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THE NEUROBIOLOGICAL BASIS OF BINGE EATING EPISODES: SEARCHING FOR NEW THERAPEUTIC APPROACHES

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INTRODUCTION

Binge Eating Disorder

Binge eating disorder (BED) is the most common eating disorder and an important public health problem worldwide that affects approximately 3% of U.S. adults in their lifetime [1]. In Europe BED is reported by <1–4%, and subthreshold eating disorders by 2–3% of women [2] with increasing percentages in the lockdown period during COVID-19 pandemic [3, 4]. BED is characterized by recurrent (≥1 per week for 3 months) and brief (usually ≤2 hours) binge eating episodes during which the subjects sense a lack of control (inability to stop eating once started) and consume larger amounts of food than most people would eat under similar circumstances, but they do not engage in regular inappropriate compensatory behaviors as in bulimia nervosa or anorexia nervosa [5]. Moreover, the following conditions may occur in the binge-individuals: eating until feeling uncomfortably full, eating large amounts of food without being hungry, eating alone because of the shame for the abnormal amount of food ingested and feeling disgusted with oneself, depressed, or guilty after the episodes [5].

Table 1. Binge Eating Disorder Diagnostic Criteria [5].

Binge-Eating Disorder

Diagnostic Criteria **307.51** (F50.8)

A. Recurrent episodes of binge eating. An episode of binge eating is characterized by both of the following:
   1. Eating, in a discrete period of time (e.g., within any 2-hour period), an amount of food that is definitely larger than what most people would eat in a similar period of time under similar circumstances.
   2. A sense of lack of control over eating during the episode (e.g., a feeling that one cannot stop eating or control what or how much one is eating).

B. The binge-eating episodes are associated with three (or more) of the following:
   1. Eating much more rapidly than normal.
   2. Eating until feeling uncomfortably full.
   3. Eating large amounts of food when not feeling physically hungry.
   4. Eating alone because of feeling embarrassed by how much one is eating.
   5. Feeling disgusted with oneself, depressed, or very guilty afterward.

C. Marked distress regarding binge eating is present.

D. The binge eating occurs, on average, at least once a week for 3 months.

E. The binge eating is not associated with the recurrent use of inappropriate compensatory behavior as in bulimia nervosa and does not occur exclusively during the course of bulimia nervosa or anorexia nervosa.

Specify if:

In partial remission: After full criteria for binge-eating disorder were previously met, binge eating occurs at an average frequency of less than one episode per week for a sustained period of time.

In full remission: After full criteria for binge-eating disorder were previously met, none of the criteria have been met for a sustained period of time.

Specify current severity:

The minimum level of severity is based on the frequency of episodes of binge eating (see below). The level of severity may be increased to reflect other symptoms and the degree of functional disability.

Mild: 1–3 binge-eating episodes per week.

Moderate: 4–7 binge-eating episodes per week.

Severe: 8–13 binge-eating episodes per week.

Extreme: 14 or more binge-eating episodes per week.

BED occurs with greater prevalence in women than in men and it has been assumed that biological differences between the two sexes may affect the development of this eating disorder [6-8]. Among biological differences, the hormone estradiol is considered as a modulator of this dietary behavior [9, 10], also noted in preclinical models [11]. In addition to sex hormones, several hormones are considered as key elements in the development of these eating disorders [12], particularly leptin [13], ghrelin [14] and glucagon-like peptide-1 (GLP-1) [15].

The type of food consumed during binge episodes varies across individuals, although normally the preference is directed towards highly palatable foods (HPF), typically rich in fats, sugars, or both [16-18]. Individuals with a preference for bingeing on sweet foods tend to binge more frequently, affecting vulnerability to binge eating and overeating [16, 18]. This altered eating behavior can lead to obesity and a whole range of comorbidities such as diabetes mellitus, hypertension, metabolic syndrome, psychological distress, anxiety disorders, mood disorders, and other conditions with high health risk for individuals suffering from BED [7, 19-21].

The consumption of highly caloric dense foods could be influenced by the emotion distress and negative feelings experienced by the subjects, thus having a correlation with environmental factors [22, 23]. BED develops along a complex etiology involving psychological risk factors, sociocultural influences, and both environmental and genetic factors and predispositions [23-26]. Regarding environmental factors, standards may lead to self-criticism and negative affect particularly in women, which experience more often weight stigmatization than man, and for this reason engaging in dietary restrain to maintain their weight [27]. The combination of dieting and stress is a common trigger for BED [28, 29], and in the appetite control are involved a complex integration of several neural circuits embeding the mesocorticolimbic dopamine (DA) system [30, 31], and brain serotonin (5-HT) and noradrenaline (NA) signaling [32-34]. The consumption of sugars and fats has notable positive effects on affect and has stress-reducing properties [35]. The principal components of the stress neuraxis are the hypothalamic-pituitary-adrenal (HPA) and the brain NA pathways [36, 37]. Coordination among the NA pathways and HPA axis is needed to elicit an appropriate response to emotional stress [38], and the alteration in NA circuitry is involved in the pathology of stress as well as

feeding behaviors [39]. As regards 5-HT, in previous studies, selective 5-HT reuptake inhibitors or drugs enhancing both NA and 5-HT neurotransmission have demonstrated efficacy in reducing the frequency of binge eating, and were also able to affect depression and anxiety disorders considered psychiatric BED comorbidity [7, 40]. Therefore, given the high comorbidity and genetic correlations with many psychiatric disorders, it seems possible that novel medications like antidepressants and antipsychotics might also potentially influence and be effective in the treatment of eating disorders [41].

Personality traits present in BED individuals are perfectionism, impulsivity and sensation seeking, this latter considered as riskier decision-making [42, 43] and symptoms like body dissatisfaction and weight preoccupation [44]. The impulsivity trait may be the main factor that contributes to the feeling of loss of control over eating experienced during the episodes by individuals with BED [45, 46]. Altogether, a novel treatment suitable for binge eating behaviour should provide not only for the reduction of the frequency of binge episodes characterizing this eating disorder, but also for the disordered eating–related cognitions and mood regulation behind this feeding disorder, thus a comprehensive approach that includes the psychological and behavioral components ("psychological") with the pharmacologic pattern.

The neurobiological processes involved in BED

Despite being highly frequent, the etiology and the neural processes that characterize binge eating behavior are not completely understood. Generally, studies that focused on the neurobiological mechanims underlying BED, investigated primarly genes and proteins related to the homeostatic control of hunger and the reward system. Among the homeostatic pathways, great interest has been addressed to leptin, melanocortin and

ghrelin systems, while for reward-related neural circuits, several studies investigated the role of central neurotransmission of DA, 5-HT, NA, opioid and the endogenous cannabinoid system (ECB). These pathways are very relevant because the balance between homeostatic and non-homeostatic controls, is essential for a healthy feeding behavior [47, 48]. However, it is not completely understood if alterations at the level to these pathways are a cause or an effect for the development of BED.

In this eating disorder, a major role is played by the impulsive and compulsive component along with a higher sensitivity to food reward, and especially the mesocorticolimbic DA system appears to be involved. Indeed, the mesocorticolimbic DA system includes both the mesolimbic and mesocortical structures, and they represent key pathways that are disrupted in addictive behaviors [49]. The main brain areas involved are the prefrontal cortex (PFC), amygdala (AMY), nucleus accumbens (Acb), and ventral tegmental area (VTA). Both pathways originate primarily in the VTA that projects to the Acb, and is a part of complex circuits involving AMY, hippocampus (HIPP), and the bed nucleus of the stria terminalis. In contrast, the mesocortical pathway projects primarily to the PFC [50].

