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Transcriptional regulation of the endocannabinoid system in a rat model of binge-eating behavior reveals a selective modulation of the hypothalamic fatty acid amide hydrolase gene

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Abstract

Objective: Binge-eating episodes are recurrent and are defining features of several eating disorders. Thus binge-eating episodes might influence eating disorder development of which exact underlying mechanisms are still largely unknown.

Methods: Here we focused on the transcriptional regulation of the endocannabinoid system, a potent regulator of feeding behavior, in relevant rat brain regions, using a rat model in which a history of intermittent food restriction and a frustration stress induce binge-like palatable food consumption.

Results: We observed a selective down-regulation of fatty acid amide hydrolase (*faah*) gene expression in the hypothalamus of rats showing the binge-eating behavior with a consistent reduction in histone 3 acetylation at lysine 4 of the gene promoter. No relevant changes were detected for any other endocannabinoid system components in any brain regions under study, as well as for the other epigenetic mechanisms investigated (DNA methylation and histone 3 lysine 27 methylation) at the faah gene promoter.

Discussion: Our findings suggest that faah transcriptional regulation is a potential biomarker of binge-eating episodes, with a relevant role in the homeostatic regulation of food intake.

KEYWORDS

binge-eating, endocannabionid system, epigenetic mechanisms, food restriction, frustration stress, gene expression

1 | INTRODUCTION

Binge-eating (BE) is generally defined as a loss of control in eating unusually large amounts of food in a discrete period of time. BE is a key symptom in bulimia nervosa and BE disorder, and occurs

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commonly among individuals with anorexia nervosa (American Psychiatric Association, 2013). Overeating can result from a negative emotional state that shares many characteristics with addictive behaviors (D'Addario et al., 2014). Due to the association of BE with emotion dysregulation (Fairburn, Cooper, & Shafran, 2003; Heatherton & Baumeister, 1991), BE is of great public and clinical concern.

Multiple neurotransmitter systems known to modulate homeostatic eating may contribute to BE behaviors, and so far a major focus has been placed on the role of dopaminergic and opioid systems (Avena & Bocarsly, 2012; Avena, Rada, & Hoebel, 2008; Bello, Yeh, Verpeut, & Walters, 2014; Mathes, Brownley, Mo, & Bulik, 2009). However, among endogenous systems, increasing evidence suggests that the endocannabinoid system (ECS) contributes to the modulation of energy balance by controlling food intake and hedonic eating as a negative feedback system that opposes anxiety (Coccurello & Maccarrone, 2018). The ECS is formed by endocannabinoids (eCBs), among which the two prototypes are the ethanolamine ("anandamide") and the glycerol ester (2-arachidonoylglycerol, 2-AG) of arachidonic acid, and their selective metabolic enzymes: *N*-acyl-phosphatidylethanolamines hydrolyzing phospholipase D (NAPE-PLD) and fatty acid amide hydrolase (FAAH), for anandamide biosynthesis and degradation; *sn*-1-specific diacylglycerol lipase (DAGL) and monoacylglycerol lipase (MAGL), for 2-AG biosynthesis and degradation. In addition, ECS comprises two seven *trans*-membrane G-protein-coupled type-1 and type-2 cannabinoid receptors (CB_1 and CB_2), located in both the central nervous system and at the periphery (for recent reviews see Maccarrone, 2017; Baggelaar, Maccarrone, & van der Stelt, 2018).

Until now, few studies have addressed the potential role of ECS in BE. It is known for instance that higher levels of peripheral eCBs increase food intake in obese animals and humans (Matias et al., 2006). It has been also reported that eCBs can modulate hedonic eating not only in obese individuals but also in normal weight healthy volunteers (Monteleone et al., 2016). Another study in an animal model where BE was induced in female rats by limited access to highly palatable food, supported a relevant role for CB_1 receptor, because its selective antagonist/inverse agonist rimonabant reduced BE behavior in female rats (Scherma et al., 2013). In line with this, genetic association studies identified genes involved in human eating disorders including some ECS genes, such as *faah*, *cnr1* and *cnr2* (coding for FAAH, CB_1 and CB_2, respectively), and G-protein-coupled receptor 55 (GPR55) that binds eCBs (Ishiguro et al., 2010; Ishiguro et al., 2011; Lutter et al., 2017; Monteleone & Maj, 2008).

Feeding behavior is essential in order to maintain adequate energy stores and brain functions that drive highly regulated mechanisms responsible for homeostatic and hedonic pathways (Berthoud, 2012; DiLeone, Taylor, & Picciotto, 2012; Johnson, 2013; Schneeberger, Gomis, & Claret, 2014; Yeo & Heisler, 2012). Thus, it is relevant to consider the role of those brain regions where dysfunction might lead to the development of eating disorders and/or obesity: the hypothalamus, that primarily receives internal signals, for appetite regulation (Bazhan & Zelena, 2013); the amygdala complex and mesolimbic system primarily process external signals. The amygdala complex regulates emotions and controls fear responses, and it might drive food-related behavior and excessive eating of highly palatable food (Blasio et al., 2013; Bohon & Stice, 2012; Galarce, McDannald, & Holland, 2010; Gallagher & Chiba, 1996; Holland & Gallagher, 2003; Pringle, Ashworth, Harmer, Norbury, & Cooper, 2011). On the other hand, the mesolimbic system represents the reward system encoding the hedonic properties of highly palatable food. This system includes the ventral tegmental area that integrates complex central and peripheral inputs (Watabe-Uchida, Zhu, Ogawa, Vamanrao, & Uchida, 2012) and plays a role in motivation for stress-induced feeding of palatable food (Meye & Adan, 2014), which projects to the nucleus accumbens and ventral striatum; the latter two regions are already known to be relevant in reward-seeking behaviors, such as those observed in eating disorders (Wang et al., 2011; Wise, 2012).

