection of heterogenous brain regions might have limited insight into the specific circuits.

The areas where palatable food withdrawal produced the greatest changes were the AMY and ACC, brain areas involved in the control of emotions and motivation. Diet exposure and withdrawal reduced the expression of endocannabinoid production enzymes and, in the case of the ACC, also the expression of brain cannabinoid receptors. Previous studies have demonstrated that the reduction of endocannabinoid signaling can be associated with anxiety due to cannabinoid modulation of glutamatergic and GABAergic transmission to central amygdala (Robbe et al., 2001; Sánchez-Marín et al., 2022; Schoffelmeer et al., 2006; Serrano et al., 2018). But, most interestingly, the changes in production enzymes were different regarding the 2-AG and the NAEs biosynthetic pathways. Both diet and treatment affected more 2-AG biosynthetic pathway (DAGLα and β enzymes), while the enzyme for AEA biosynthesis, NAPE-PLD, was influenced by food withdrawal only in the AMY. This specificity is interesting, because a decrease in NAPE-PLD activity in the AMY might be associated with lower AEA production in this area and activation of other mechanisms of anxiogenesis that might enhance further AEA hydrolysis, causing a cycle that promotes anxiety as suggested by (Gray et al., 2015). Moreover, the shift from cafeteria diet to standard CHOW reduces the ingested amount of fatty acids and might alter AEA precursor biosynthesis, potentially limiting the generation of AEA (Berger et al., 2001; Carta et al., 2020). With respect to 2-AG biosynthesis, the reduced expression of both DAGLα and β enzymes might contribute to the anxiety phenotype observed, since augmentation of 2-AG signaling has been found to generate anxiety-like behaviors. For example, a marked deficit in 2-AG release in the central nucleus of the amygdala was observed in alcohol dependent rodents along alcohol abstinence (Serrano et al., 2018). However, 2-AG works through a different pathway than AEA, and their effects are not additive, suggesting a complex mechanism for mood regulation that needs further investigation. The main conclusion from these results is that withdrawal from palatable food modifies the activity of the ECS likely contributing to the anxiety phenotype observed in cafeteria-fed VEH animals (Bedse et al., 2018).

This study suggests an effective treatment for anxiety related to food abstinence, demonstrating that ECS can be a target. Data obtained from behavioral tests confirm that FAAH inhibition through PF-3845 administration exerts an anxiolytic effect consistent with existing literature (Bedse et al., 2018; Kinsey et al., 2011; Naidu et al., 2007) and that pharmacological treatment partially restores the dysregulated ECS protein expression observed in the brain of animals withdrawn from palatable diet. Most importantly, PF-3845 administration affects neither body weight nor food consumption in abstinent rats at the end of the experiment, when they resembled the animals that never experienced palatable food withdrawal. However, food intake fluctuations were detected in treated rats, results that will be interesting to investigate thoroughly in the future. Currently, no data are available regarding the effect of PF-3845 on metabolism and on body weight or food intake (Matheson et al., 2021; Ramesh et al., 2011, 2013). The increase of *N*-acylethanolamides, such as AEA, PEA, and OEA after PF-3845 injections (Ahn, Johnson, & Cravatt, 2009; Ahn, Johnson, Mileni, et al., 2009) could influence the decrease in food consumption. In this context it is important to highlight that especially in the first 12 days of abstinence, when palatable foods were replaced by chow only, CAF vehicle and CAF PF-3845 ate less than control group rats, fed with standard food throughout the entire study. This finding is in accordance with other works (Rogers, 1985; South et al., 2014; Teegarden & Bale, 2007) and suggests that previous exposure to cafeteria diet alters feeding patterns, reducing the motivation to consume less palatable items in the absence of variety. Moreover this study indicates that PF-3845 treatment can significantly reduce the DAGLα expression in HYPO of abstinent rats: inhibition of DAGLα in HYPO has been related to altered response of hypothalamic tanycytes to neuropeptides and altered modulation of orexigenic and anorexigenic neurons (Palma-Chavez et al., 2019).

PF-3845 acts mainly by exerting an anxiolytic-like effect: abstinent and treated animals displayed a normalization of all the analyzed parameters at levels comparable to the control group in both the EPM and OFT. Several mechanisms have been hypothesized for PF-3845's

anxiolytic action: FAAH inhibition exerts anti-anxiety effects through long-time depression (Duan et al., 2017), normalization of glutamatergic transmission in AMY (Natividad et al., 2017), and normalization of AEA levels in limbic areas (Bedse et al., 2017). Moreover, PF-3845 increases neuronal survival and decreases neuroinflammation that can be associated with anxiety (Tchantchou et al., 2014).