In binge-eating animal models it was shown an increase in DA release in response to HPF [51], with region-specific upregulation in the DA transporter, downregulated striatal DA receptors (dopamine D2 receptors, D2Rs) and increased DA turnover [46, 52, 53]. Notably, the approach of lentivirus-induced knockdown of striatal D2Rs in obese rats leads to compulsive food-seeking behaviors, indicating a role for this receptor in addiction-like reward deficits [54]. In addition, in non obese sugar-bingeing rodents, it was observed a decreased dorsal striatal D2Rs levels, along with increased μ-opioid and D1Rs levels in the Acb [53, 55]. Moreover, besides the role played by the dopaminergic

system in the reward-related food consumption, there is also the involvement of the opioid neurotransmission, crucial for hedonic aspects of reward [56]. Although the blockage of μ-opioid receptors in animal models showed a decrease in food intake, clinical studies exploring the effect of the μ-opioid antagonist showed no efficacy in reducing binge eating in BED individuals [57, 58].

ECB system has been implicated in the neurobiological mechanisms of BED [59] for its ability to influence the motivation for natural rewarding stimuli and to modulate the palatability and food preference [60], via direct connections with dopaminergic and opioid pathways, and especially activating mesolimbic DA transmission. Indeed, CB1 receptors (CB1Rs) are particularly abundant in regions with known involvement in reward, including PFC, AMY, Acb, cingulate cortex, caudate putamen (CPu), VTA and lateral hypothalamus [61]. ECB play a key role in fine-tuning the activity of the VTA-Acb DA projection and its influence on approach and avoidance behaviors that characterized reward acquisition [62].

CB1R modulates directly the mesolimbic DA response to natural reward [63] and the antagonism of CB1R blocked the enhance of DA release in Acb. Rimonabant, a CB1R antagonist was demonstrated to be able to reduce the excessive overeating, and to suppress the self-administration of HPF and the risk-taking behavior of compulsive eating in animal models. However, in clinical studies, despite benefits of binge episodes reduction, this drug presented severe psychiatric side effects that led to the market withdrawal of Rimonabant as an anti-obesity drug [64-67].

A desirable long-term treatment of this complex eating disorder consists in a combination of several factors, such as the reduction of binge eating compulsiveness, decreased food intake and an effect on brain reward circuitry, with limited and mild side effects. Thus,

future studies are needed to better understand of the neurobiological alterations underlying binge eating behavior, and to the develop novel pharmacotherapies that might influence these systems.

Lisdexamfetamine Dimesylate

BED remains underrecognized and undertreated, and currently, cognitive behavior therapy (CBT) and interpersonal therapy (IPT) are considered as the first-line treatments effective in reducing binge eating symptoms and the associated psychopathology, even though these approaches lack efficacy for weight loss in these patients [68]. However, in the last years, progresses have been made in the undestanding of genetics, neurobiological, neuropsychological and neurophysiological mechanisms underlying binge eating behavior, which have consequently influenced and supported the development of new potential treatments for BED [46, 47]. In particular, several line of evidence suggested common neurobiological basis for BED and Substance Use Disorder, leading to the concept of food addiction [55, 69], and individuals with BED seem to show greater cognitive attentional biases towards food, reduced reward sensitivities, and altered brain activation in regions associated with impulsivity and compulsivity, including the mesocorticolimbic DA system, compared to individuals without BED [46, 47]. Several classes of pharmacological agents have been tested and explored in the treatment of BED, such as antidepressants, antiepileptics, weight-loss agents, drugs approved for Attention-Deficit/Hyperactivity Disorder (ADHD) and antiaddiction agents among the others [68, 70].

In the 2015, the U.S. Food and Drug Administration reported the approval of Lisdexamfetamine Dimesylate (LDX) (Vyvanse®) as the first medication for the

treatment of adults with moderate to severe BED [71], expanding the already approved use of this drug in ADHD [72]. LDX is a prodrug, in which the naturally occurring amino acid l-lysine is covalently linked to D-amphetamine, and it is metabolized into the inactive l-lysine and the active D-amphetamine by an enzymatic conversion, that primarly occurs in the red blood cells (Fig. 1) [73].

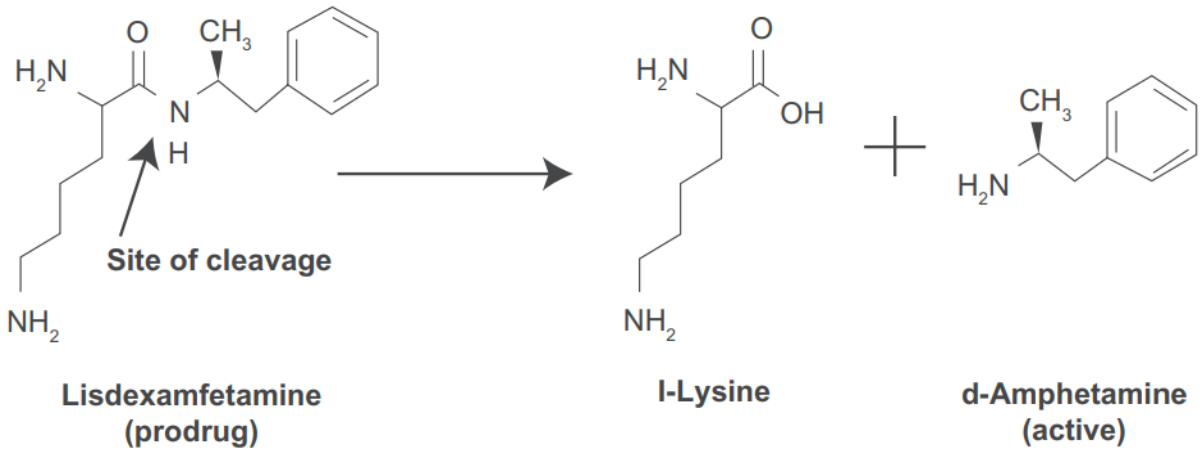

Figure 1. Enzimatic conversion of LDX in the inactive l-lysine and the active metobolite D-Amphetamine [73].

Pharmacologically, D-amphetamine is a moderately potent inhibitor of DA transporter, NA transporter, and vesicular monoamine transporter 2, with much weaker affinity for 5-HT transporter; in addition, D-amphetamine is a weak inhibitor of monoamine oxidases, and the general effect of these multiple activities is the increase in catecholamine availability in the extracellular space, by a reversal of catecholamine transport out of the neuron terminal [74]. Thus, LDX, in common with most drugs used for ADHD, facilitates the function of brain DA and/or NA neurotransmitter systems, with the consequent ability

of improving impulsivity, through the modulation of the corticostriatal circuits, which are broadly involved in reward sensitivity and inhibitory control [74, 75].

One of the first study that investigated the effects of LDX, in a preclinical model of binge-eating behaviour, proved that it dose-dependently decreased the consumption of chocolate by 15%–71% during the 2 h binge session, at doses $\geqslant$0.3 mg kg^{-1}, without a significant reduction in the intake of normal chow [76]. Furthermore, recently, Presby et al. reported that intraperitoneal injection (i.p.) of LDX, in female rats, produced a robust soppression of both chocolate and chow intake in the animal exposed to the more palatable food [77], using experimental conditions similar to those previously used by Vickers et al. [76], and in addition, LDX reduced food-reinforced lever pressing, in a task in which animals had a choice between working for chocolate flavored pellets and consuming an available but less preferred standard chow [77].