Based on this background, here we aimed at evaluating the transcriptional regulation of genes of different ECS components in distinct brain regions in a well-characterized animal model of binge-eating behavior, based on the exposure of rats to both food restriction and stress (Cifani, Polidori, Melotto, Ciccocioppo, & Massi, 2009), in order to identify possible molecular alterations which might drive the induction of this phenotype.

It should be noted that brain functions could differ between individuals according to environmental factors, including malnutrition and stress. Under the influence of these external factors, epigenetic mechanisms regulate the expression of genes, which, successively, alter the long-term risk of developing a pathological state, including eating disorders (Campbell, Mill, Uher, & Schmidt, 2011). Therefore, we decided to assess whether regulation of ECS genes could possibly engage epigenetic mechanisms, namely DNA methylation and histone modifications contributing to the dysregulated feeding patterns and thus to the generation of BE episodes. It is known that DNA methylation occurs at position 5 of the cytosine pyrimidine ring of a cytosine preceding a guanine (CpG site) (Chen et al., 1991), often found in small clusters of DNA named "CpG islands" (Bird, 1986). The latter ones ultimately appear to be relevant in preventing access and binding of transcription factors to regulatory elements, and lead to gene silencing (Jaenisch & Bird, 2003). Instead histone modifications are covalent post-translational modifications of histone tails, such as methylation and acetylation of lysine, that alter chromatin structure and function and are associated with transcriptional activation or repression (Henikoff & Matzke, 1997).

2 | MATERIAL AND METHODS

2.1 | Animals and diet composition

A total of 64 (n = 8 each group) selected female Sprague–Dawley rats (32 for testing food intake after stress procedure, and 32 for molecular biology studies) weighing approximately 230 g at the beginning of the experiment, were individually housed and kept in a temperature- and humidity-controlled room with a 12-h light/dark cycle (lights on at 8:00 am), with free access to food and water for 2 weeks before the experiments. All experiments were carried out in accordance with the European directive 2010/63/UE governing animal welfare and protection and with the Italian Legislative Decree 116 of January 27, 1992. We used young female rats in consideration of the high

prevalence of binge eating disorders in adolescent and young adult females (Hudson, Hiripi, Pope Jr., & Kessler, 2007; Kjelsås, Bjørnstrøm, & Götestam, 2004; Spitzer et al., 1993).

Rats were fed with standard food pellets, 4RF18 (Mucedola, Milan, Italy). The palatable food was offered as a paste, composed of 52% Nutella (Ferrero, Torino, Italy) chocolate cream, 33% food pellets, and 15% water.

Standard pellets were offered inside a metallic grid container that was hung on the anterior wall of the cage; it was removed from the cage to measure its weight in order to determine food pellet intake. Palatable food was offered in a china coffee cup (6 cm × 5.8 cm); the handle of the cup was inserted into the metallic grid of the anterior wall of the cage and fixed to the wall. Body weights and food intake were recorded daily.

2.2 | Binge-eating experimental procedure

The BE experimental procedure was accurately described by Cifani et al. (2009). Rats were kept in individual cages with metallic walls; the floor and the front wall were made of metallic grids. The dimensions of the cage floor were 30 cm × 30 cm; the cage was 30 cm high. A front door (30 cm × 20 cm) consisting of a metallic grid was present in the anterior wall of the cage to obtain access to the inside of the cage; the remaining part of the front wall was equipped with a drinking burette.

Briefly, after 2 weeks of standard food exposure, rats were divided in two groups and were exposed (or not) to three 8-day cycles of food restriction and during two of these cycles they were given access to palatable food for 2 hr during the light cycle (on days 5–6 and 13–14). On day 25, the final test day, each group was exposed or not to stress (15 min) (see Figure 1 for details). During the stress procedure for 15 min the china coffee cup containing palatable food was placed inside a metallic grid container that was hanged up on the anterior wall of the cage. In these conditions, the animal was able to see the cup and the palatable food itself and could smell its odor. In this 15 min period the rat engaged in repeated movements of the forepaws, head and trunk aimed at obtaining the palatable food, of which it was unable to obtain. This procedure was adopted to generate a mild stressful condition (that we refer as a frustration stress), and we previously showed it causes a significant increase in serum corticosterone levels in both groups of animals subjected to the stressor (Cifani, Micioni Di Bonaventura, Ciccocioppo, & Massi, 2013; Cifani, Micioni Di Bonaventura, Vitale, Ciccocioppo, & Massi, 2010; Micioni Di Bonaventura, Cifani, Vitale, & Massi, 2012). After 15 min, the palatable food cup was placed inside the cage and chow and palatable food intake were assessed for 2 hr, in stressed and nonstressed rats. These animals never engaged in competing behaviors, but continuously remained over the cup containing highly palatable food and focused their attention on the intake. In our model, we can define BE as significantly higher palatable food consumption during the 2 hr test in the repeated restriction plus frustration stress condition than in the other experimental conditions. We found in a previous study that stress-induced BE in our model is not observed during the estrous phase (Alboni et al., 2017; Micioni Di Bonaventura, Lutz et al., 2017). Therefore, we determined the estrous cycle phase in a blind manner to the all-experimental conditions and we excluded rats that were in this phase from the statistical analysis. In animals used for molecular biology studies, at the 25th day, rats exposed or not to the stress procedure were sacrificed and brains quickly removed. The whole hypothalamus, the amygdala complex, the nucleus accumbens, the ventral tegmental area, and the caudate putamen were bilaterally dissected.