In the present study, PF-3845 administration affected the expression of the ECS in the AMY, reversing the changes induced by diet exposure and abstinence. In particular, FAAH inhibition enhances 2-AG degradation by significantly increasing MAGL expression, and reduces 2-AG synthesis through a significant decrease of DAGLβ. We speculate that 2-AG signaling decreases in the AMY, reducing GABA signaling associated with anxiety-like behavior, as suggested by Di et al. (2016). In addition, PF-3845 causes significant increase of the expression of CB2 receptor in PFC, whose stimulation has been linked to anxiolytic-like (Ivy et al., 2020) and neuroprotective effects (Youssef et al., 2019). As a matter of fact, we also demonstrated that CB2 expression in PFC positively correlates with zone entries in OF, confirming its role in anxiolysis. FAAH inhibition also induced a significant reduction of CB1 receptor expression, whose activation has been linked both to anxiolytic and anxiogenic response in a dose-dependent manner (Rubino et al., 2008). These results are consistent with existing research on the role of PF-3845 in the modulation of the ECS in the brain (Bedse et al., 2018; Cifani, Avagliano, et al., 2020; Hruba et al., 2015), suggesting that PF-3845 increases brain acylethanolamides tone. Of course, it is important to emphasize that PF-3845 was chronically administered in the current study; thus, some of the effects shown in our study might reflect a compensation to chronic FAAH inhibition.

In summary, the results of this study suggest that PF-3845 may be a potential candidate to treat anxiety associated with food restriction. In this preclinical study, PF-3845 reversed the anxiety-like behavior caused by diet exposure and abstinence and restored (selectively in abstinent animals) altered emotional behavior and expression of ECS proteins in key brain areas associated with anxiety.

4.1 | Limitations

Although both behavioral data and changes in the expression of ECS signaling proteins support the hypothesis that changes in the ECS following cafeteria diet withdrawal relate to the development of anxiety, we did not directly measure endocannabinoid release in vivo from brain structures, especially from the AMY. We did not measure 2-AG/AEA concentrations in tissue samples since they are poor indicators of the extracellular signaling of ECS. The intracellular concentrations of 2-AG and AEA exceed in more than one order of magnitude the extracellular ones, indicating that the role of these molecules must be assessed via microdialysis. Moreover, the NAPE-PLD pathway appears less affected than DAGLα and β, indicating that the contribution of non-cannabinoid acylethanolamides (OEA, PEA, etc.) is less relevant than that of 2-AG. Future studies should explore the effect of chronic PF-3845 administration on 2-AG signaling (Tchantchou et al., 2014) and whether MAGL inhibitors result in a similar impact on anxiety associated to food withdrawal. Moreover, performing a time course study would be interesting, in order to evaluate the gradual development of negative mood in abstinent rats, providing evidence for adaptive changes affecting both the endocannabinoid system and anxiety-related responses such as glucocorticoid secretion. Finally, the current study included only male rats. Studies in females, especially considering the influence of the estrous cycle on anxiety, feeding behavior and modulation of the ECS, are necessary to further explore the pharmacological treatment of diet-associated anxiety with FAAH inhibitors.

AUTHOR CONTRIBUTIONS

Marialuisa de Ceglia: Data curation; formal analysis; investigation; methodology; validation; visualization; writing – original draft; writing – review and editing. **Maria Vittoria Micioni Di Bonaventura:** Data curation; formal analysis; investigation; methodology; validation; visualization; writing – original draft; writing – review and editing. **Adele Romano:** Data curation; formal analysis; investigation; methodology; validation; writing – original draft; writing – review and editing. **Marzia Friuli:** Data curation; formal analysis; investigation; methodology. **Emanuela Micioni Di Bonaventura:** Data curation; formal analysis; investigation; methodology. **Ana Luisa Gavito:** Data curation; formal analysis; investigation; methodology. **Luca Botticelli:** Data curation; formal analysis; investigation; methodology. **Silvana Gaetani:** Conceptualization; data curation; formal analysis; funding acquisition; investigation; methodology; project administration; supervision; validation; visualization; writing – original draft; writing – review and editing. **Fernando Rodriguez de Fonseca:** Conceptualization; data curation; formal analysis; funding acquisition; investigation; methodology; project administration; supervision; validation; visualization; writing – original draft; writing – review and editing. **Carlo Cifani:** Conceptualization; data curation; formal analysis; funding acquisition; investigation; methodology; project administration; supervision; validation; visualization; writing – original draft; writing – review and editing.

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DATA AVAILABILITY STATEMENT

Raw data were generated at School of Pharmacy, Pharmacology Unit, University of Camerino; Department of Physiology and Pharmacology "V. Erspamer," Sapienza University of Rome; UGC Salud Mental, Instituto de Investigación Biomédica de Málaga (IBIMA), Universidad de Málaga-Hospital Universitario Regional de Málaga. Derived data

supporting the findings of this study are available from the senior authors SG, FRF, CC on request.

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