Interestingly, LDX was demonstrated not only able to reduce the binge episodes frequency of HPF intake in both preclinical and clinical studies, but also to affect the impulsive aspect of BED [77-79]. Indeed, intolerance of delayed reward and enhanced delay-discounting are established indices of impulsive choice in psychiatric and eating disorders [80, 81], and BED can be considered as a classical impulse control disorder [82]. In this context, LDX administration was found to reduce delay-discounting in binge-eating rats, that normally showed a strong trend to the immediate reward [83]. These effects of the drug are in accordance with the clinical studies from McElroy et al., in which LDX (50 or 70 mg/day) markedly decreased the number of binge-eating days in subjects with BED versus placebo [71, 84].

Furthermore, greater improvements compared to the placebo were maintained for the duration of the 12-weeks supporting the relatively rapid efficacy of LDX in reducing both

binge eating behavior and binge eating-related psychopathology, and LDX also was able to decrease obsessive-compulsive and impulsive features of BED [79].

However, as a CNS stimulant, LDX has an high potential of abuse and dependence, and the most common adverse effects associated with its use are dry mouth, insomnia, decreased appetite, constipation, feeling jittery, anxiety and increased heart rate, [82]. The presence of several side effects at the level of the heart system discourages the use of the drug in patients suffering from cardiomyopathies [85, 86].

Thus, considering the mentioned adverse effects commonly observed with LDX use, and the high incidence of BED, safer and innovative pharmacological approaches are needed for the treatment of this altered feeding behavior, and animal models offer an important strategy for the development, characterization, and preclinical evaluation of the potential efficacy of novel compounds to treat BED.

THE AIMS OF THE STUDIES

The scientific activity has been primarily focused on the understanding of the neurobiological processes involved in the compulsive-like eating and in the evaluation of the effects of several compounds in a preclinical model of binge eating behavior developed by Cifani et al., [87] in female rats.

The compounds selected were: oleoylethanolamide (OEA) and A_{2A} adenosine receptor (A_{2A}AR) agonists; OEA is an endocannabinoid-like lipid chosen for its ability to induce satiety, hypophagia and reduce fat mass in rodents, while the A_{2A}AR agonists were tested considering that these drugs can affect compulsive alcohol consumption in Marchigian-Sardinian alcohol-preferring rats.

In the first part of the thesis it was evaluated the potential effect of OEA in this well-characterized animal model of binge eating. Then, there was also investigated the possibility that OEA may reduce food intake by influencing DA, 5-HT and NA signaling within brain areas involved in homeostatic and hedonic processes; moreover, the influence of OEA administration was explored on oxytocin and corticotropin-releasing factor (CRF) levels and, finally, mapping the expression of c-Fos, the decreased and increased activation of brain areas were measured, key brain regions involved in stress-responce and in the control of food intake.

The topic of the second part is focused on the evaluation of A_{2A}AR agonists. Indeed, the adenosine neurotransmission appears to be involved in the regulation of appetite and in reward mechanisms, including voluntary ethanol drinking and HPF intake. Moreover, given the known influence of adenosine on DA signaling, the epigenetic regulation of A_{2A}AR and D2R genes were also investigated in the amygdaloid complex.

OLEOYLETHANOLAMIDE DECREASES FRUSTRATION STRESS-INDUCED BINGE-LIKE EATING IN FEMALE RATS: A NOVEL POTENTIAL TREATMENT FOR BINGE EATING DISORDER

INTRODUCTION

OEA is a well-established anorectic lipid mediator regulating feeding and body weight, through activation of the peroxisome proliferator-activated receptor-α (PPAR-α), a nuclear receptor involved in several aspects of lipid metabolism and energy balance, while it does not bind to the cannabinoid receptors [88-90]. OEA is a N-acylethanolamine synthesized in the proximal small intestine from the precursor molecule, oleic acid [91, 92], and it seems to be involved in the regulaton of satiety, since OEA levels decrease during food deprivation and increase upon refeeding, thus suggesting its participation in the appetite control in rodents [88]. The synthesis and deactivation of the molecule is described in the Figure 2.

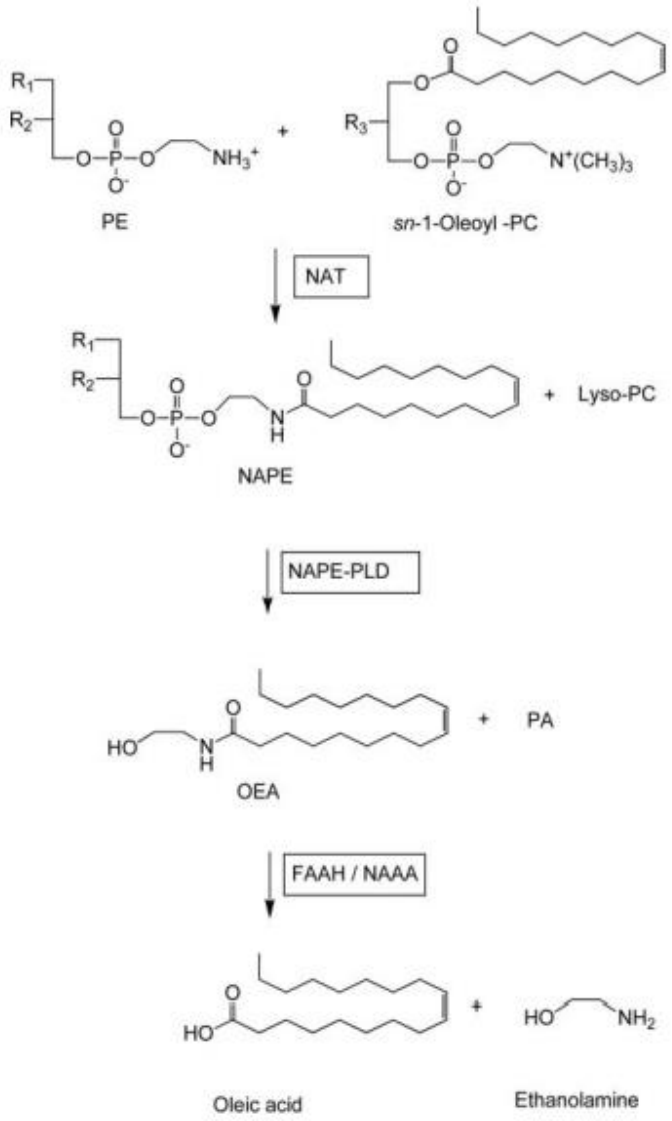

Figure 2. Biochemical pathway responsible for OEA synthesis and degradation.

PE, phosphatidylethanolamine; PC, phosphatidylcholine; PA, phosphatidic acid; NAT, N-acyltransferase; NAPE, N-acyl phosphatidylethanolamine; NAPE-PLD, NAPE-specific phospholipase D; FAAH, fatty-acid amide hydrolase; NAAA, N-acylethanolamine-hydrolyzing acid amidase [91].