2.3 | Analysis of gene expression by quantitative real-time reverse transcription-polymerase chain reaction (qRT-PCR)

Stress and food restriction-induced changes in the expression of ECS genes in several brain regions were assessed by qRT-PCR as described previously (D'Addario et al., 2017; Pucci et al., 2016). Specifically, expression of *cnr1*, *cnr2*, the gene coding for CB receptors, CB_3 (GPR55) receptors and TRPV1 was analyzed as well as the expression of the enzymes involved on biosynthesis (DAGL and NAPE-PLD) and degradation (MAGL and FAAH) of eCBs. Total RNA from selected brain regions of 32 selected female rats (as mentioned above: submitted or not to the food restriction cycles and exposed or not to the stress at day 25) was isolated using TRIzol reagent (Life Technologies), following the manufacturer's recommended protocol. The integrity of purified RNA was determined by RNA absorbance at different wavelengths. In order to ascertain whether the extract was contaminated by unwanted molecules, the absorbance ratio at 260/280 nm was used to assess protein contamination, whereas that at 260/230 nm revealed other contaminants like phenol, guanidine, or carbohydrates. Starting with 0.5 μg of RNA, complementary DNA (cDNA) was prepared using the RevertAid RT Reverse Transcription Kit (Thermo Scientific). Random hexamers and oligo-dT primers were used in the RT reaction in an unbiased manner. The relative abundance of each

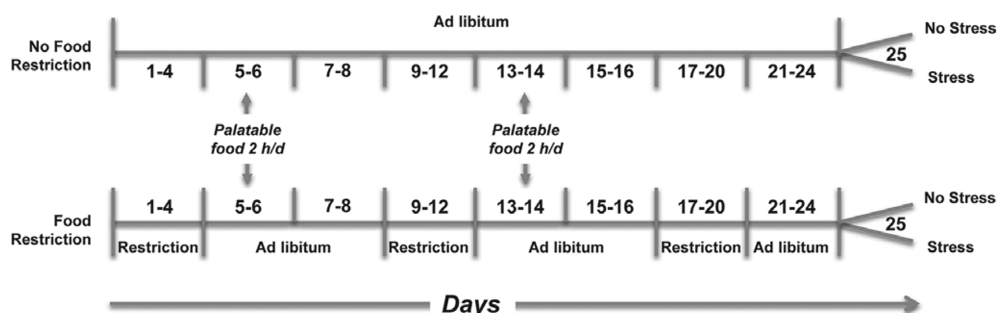

FIGURE 1 Timeline of the experimental procedure of the BE behavior animal model

mRNA species was assessed by qRT-PCR, using SensiFAST SYBR Low-ROX kit (Bioline) on a 7,500 Fast Real-Time PCR system (Thermo Scientific). The primers used for the amplification were reported in Supporting Information Table S1 and all the data were normalized to the endogenous reference gene β-actin and GAPDH.

2.4 | DNA methylation analysis by pyrosequencing

Genomic DNA was extracted from tissues by using TRIzol Reagent (Life Technologies) and was subjected to bisulfite modification by means of a commercially available modification kit (Zymo Research). The schematic of CpG island in FAAH promoter regions and the details of pyrosequencing assay are illustrated in Figure 2 and Supporting Information Table S2. Bisulfite treated DNA was amplified by PyroMark PCR Kit (Qiagen) according to the manufacturer's protocol. Polymerase chain reaction conditions were as follows: 95°C for 15 min, followed by 45 cycles of 94°C for 30 s, 56°C for 30 s, 72°C for 30 s, and finally, 72°C for 10 min. Polymerase chain reaction products were verified by agarose electrophoresis. Pyrosequencing methylation analysis was conducted using the PyroMark Q24 (Qiagen). The level of methylation was analyzed using PyroMark Q24 Software (Qiagen), which calculates the methylation percentage mC/(mC + C) (where mC is methylated cytosine and C is unmethylated cytosine), for each CpG site, allowing quantitative comparisons.

2.5 | Chromatin immunoprecipitation (ChIP)

Chromatin immunoprecipitation was performed as previously described by D'Addario et al. (2013). Tissues were fixed in 1% formaldehyde solution enriched with broad-range protease inhibitor cocktail (PIC) (Roche Diagnostics) and sodium butyrate (Sigma, St. Louis, MO, USA) and incubated at room temperature for 5 min followed by the addition of 2 mL of 10× glycine to quench the unused formaldehyde.