The satiety effect of OEA is also highlighted by the physiological regulation of its biosynthesis in the small intestine, that is influenced both by the natural circadian feeding rhythms and by forced starvation [90, 93]. The pharmacological administration of OEA, orally or i.p., inhibits food intake by delaying meal initiation and increasing the interval

between successive meals without effect on meal sizes [90, 93]. *In vivo* experiments have revealed that OEA is able to mimic the actions of the synthetic agonists of PPAR-α [94], and that genetic deletion of PPAR-α abrogates the hypophagic actions of OEA [89], and it has been proposed that this lipid messanger might exert its anorexic effects through a mechanism mediated by the vagus nerve [95]. A lack of the satiety response to OEA is observed when, in rodents, a surgical disconnection of the vagus nerve is performed, or when the animals are treated with neurotoxic doses of capsaicin, which deprives of peripheral vagal and non-vagal sensory fibers [88]. OEA was able to reduce food consumption after systemic administration, but not after infusion into the brain ventricles. Futhermore, OEA stimulates transcription of c-Fos in the nucleus of the solitary tract [88, 96], and local enhancement of OEA production in the small intestine is sufficient to affect feeding behavior in rats, similarly to that elicited by systemic administration of exogenous OEA [97].

Therefore, OEA regulates food intake in rodents by selectively activating intestinal PPAR-α and this is associated with the activation of key brain areas, including the nucleus of the solitary tract, the tuberomammillary nucleus [98], the area postrema [99], and the paraventricular nucleus (PVN) of the hypothalamus (HYPO) [96], where histaminergic [100], noradrenergic [101], and oxytocinergic [102] neurons play an important role.

Indeed, studies have revealed that systemic injection of OEA causes an increased expression of oxytocin, in the magnocellular neurons of the PVN and in the supraoptic nucleus of the HYPO and, at the same time, the administration increases brain histamine release, highlighting that the hypophagic effect requires both the integrity of the brain histamine system, since the hypophagic effects of OEA are blunted in brain histamine-deficient mice, and the mediation by oxytocin to fully exert its anorexic action [98, 102].

Intriguingly, it has been demonstrated that OEA treatment is also able to restore a physiologic sensitivity to the rewarding properties of fat in high-fat-fed mice, by restoring the DA release [103], and OEA was futher investigated for a potential antidepressant-like action [104, 105] for the ability to increase cerebral levels of NA and 5-HT [105]. The experiments showed that oral OEA treatment significantly decreased the immobility time in the tests and regulate the central monoamine neurotransmitters [105] and BDNF in the HIPP and cortex [104]. Knowing the involvement of oxytocin and histamine in depression [106, 107], a possible link between these neurotransmitters and OEA was investigated in this psychiatric disorder, finding that the beneficial effects of OEA, by means of oxytocin, presumably requires the histaminergic neurotransmission, thus, the integrity of the brain histamine system is needed to its antidepressant-like effect [108, 109].

In this study, we used a rat model of binge-like palatable food consumption [87] to test the hypothesis that OEA might be a novel target for BED treatment. In this model, young female rats are subjected to three 8-day cycles of intermittent food restriction/refeeding. On the day of the experiment, these rats show binge-like HPF consumption after the exposure to a 15-min "*frustration stress*", consisting of the sight and smell of HPF placed out of reach [87]. We will refer in the text to dietary-restricted (R) vs not-restricted (NR) rats and exposed-to stress (S) vs not-exposed-to stress (NS) rats.

In this model, we investigated the anti-binging acute effects of OEA (2.5, 5, or 10 mg kg^{-1}, i.p.) on HPF intake, and we analyzed the neurobiological basis of these effects by focusing on different endpoints. These include the brain pattern of c-Fos expression, DA extracellular release in the shell of the nucleus accumbens (AcbSh), monoamine tissue concentrations/turnovers in selected brain regions, corticotropin-releasing factor (CRF),

and oxytocin mRNA levels in the central amygdala (CeA) and PVN and, finally, oxytocin receptor immunoreactivity in selected brain areas.

In all these neurochemical analyses, we focused our attention on the stressed groups (R + S vs NR + S), comparing the effects of OEA vs vehicle treatment. The rationale of this choice is based on the observation that intermittent caloric restriction is the predisposing condition that allows stress to act as a trigger (R + S), whereas the *ad libitum* feeding condition represents the baseline control, in which stress is ineffective (NR + S), thus also providing the control for the stress effect.

MATERIALS AND METHODS

Binge eating model

One-hundred and eighty-four female Sprague Dawley rats (Charles River, Italy), 200–225 g at the beginning of the experiments, were housed under a 12-h light/dark cycle (lights on at 8:00 a.m.), at constant temperature (20–22° C) and humidity (45–55%), and with access to food and water ad libitum for 2 weeks before the experiments. According to the dietary schedule, the rats were given standard food pellets (4RF18, Mucedola, 2.6 kcal/g) or HPF (3.63 kcal/g) consisting of a paste prepared by mixing Nutella (Ferrero®) chocolate cream (5.33 kcal/g; 56, 31, and 7% from carbohydrate, fat, and protein, respectively), grounded food pellets (4RF18), and water in the following w/w/w percent ratio: 52% Nutella, 33% food pellets, and 15% water. The procedure for binge eating induction was performed according to our previous studies [36, 87, 110]. Briefly, two groups of female rats were housed individually in metal cages (30 × 30 × 30 cm) and exposed (or not exposed) for 24 days to three 8-day cycles of intermittent food restriction

(66% of chow intake on days 1–4 and free feeding on days 5–8 of each cycle), during which they were given access to HPF for 2 h during the light cycle, between 10:00 a.m. and 12:00 a.m. (2 h after the onset of the light cycle) on days 5–6 and 13–14 of the first two cycles (total of four exposures). Although this intermittent caloric restriction caused body weight fluctuations during the three cycles, on the test day, similar body weights were detected in all rats (Fig. 3).

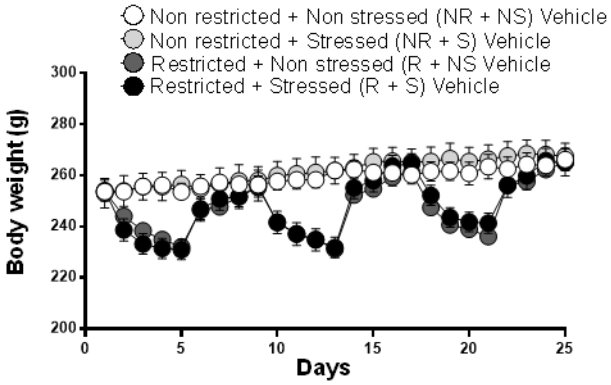

Figure 3. Mean ± SEM body weight (g) of female rats exposed or not exposed to repeated intermittent cycles of food restriction/refeeding [111].

On the test day (day 25), at 10 a.m., half of the rats in each group were subjected to a 15-min *frustration stress*, consisting of the exposure to HPF placed out of reach. During this 15-min period, the rats could smell and see the HPF and repeatedly attempted to reach it. The second half of rats in each group were not exposed to the stress manipulation. Therefore, we will refer in this manuscript to dietary restricted (R) vs non restricted (NR) rats and exposed to stress (S) vs non exposed to stress (NS) rats. After 15 min of stress exposure, the HPF was placed inside the cage for all rats. In accordance with our previous studies, binge eating behavior occurred in R + S rats, as demonstrated by the immediate and persistent consumption of a larger amount of HPF within the first 15-min access, with respect to the other groups (Fig. 4).