Tissues were sonicated to shear the chromatin to a manageable size. After sonication, chromatin–protein complexes were immunoprecipitated with 1 μg of anti-H3K9ac and anti-H3K27me3 antibodies (Cell Signaling Technology) and 20 μL of fully re-suspended protein A/G magnetic beads. Beads were then washed two times with sonication buffer, and DNA was eluted in elution buffer. Cross-links were reversed overnight.

Immunoprecipitated and input DNA were purified by a Gel and PCR Clean-up kit (Macherey-Nagel), and the relative abundance was assessed by RT-qPCR using the SensiFAST SYBR Low-ROX kit (Bioline) with the following program: 10 min at 95°C for initial denaturation, 15 s at 95°C, 1 min at 60°C, for 40 cycles, followed by 5 min at 72°C for final extension. Each sample was assayed in triplicate, and the fold enrichment ratio was calculated as the value of the ChIP sample vs. the corresponding input sample. The primers used for these studies are listed in Supporting Information Table S2.

2.6 | Statistical analysis

Data were expressed as mean ± *SEM* and each value indicates the mean of the values per group. Data from Palatable food intake, gene expression and ChIP samples were statistically analyzed by two-way analysis of variance (ANOVA) followed by Bonferroni test as post hoc analysis for further examination of group differences, using GraphPad Software for science, version 6 (San Diego, CA). Moreover, DNA methylation at each CpG site was analyzed using the Mann–Whitney test and Bonferroni correction was used for the multiple comparisons. The *p*-values <.05 were considered to be statistically significant.

3 | RESULTS

3.1 | Binge-eating behavior in rats exposed to food restriction and binge-eating test

As reported in previous studies (Micioni Di Bonaventura et al., 2012; Micioni Di Bonaventura et al., 2014; Pucci et al., 2016), we found that rats lose weight during the food restriction period but they regain it during the subsequent free-food period. On the last day of the BE

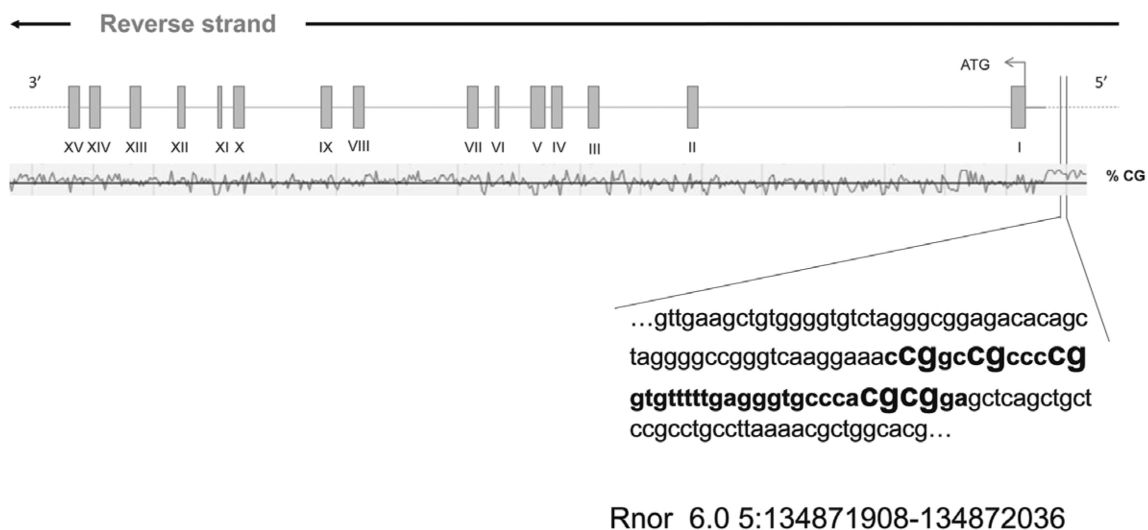

FIGURE 2 Schematic representation of the rat *faah* gene and the 5′ upstream region. Transcription start site position, translation start code (ATG), exons and introns are depicted. Primers used to analyze histone modifications (in italics) as well as region sequenced to analyze DNA methylation levels of the 5 CpG sites (in bold) are also shown

experimental procedure, the body weight of restricted rats was not statistically different from those not restricted (data here are not shown to avoid redundancy with previous articles). By the last day of refeeding (24 hr before the binge eating test), body weight and food intake of restricted rats were not statistically different from those of nonrestricted rats, thus eliminating the potentially confounding influence of hunger or energy deficit.

At the 15 min time-point of food consumption, two-way ANOVA, which included the between-subject factors of history of intermittent food restriction (no, yes), and stress during testing (no, yes), showed a significant interaction among the two factors [$F_{(1, 28)} = 5.14$, $p < .05$]. Post hoc comparisons indicated that palatable food intake of the restricted and stressed group was markedly higher than in the other groups (not restricted and not stressed: 91.1 ± 9.8 kcal/kg; not restricted and stressed: 94.1 ± 7.1 kcal/kg; restricted and not stressed: 96.4 ± 10.9 kcal/kg; restricted and stressed: 137.9 ± 14.3).

ANOVA after the 2 hr cumulative palatable food showed a two-way interaction (food restriction, stress) [$F_{(1, 28)} = 4.2$, $p < .05$]. Post hoc test revealed that, at this time-point, food intake was significantly increased in the restricted and stressed group in comparison to the other control groups (not restricted and not stressed: 163.4 ± 11.3 kcal/kg; not restricted and stressed: 171.6 ± 13.8 kcal/kg; restricted and not stressed: 163.8 ± 7.0 kcal/kg; restricted and stressed: 214.5 ± 7.7).

3.2 | Regulation of ECS in rats exposed to food restriction and the stress procedure

Gene expression analysis for selected brain regions allowed for the evaluation of whether or not stress and cycles of intermittent food restriction may determine change in the regulation of the ECS system.

Overall changes in ECS mRNA levels are reported in Table 1 and Figure 3 (in regard to specifically *faah* in the hypothalamus). Statistical analysis by two-way ANOVA showed that food restriction and stress did not determine changes of any ECS components in the ventral tegmental area, caudate putamen, amygdala complex and nucleus accumbens (Table 1a–d). The first relevant finding of this study is that we observed selective alterations of *faah* gene expression just in the hypothalamus of rats showing binge-eating behavior (Table 1e and Figure 3). The two-way ANOVA showed that mRNA levels were affected by food restriction [$F_{(1, 30)} = 5.59$, $p = .025$] and stress [$F_{(1, 30)} = 6.23$, $p = .018$], with a significant interaction between these two factors [$F_{(1, 30)} = 5.18$, $p = .030$]. Bonferroni's post-test showed a significantly lower *faah* mRNA level in restricted and stressed rats when compared with all the other groups. Post hoc group differences are indicated in Figure 3. Moreover, a *t*-test between the five brain regions, corrected by Sidak–Bonferroni multiple comparisons, showed in the hypothalamus of rats exposed to restriction and stress a significant decrease of *faah* gene expression in respect to the other groups ($p < .001$) (data are not shown).

In order to evaluate if epigenetic mechanisms might account for gene expression changes, we analyzed DNA methylation (Figures 2 and 4 and Table 2) and specific histone modifications (Figure 5) at the *faah* promoter region.

As reported in Table 2 and Figure 4, the DNA methylation analysis of each CpG site as well as in the average of five sites present at the *faah* gene promoter did not show significant changes in the hypothalamic region. The two-way ANOVA showed that the DNA methylation was not affected by restriction and stress nor was there an interaction between these two factors.

The analysis on H3 modification at the gene promoter showed H3K9Ac levels were affected by stress [$F_{(1, 19)} = 11.10$, $p = .003$] but not by restriction [$F_{(1, 19)} = 2.93$, $p = .072$], without a significant interaction between these two factors [$F_{(1, 19)} = 3.63$, $p = .024$]. Bonferroni's post-test indicated a significant decrease of H3K9Ac at *faah* promoter region in stressed rats which have undergone restriction or not when compared with not stressed and not restricted animals ($p < .05$) (Figure 5a). H3K27me3 levels were not significantly different in the *faah* promoter region between all groups in the examined area (Figure 5b). No changes of histone deacetylases (HDACs) (1–11) mRNA levels were observed (Supporting Information Figure S1).

4 | DISCUSSION

In this work, the combination of stress and repeated food restriction induced binge-eating behavior for highly palatable food in female rats as shown previously (Cifani et al., 2009; Piccoli et al., 2012), where regulation of nociceptin/orphanin FQ and corticotropin-releasing factor genes was reported as a potential contributor (Micioni Di Bonaventura et al., 2013; Micioni Di Bonaventura et al., 2017; Pucci et al., 2016). Here, we extended our previous investigations to the role of ECS gene regulation in the same animal model. Of note, to the best of our knowledge, this is the first study focusing on ECS component gene regulation in different brain regions in a well-characterized animal model of binge-eating behavior. In this context, it seems of paramount importance that among the different ECS genes, selective changes could be observed only in the expression of *faah* (that encodes for FAAH) in the hypothalamus of the BE group (i.e., rats under restriction and stress). Remarkably, in the same brain area the homeostatic and hedonic circuits regulating eating are most likely integrated.

FAAH is widely distributed in organs involved in food intake and energy balance (Ueda & Yamamoto, 2000), and it has been recognized as the main enzyme responsible for anandamide degradation, both in vitro and in vivo (Maccarrone, 2017). Modulation of its expression would thus result in changes in anandamide content. Interestingly, it is known that anandamide administration in the hypothalamus, both systemically (Williams & Kirkham, 1999) and locally (Jamshidi & Taylor, 2001), stimulates appetite, and fasting increases anandamide levels (Fu et al., 2007).