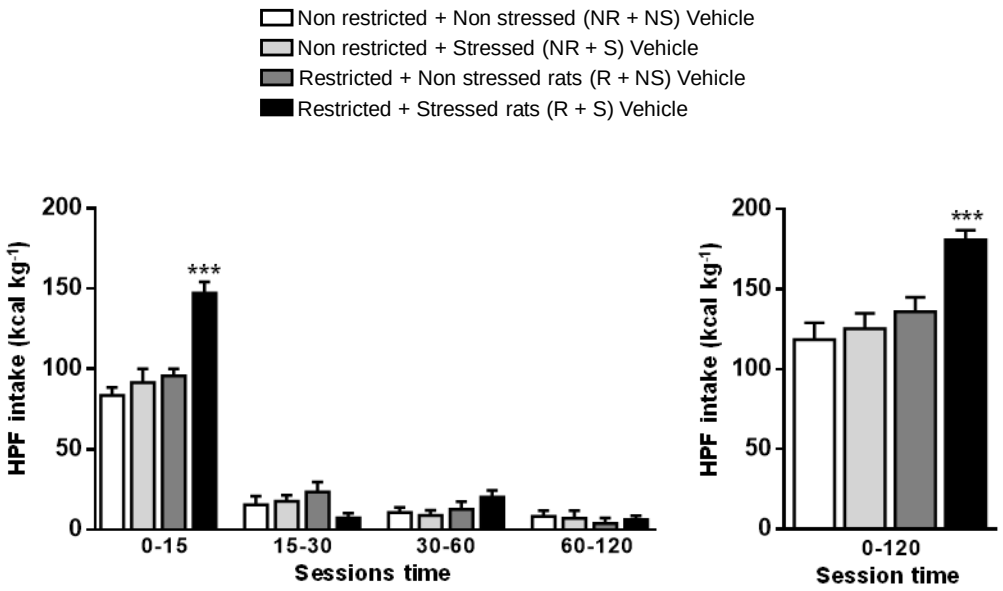

Figure 4. Mean ± SEM palatable food intake (kcal kg^{-1}) at different sessions time (0-15, 15-30, 30-60, 60-120 min) during testing (left) and total 120 min palatable food intake (right) in the vehicle-injected rats in EXP.1. ***P < 0.001, different from the other three groups; N = 6 - 8 per group [111].

The statistical analyses of HPF intakes during the test day demonstrated a significant interaction among the three factors (food restriction x stress x sessions time) (Finteraction = 6.902, df = 3/78, P < 0.001). Post hoc tests revealed a significant (P < 0.001) increase in HPF consumption at the 0-15 min time point in rats with a history of food restriction and exposed to *frustration stress* (R + S), as compared to the other groups, while no change in HPF consumption was observed during the other sessions time of the test (15–30; 30-60; 60-120 min) among all groups (Fig. 4, left panel). One-way ANOVA of the 120 min cumulative HPF intake showed a two-way interaction (food restriction x stress) (Finteraction = 4.460, df = 1/26, P < 0.05) and the post hoc analyses (P < 0.001) revealed that R + S rats were the only group showing increased HPF intake with respect to the other groups (Fig. 4, right panel). These results demonstrated that stress exposure was

able to trigger a binge-like behavior in R + S rats, which consumed a large amount of HPF within a short period of time, while it had no effect on HPF intake in rats that did not have history of food restriction. Vaginal smears were collected at the end of the experiments to exclude from the results rats in the estrous phase, since we previously observed that binge eating does not occur during the estrous phase of female rats [11, 112]. The experimental procedure is depicted in Figure 5.

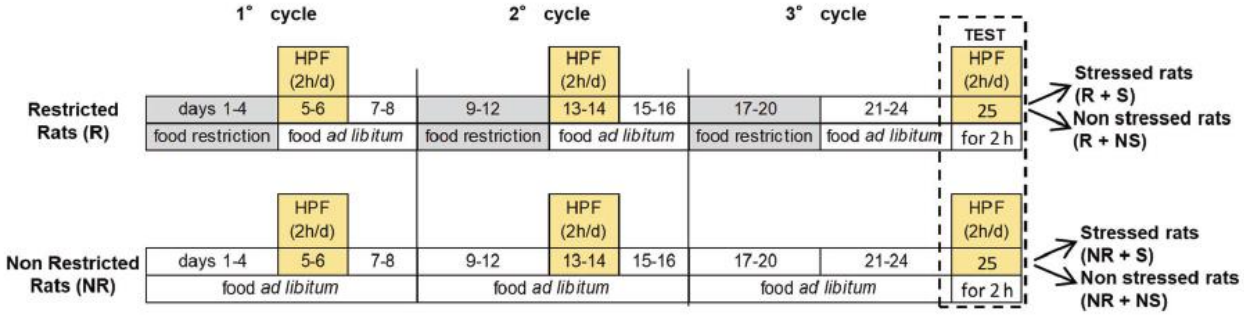

Figure 5. Female rats were exposed (restricted rats, R) or not exposed (non restricted, NR) to three 8-day cycles of intermittent food restriction (66% of chow intake), occurring on days 1–4, and freefeeding condition allowed on days 5–8 of each cycle. During the ad libitum condition of days 5–6 and 13–14 of the first two cycles, both NR and R rats were given access to HPF for 2 h during the light phase. On day 25, both R and NR rats were exposed (R + S and NR + S) or not exposed (R + NS and NR + NS) to *frustration stress* [111].

This paradigm was used in four different experiments, in which the consumption of the HPF was allowed for 120 min, 60 min, or 0 min, depending on the endpoints analyzed (Fig. 6). All experiments were carried out in accordance with 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.