Indeed, pharmacological inhibition or genetic ablation of FAAH has been associated with outcomes that are relevant also for binge-eating behavior. For instance, FAAH deficiency enhanced motivation for food (Tourino, Oveisi, Lockney, Piomelli, & Maldonado, 2010) and also increased operant self-administration of ethanol in alcohol-preferring rats (Cippitelli et al., 2008; Hansson et al., 2007). These findings support our data showing reduced *faah* expression in stress-driven binge-eating behavior, which might be related also to the

TABLE 1 Gene expression of ECS elements (receptors and metabolic enzymes) in different brain regions (a: Ventral tegmental area; b: Caudate putamen; c: Amygdala complex; d: Nucleus accumbens; e: Hypothalamus) of rats exposed (or not) to restriction and stress (N = 6–8), reported as 2^{-DDCt} values calculated by Delta–Delta Ct (DDCt) method versus healthy controls posed equal to 1

	History of food restriction			
Ventral tegmental area	**No**		**Yes**	
Stress	**No**	**Yes**	**No**	**Yes**
Cnr1	1.08 ± 0.19	1.04 ± 0.25	0.92 ± 0.25	0.58 ± 0.19
Cnr2	0.94 ± 0.31	1.61 ± 0.28	1.16 ± 0.24	2.15 ± 0.38
Gpr55	N.D.	N.D.	N.D.	N.D.
Trpv1	1.14 ± 0.30	0.75 ± 0.29	0.97 ± 0.23	0.36 ± 0.12
Nape-Pld	1.04 ± 0.15	2.21 ± 0.54	1.52 ± 0.14	2.18 ± 0.55
Faah	1.08 ± 0.21	1.81 ± 0.62	1.60 ± 0.27	1.83 ± 0.25
Dagl	1.13 ± 0.26	1.92 ± 0.32	1.55 ± 0.16	1.61 ± 0.22
Magl	1.09 ± 0.20	1.40 ± 0.42	1.14 ± 0.29	1.48 ± 0.21
	History of food restriction			
Caudate putamen	**No**		**Yes**	
Stress	**No**	**Yes**	**No**	**Yes**
Cnr1	1.21 ± 0.26	0.84 ± 0.18	0.93 ± 0.25	0.95 ± 0.20
Cnr2	1.04 ± 0.15	0.62 ± 0.20	0.78 ± 0.31	0.39 ± 0.07
Gpr55	1.12 ± 0.25	0.54 ± 0.18	0.65 ± 0.36	0.59 ± 0.11
Trpv1	1.01 ± 0.08	0.68 ± 0.09	0.69 ± 0.21	0.38 ± 0.05
Nape-Pld	1.05 ± 0.14	1.49 ± 0.06	1.13 ± 0.11	1.65 ± 0.27
Faah	1.04 ± 0.12	0.94 ± 0.08	0.85 ± 0.18	0.86 ± 0.12
Dagl	1.02 ± 0.10	1.23 ± 0.10	0.65 ± 0.08	0.99 ± 0.12
Magl	1.04 ± 0.13	0.99 ± 0.14	0.94 ± 0.09	1.26 ± 0.16
	History of food restriction			
Amygdala complex	**No**		**Yes**	
Stress	**No**	**Yes**	**No**	**Yes**
Cnr1	1.08 ± 0.21	0.98 ± 0.19	0.62 ± 0.09	0.80 ± 0.21
Cnr2	1.13 ± 0.32	0.57 ± 0.30	1.10 ± 0.23	1.17 ± 0.27
Gpr55	1.00 ± 0.05	0.56 ± 0.22	1.46 ± 0.40	1.22 ± 0.36
Trpv1	1.02 ± 0.15	0.91 ± 0.29	1.05 ± 0.25	1.13 ± 0.31
Nape-Pld	1.09 ± 0.20	0.98 ± 0.12	0.93 ± 0.12	1.07 ± 0.18
Faah	1.13 ± 0.27	0.86 ± 0.19	0.79 ± 0.14	0.83 ± 0.06
Dagl	1.01 ± 0.08	1.23 ± 0.24	1.21 ± 0.12	1.00 ± 0.26
Magl	1.09 ± 0.19	0.69 ± 0.08	0.81 ± 0.07	1.16 ± 0.16
	History of food restriction			
Nucleus accumbens	**No**		**Yes**	
Stress	**No**	**Yes**	**No**	**Yes**
Cnr1	1.02 ± 0.10	0.82 ± 0.14	0.48 ± 0.09	0.86 ± 0.15
Cnr2	1.08 ± 0.20	0.64 ± 0.31	0.68 ± 0.26	1.11 ± 0.18
Gpr55	1.02 ± 0.08	0.94 ± 0.19	0.52 ± 0.14	1.34 ± 0.19
Trpv1	1.09 ± 0.21	0.74 ± 0.13	1.24 ± 0.31	1.35 ± 0.40
Nape-Pld	1.06 ± 0.17	0.67 ± 0.09	1.07 ± 0.13	1.14 ± 0.13
Faah	1.03 ± 0.11	1.10 ± 0.41	0.99 ± 0.11	1.02 ± 0.15
Dagl	1.02 ± 0.10	0.97 ± 0.22	0.89 ± 0.23	0.91 ± 0.13
Magl	1.03 ± 0.11	0.91 ± 0.21	0.73 ± 0.12	0.98 ± 0.17
	History of food restriction			
Hypothalamus	**No**		**Yes**	
Stress	**No**	**Yes**	**No**	**Yes**
Cnr1	1.02 ± 0.08	1.20 ± 0.37	0.54 ± 0.08	0.71 ± 0.12
Cnr2	1.21 ± 0.37	1.14 ± 0.33	0.95 ± 0.36	0.93 ± 0.27
Gpr55	1.22 ± 0.33	1.19 ± 0.62	0.89 ± 0.33	1.19 ± 0.28