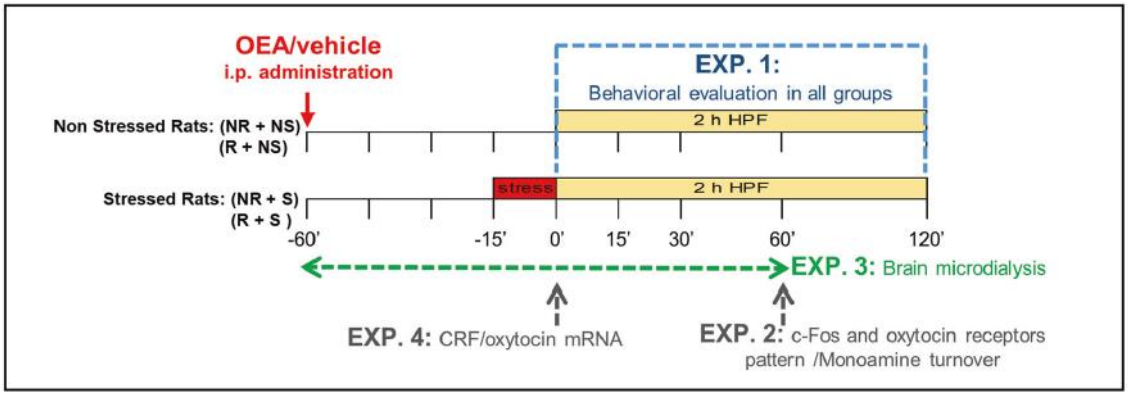

Figure 6. EXP. 1: on test day (day 25), after the third cycle, both NR and R rats were administered with vehicle (veh) or three different doses of OEA (2.5, 5, and 10 mg kg^{-1}, intraperitoneal injection (i.p.)). Forty-five minutes after treatments, both NR and R rats were exposed (stressed: NR + S and R + S) or not exposed (non stressed: NR + NS and R + NS) to a 15-min stress procedure. One hour after the respective treatments, rats were given free access to HPF for 120 min, and food intake was monitored. EXP. 2: NR + S and R + S rats were administered with veh or OEA (10 mg kg^{-1} i.p.), and were allowed to consume the HPF only for 60 min. At the end of this procedure, rats were sacrificed, their brains immediately collected for immunohistochemical evaluation of the pattern of c-Fos expression, oxytocin receptor expression, and HPLC analyses of monoamine turnover. EXP. 3: NR + S and R + S rats were administered with veh or OEA (10 mg kg^{-1}, i.p.), and underwent brain microdialysis in the AcbSh for the analysis of DA extracellular levels (the detailed paradigm of the microdialysis experiment is described in the legend of Fig. 3). EXP. 4: NR + S and R + S rats were administered with veh or OEA (10 mg kg^{-1}, i.p.), and immediately sacrificed at the end of the stress procedure. Their brains were collected for in situ hybridization analysis of CRF and oxytocin mRNA [111].

Experiment 1: Effect of OEA on stress-induced binge eating

The first set of rats (N = 144) was divided into 16 groups (N = 9 per group) in a 2 (history of intermittent food restriction: yes (R), no (NR) rats) × 2 (stress during testing: yes (S), no (NS) rats) × 4 (OEA dose: 0, 2.5, 5, and 10 mg kg^{-1}) factorial design, to evaluate the behavioral effects of OEA during the test day. To this aim, OEA or vehicle were

administered 1 h before the access to HPF; rats were exposed (or not exposed) to the 15-min *frustration stress*, and once they had access to the HPF, the intake was measured at the following time points (15, 30, 60, and 120 min). The experimental paradigm is depicted in Figure 6 (EXP. 1). After testing, 29 rats were excluded from statistical analyses because they were in the estrous phase.

Experiment 2: Effects of OEA on the pattern of c-Fos, oxytocin receptor expression, and on monoamine turnover

Previous studies demonstrated that the effect of OEA on food intake is paralleled by a selective induction of c-Fos, an immediate early gene widely used as a marker of cellular activation, at the level of the HYPO and brainstem [96, 99, 113], key regions involved in the control of feeding [114]. Here, we have expanded those findings by examining the impact of OEA (10 mg kg^{-1}) on the brain pattern of c-Fos immunostaining in response to 60 min of HPF consumption in female rats with different diet histories and exposed to acute stress (R + S vs NR + S, Fig. 4, EXP. 2). In this experiment, we tested the effects of the highest dose (OEA 10 mg kg^{-1} i.p.), based on the observations made in EXP. 1. Moreover, we evaluated whether the interaction between food restriction and stress exposure is accompanied by alteration of oxytocin receptor immunoreactivity in selected brain regions, and whether OEA treatment is able to affect this endpoint. As a further aim of this experiment (Fig. 6, EXP. 2), we analyzed the effects of OEA on tissue concentrations of monoamines (DA, 5-HT, and NA) and their main metabolites in the principal neural nodes that control different aspects of food intake in the brain. The immunohistochemistry experiment and monoamine analyses were performed according to our previous studies [99, 115].

Experiment 3: Effects of OEA on DA transmission in the AcbSh

To investigate whether OEA would decrease the central dopaminergic response to appetitive/reinforcing stimuli, we performed in vivo microdialysis experiment to evaluate DA extracellular concentration at the level of the AcbSh in R + S and NR + S rats (Fig. 6, EXP. 3), according to the protocol used in our previous study [116, 117]. To this aim, a new set of rats (N = 40) was divided into R and NR groups, according to the protocol described for EXP. 1, and underwent the procedure for microdialysis experiment.

Experiment 4: Effects of OEA on CRF and oxytocin mRNA

In situ hybridization was performed in brain slices obtained from R + S and NR + S rats according to the protocol reported in our previous studies [96, 113, 118].

Statistical analyses

All data were expressed as mean ± SEM. Feeding data showed in Fig. 4, left panel were statistically analyzed by three-way ANOVA for repeated measures, which included the intermittent food restriction (R or NR) and the *frustration stress* during testing (S or NS) as the between-subjects factors, with sessions time (0-15, 15-30, 30-60, 60-120 min) as the within-subject factor. Feeding data showed in Figure 4 right panel, were statistically analyzed by two-way ANOVA with intermittent food restriction and stress as the two factors. Feeding data showed in Figure 7 were statistically analyzed by one-way ANOVA with treatment as between-subject factor. Bonferroni's test for multiple comparisons (Systat Software 10.0) was used for post hoc analyses of all feeding data. Results obtained from immunohistochemistry, HPLC analysis of tissue monoamines and *in situ*

hybridization were statistically analyzed by two-way ANOVA, with food restriction and treatment as the two factors. Tukey's test was used as a post hoc to perform multiple comparisons. Moreover, for analysis of data obtained from the semi-quantitative densitometric analyses, because of the difference in the number of slices examined and the high degree of freedom, the error degrees of freedom were kept constant at a value based on the actual number of rats per group used in each experiment [99, 119]. The results from microdialysis experiments resulted homoscedastic and were analyzed by two-way ANOVA for repeated measures, with time as the within variable and treatment as the between variable, followed by Dunnett's and Bonferroni's post hoc tests for multiple comparisons. Overall, DA extracellular levels were calculated as percentages of baseline, which was defined as the average of the first three consecutive samples with stable level of neurotransmitters. Unpaired t-test was used to evaluate the difference between the marginal means of the first three dialysate samples (basal values). In all instances, the threshold for statistical significance was set at $P < 0.05$.

RESULTS

OEA treatment selectively prevented binge-like eating in a dose-dependent manner

We found that acute treatment with OEA, systemically administered to rats 1 h before giving access to HPF, selectively prevented binge-like eating of R + S rats, without altering feeding behavior in the other experimental groups (Fig. 7). In particular, OEA decreased *frustration stress* induced HPF overconsumption in a dose- and time-dependent manner, with the strongest and long-lasting effect observed at the dosage of 10 mg kg^{-1} i.p. (Fig. 7). The intermediate dose of OEA (5 mg kg^{-1} i.p.) was effective only at the 15-

min time point, while the lowest dose of OEA was ineffective. The results obtained from ANOVA showed a significant effect of treatment in the session time 0–15 min (Ftreatment = 29.763, df = 3/27, P < 0.001) and in 0–120 min (Ftreatment = 5.758, df = 3/27, P < 0.01). Significant differences among groups evaluated by the post hoc analyses are indicated in Fig. 7.

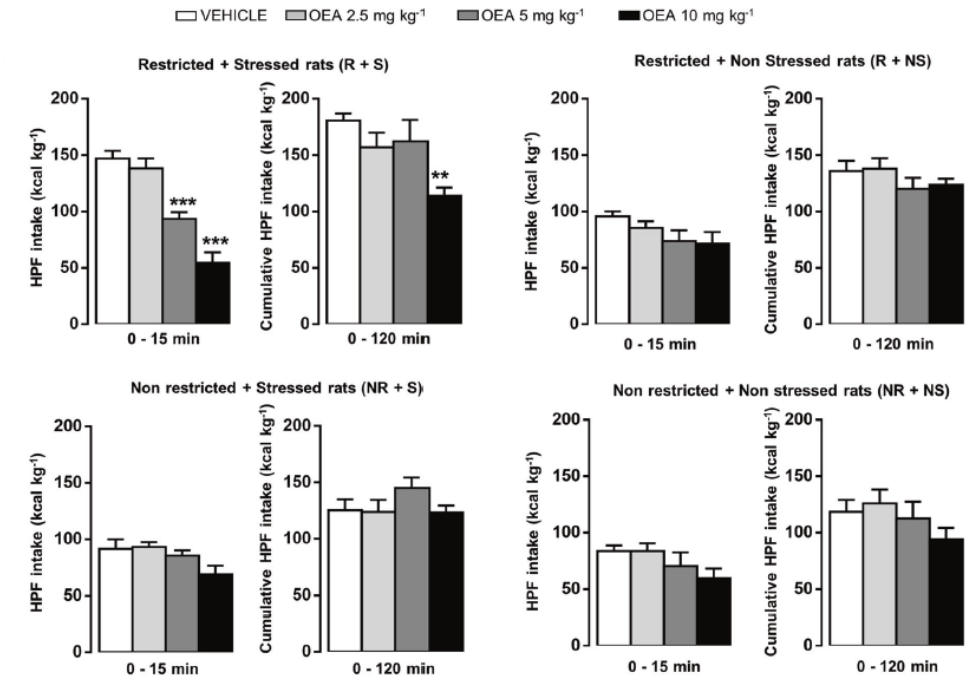

Figure 7. HPF intake (kcal $kg^{-1)}$ during the first 15 min (left) and the total 120 min (right) test session after vehicle (veh) or three different doses of OEA (2.5, 5, and 10 mg kg^{-1} i.p.) administration to R + S (restricted + stressed, N = 31), R + NS (restricted + non stressed, N = 28), NR + S (non restricted + stressed, N = 26), and NR + NS (non restricted + non stressed, N = 30). Data are expressed as mean ± SEM. **P < 0.01; ***P < 0.001 vs R + S veh (Bonferroni's test for multiple comparisons) [111].

OEA treatment affected the brain pattern of c-Fos expression in bingeing rats

The semiquantitative analyses of immunostaining optical densities revealed that the interaction between intermittent food restriction and stress exposure induced an increase of c-Fos expression in the Acb, CPu, AMY, and substantia nigra (SN) of bingeing rats (R + S veh), with respect to non-bingeing rats (NR + S veh), and that OEA treatment completely prevented such increase (Fig. 8 c, d, f, h). Conversely, c-Fos expression within the PVN, pedunculopontine nucleus (PP), and VTA (Fig. 8 e, g, i) was unchanged in bingeing rats (R + S veh), with respect to non-binging rats (NR + S veh), but significantly increased by OEA treatment (R + S-OEA vs R + S veh), which induced a similar effect also in the AMY and PP of NR + S rats (Fig. 8 f, g, respectively). No difference was observed within the ventral pallidum nucleus among all rat groups (Fig. 8 b). The results obtained from the post hoc analyses are reported in Figure 8.

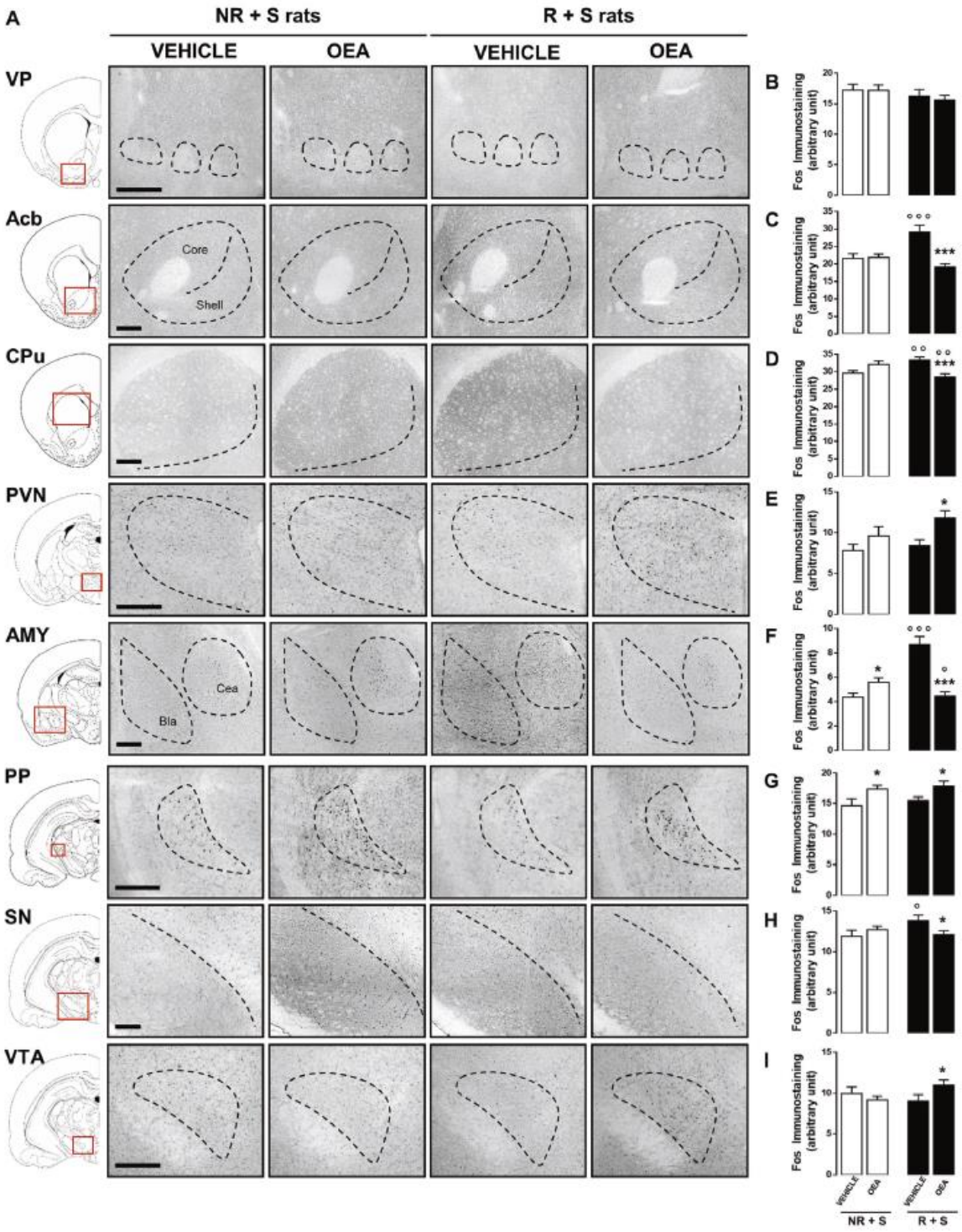

Figure 8. OEA treatment affected the brain pattern of c-Fos expression in bingeing rats. Representative photomicrographs (scale bar=500 μm, a) showing c-Fos immunostaining within the ventral pallidum (VP), nucleus accumbens (Acb), caudate putamen (CPu), paraventricular nucleus (PVN), amygdala (AMY), pedunculopontine nucleus (PP), substantia nigra (SN), and ventral tegmental area (VTA) in brain slices

collected from both NR+ S (non restricted + stressed) and R+S (restricted + stressed) rats treated with either vehicle (veh) or OEA (10mg kg^{-1}, i.p.) and sacrificed 120min after treatment. Semiquantitative densitometric analysis of c-Fos expression within the VP (b), Acb (c), CPu (d), PVN (e), AMY (f), PP (g), SN (h), and VTA (i) of NR+S and R+S rats treated with either veh or OEA (10mg kg^{-1}, i.p.) and sacrificed 120 min after treatment. Data are expressed as mean ± SEM. *P < 0.05; ***P < 0.001 vs veh in the same diet regimen group; °P<0.05; °°P < 0.01; °°°P<0.001 vs NR+S in the same treatment group (Tukey's post hoc test, N=3) [111].