(*Continues*)

TABLE 1 (Continued)

Hypothalamus	History of food restriction			
	No		Yes	
Stress	No	Yes	No	Yes
Trpv1	1.24 ± 0.31	1.68 ± 0.62	1.27 ± 0.48	1.92 ± 0.31
Nape-Pld	1.18 ± 0.23	0.89 ± 0.26	0.65 ± 0.03	0.97 ± 0.27
Faah	1.06 ± 0.16	1.05 ± 0.23	0.94 ± 0.14	**0.42 ± 0.05**
Dagl	1.30 ± 0.37	0.78 ± 0.26	0.79 ± 0.30	1.30 ± 0.40
Magl	1.14 ± 0.26	1.21 ± 0.32	1.52 ± 0.76	1.76 ± 0.33

Expression was normalized to the mean of GAPDH and β-actin housekeeping genes, and data are reported as mean ± *SEM*. N.D. stands for not detectable.

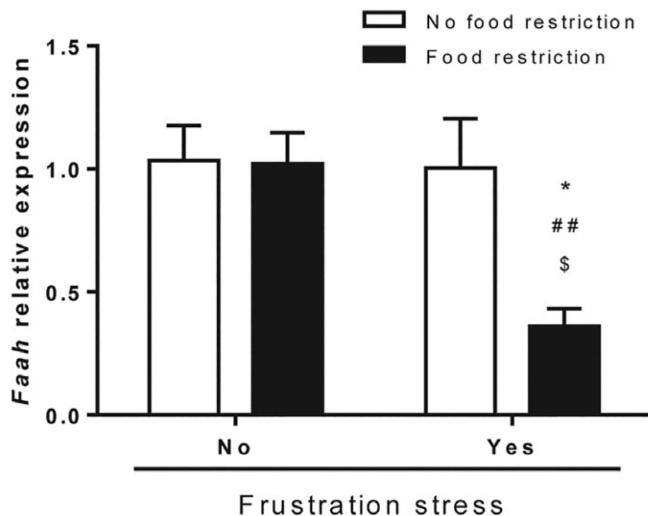

FIGURE 3 *Faah* relative gene expression in the hypothalamus of rats exposed (or not) to restriction and stress. Data were reported as 2^{-DDCt} values calculated by Delta–Delta Ct (DDCt) method versus control animals (no restricted and no stressed) posed equal to 1. Expression was normalized to GAPDH and β-actin, and data were reported as mean ± *SEM* (*N* = 6–8). **p* < .05 vs. no food restriction and no stress. ##*p* < .01 vs. food restriction and no frustration stress. $*p* < .05 vs. no food restriction and stress

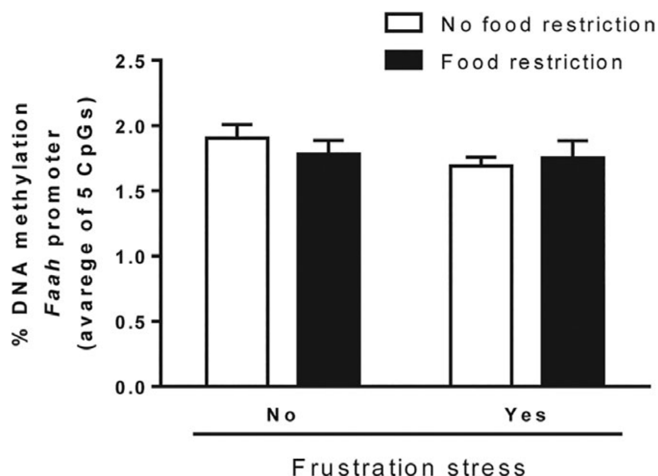

FIGURE 4 Amount of methylated DNA in the promoter region of *faah* in the hypothalamus. Bars represent the mean of the % of DNA methylation values of the average (AVE) of the 5 CpG sites ± *SEM* (*N* = 6–8)

rewarding effects of the highly palatable food. Moreover, our *faah* data potentially leading to higher eCB levels might be consistent with the study showing reduced BE behavior observed in female rats evoked by rimonabant as mentioned in the introduction section (Scherma et al., 2013). As mentioned above, in our previous study, we also evaluated the role of the crf system using the same animal model and we observed in the hypothalamus an increase in crf mRNA levels in BE rats. These data are interesting considering that it appears the combination of restriction and stress should induce the increase of crf, well-known to be the primary mediator of stress responses in mammals, and that its signal might remain elevated after cessation of the stressor with the availability of the highly palatable food. Incidentally, a crucial involvement of the ECS in stress-related emotional symptoms has been reported in different experimental paradigms (Hill et al., 2009), and here we add a new piece of evidence in this regard.

Another major finding of our study is the epigenetic regulation of *faah* gene only under the experimental conditions used. Most of the studies on epigenetics focused on DNA methylation changes at candidate gene promoters, and the majority of them addressed anorexia or bulimia nervosa. To date, reports on epigenetic changes in BE are missing (Yilmaz, Hardaway, & Bulik, 2015). Here we show that DNA methylation and histone methylation apparently are not involved in the alterations of gene expression; however, a selective reduction was observed in rats subjected to stress for H3K9ac, an epigenetic marker associated to gene silencing. Others studies have recently investigated the epigenetic regulation of ECS components under both physiological and pathological conditions (for a review see D'Addario, Di Francesco, Pucci, Finazzi Agrò, & Maccarrone, 2013); however, to the best of our knowledge, this is the first study showing the involvement of histone acetylation in *faah* regulation. Indeed, so far most of the attention has been focused on DNA methylation alterations that occur at the gene promoter (D'Addario et al., 2012; Grimaldi et al., 2012). There is only one more report showing decreased histone acetylation of genes of ECS components, and this addressed *cnr1* transcriptional repression in a mouse model of Huntington's disease (Sadri-Vakili et al., 2007).

Even though to date accumulated evidence suggests that stress-related disorders are associated with epigenetic modulations, present findings on the epigenetic regulation of a specific gene promoter (connected to a specific stress that, in turn, could induce binge-eating) is unprecedented. We also analyzed the possible role of HDACs regulation, but we failed to show overall alterations in any of the 12 HDAC isoforms in the hypothalamus. These findings further support the

TABLE 2 DNA methylation changes at *faah* gene promoter in the hypothalamus of the four groups of rats restricted and stressed or not

faah		History of food restriction									
		No					Yes				
Frustration stress	No	1.22 ± 0.10	1.38 ± 0.14	1.78 ± 0.11	4.53 ± 0.23	0.61 ± 0.02	1.01 ± 0.07	1.45 ± 0.26	1.74 ± 0.14	4.12 ± 0.16	0.58 ± 0.07
	Yes	0.99 ± 0.13	1.20 ± 0.08	1.72 ± 0.06	4.01 ± 0.19	0.52 ± 0.02	0.89 ± 0.08	1.09 ± 0.13	1.95 ± 0.30	4.33 ± 0.42	0.49 ± 0.03
CpG sites		1	2	3	4	5	1	2	3	4	5

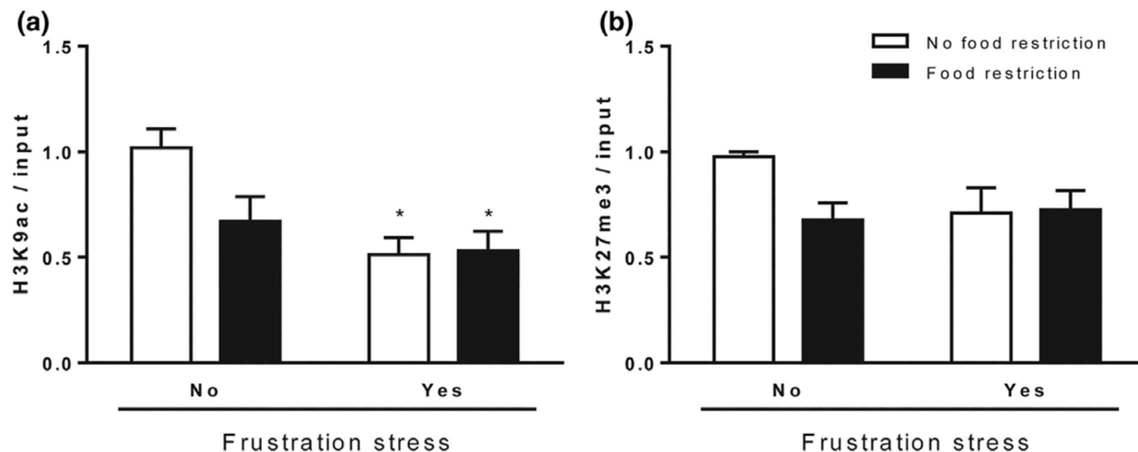

FIGURE 5 *Faah* promoter histone modifications. RT-qPCR analyses of H3K9ac (a) and H3K27me3 (b) immunoprecipitated DNA fragments at *faah* promoter in rat hypothalamus. ChIP histogram shows the levels of specific histone modifications, normalized to total input DNA, in rats exposed (or not) at restriction and stress. Data were expressed as means ± *SEM* of 6–8 animals for each group. $^*p < .05$ vs. no food restriction and no stress

hypothesis that possible chromatin remodeling in BE is confined at specific gene promoters, such as *faah*, without global genomic effects, at least under our experimental conditions.

It is important to mention limitations of our study. First of all, our animal model is based on the occurrence of a single episode of BE and that the molecular observations occur only when there is a history of caloric restriction. Further studies are needed to ascertain whether these observed alterations would occur also after highly palatable food intake as well as after several episodes, or whether there might be other mechanisms involved in ECS regulation. Another limitation of our study is the paucity of samples, which made it impossible to assess anandamide levels.

In conclusion, our findings reveal down-regulation of gene expression of a distinct ECS element like *faah*, in a specific brain region like the hypothalamus that is relevant for eating disorders. Gene alterations are clearly associated with histone acetylation, an epigenetic mark linked to gene activation, without engagement of other regulatory mechanisms of gene transcription. Therefore, it can be suggested that the *faah* gene is a potential biomarker of binge-eating episodes, with a relevant role in the homeostatic regulation of food intake rather than in the control of hedonia when the latter is considered an allostatic form of addiction like the one observed in substance abuse.

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CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

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