OEA treatment affected monoaminergic system in bingeing rats

The results (Table 2) revealed that OEA treatment affected mainly monoaminergic tissue concentration/turnover in bingeing rats, rather than in NR + S rats. In fact, in NR + S rats, the effects of OEA treatment included only an increase in NA and DA concentration within the HYPO and VTA, respectively, and an increase of 5-HT turnover in the Acb. Analyzing the results obtained from vehicle-administered rats, bingeing rats (R + S veh) showed an increased DA turnover in the medial prefrontal cortex (mPFC) and AMY, as well as increased 5-HT turnover and 5-HT tissue concentration in the AMY and HYPO, respectively, as compared with non-bingeing rats (NR + S veh). The increased turnovers observed in the mPFC and AMY of bingeing rats resulted in complete prevention by OEA treatment, which increased DA and 5-HT concentrations in the mPFC. The latter effect was accompanied by a decrease of 5-HT turnover in the mPFC of R + S OEA rats, with respect to their vehicle-treated controls. DA tissue concentrations were affected by OEA treatment also in Acb (where it decreased) and VTA (where it increased) of R + S rats, without producing any effect on DA turnover. Similarly, OEA administration to R + S rats caused a marked increase of 5-HT tissue concentrations in Acb, HIPP, VTA, and locus coeruleus (LC), without affecting 5-HT turnover in these areas. Finally, OEA

treatment caused a significant increase of NA concentration in the CPu, HYPO, VTA, and LC.

Table 2. Tissue monoamine concentrations (ng mg^{-1} of wet tissue) and monoamine turnover in selected brain areas [111].

		NR + S rats		R + S rats	
		VEH	OEA	VEH	OEA
mPFC	DA	0.05 ± 0.01	0.05 ± 0.005	**0.03 ± 0.002**	**0.06 ± 0.01** **↑
	DOPAC+HVA/DA	**3.07 ± 0.16**	2.69 ± 0.52	**4.48 ± 0.41** °↑	**2.11 ± 0.19** ***↓
	NA	0.20 ± 0.03	0.20 ± 0.02	0.20 ± 0.02	0.19 ± 0.03
	5HT	0.08 ± 0.01	0.09 ± 0.01	**0.07 ± 0.01**	**0.14 ± 0.03** *↑
	5HIAA/5HT	4.13 ± 0.67	3.54 ± 0.80	**5.15 ± 1.11**	**2.44 ± 0.31** *↓
Acb	DA	1.40 ± 0.28	0.81 ± 0.16	**1.29 ± 0.18**	**0.64 ± 0.16** *↓
	DOPAC+HVA/DA	0.74 ± 0.23	0.96 ± 0.13	1.02 ± 0.16	0.86 ± 0.22
	NA	0.12 ± 0.03	0.12 ± 0.03	0.12 ± 0.02	0.12 ± 0.02
	5HT	0.07 ± 0.02	0.06 ± 0.01	**0.06 ± 0.01**	**0.13 ± 0.03** *↑
	5HIAA/5HT	**0.63 ± 0.08**	**0.99 ± 0.17** *↑	0.68 ± 0.06	0.42 ± 0.08
CPu	DA	2.56 ± 0.52	2.74 ± 0.64	2.34 ± 0.36	2.18 ± 0.52
	DOPAC+HVA/DA	0.59 ± 0.14	0.65 ± 0.14	0.69 ± 0.08	0.48 ± 0.06
	NA	0.07 ± 0.01	0.10 ± 0.01	**0.10 ± 0.01**	**0.14 ± 0.01** *↑
	5HT	0.15 ± 0.02	0.11 ± 0.02	0.12 ± 0.02	0.12 ± 0.02
	5HIAA/5HT	2.43 ± 0.51	3.21 ± 0.62	2.87 ± 0.65	2.77 ± 0.76
HYPO	DA	0.28 ± 0.03	0.39 ± 0.11	0.25 ± 0.06	0.35 ± 0.03
	DOPAC+HVA/DA	0.52 ± 0.10	0.48 ± 0.06	0.50 ± 0.07	0.36 ± 0.05
	NA	**0.90 ± 0.07**	**1.45 ± 0.11** *↑	**1.06 ± 0.14**	**1.56 ± 0.21** *↑
	5HT	**0.24 ± 0.03**	0.32 ± 0.04	**0.44 ± 0.06** °↑	0.59 ± 0.07
	5HIAA/5HT	3.13 ± 0.61	2.30 ± 0.88	2.17 ± 0.24	1.23 ± 0.18
AMY	DA	0.40 ± 0.13	0.43 ± 0.07	0.59 ± 0.24	0.36 ± 0.09
	DOPAC+HVA/DA	**0.59 ± 0.09**	0.46 ± 0.08	**0.92 ± 0.16** °↑	**0.47 ± 0.07** **↓
	NA	0.24 ± 0.05	0.23 ± 0.04	0.28 ± 0.08	0.19 ± 0.03
	5HT	0.10 ± 0.03	0.16 ± 0.04	0.07 ± 0.02	0.10 ± 0.03
	5HIAA/5HT	**0.94 ± 0.41**	1.39 ± 0.36	**3.16 ± 1.22** °↑	**1.16 ± 0.37** *↓
HIPP	DA	0.02 ± 0.01	0.03 ± 0.01	0.02 ± 0.01	0.03 ± 0.01
	DOPAC+HVA/DA	3.11 ± 0.75	3.86 ± 1.06	3.67 ± 1.00	2.88 ± 0.84
	NA	0.22 ± 0.04	0.20 ± 0.02	0.23 ± 0.04	0.27 ± 0.03
	5HT	0.11 ± 0.02	0.12 ± 0.03	**0.09 ± 0.02**	**0.22 ± 0.05** *↑
	5HIAA/5HT	4.27 ± 0.73	3.85 ± 0.51	4.83 ± 0.75	4.08 ± 0.42
SN	DA	0.18 ± 0.03	0.17 ± 0.03	0.13 ± 0.03	0.15 ± 0.01
	DOPAC+HVA/DA	0.93 ± 0.09	0.94 ± 0.07	1.07 ± 0.10	0.73 ± 0.10
	NA	0.17 ± 0.03	0.23 ± 0.05	0.19 ± 0.03	0.24 ± 0.02
	5HT	0.19 ± 0.05	0.26 ± 0.07	0.14 ± 0.04	0.24 ± 0.03
	5HIAA/5HT	3.11 ± 0.90	3.60 ± 0.86	2.90 ± 0.55	1.30 ± 0.21
VTA	DA	**0.17 ± 0.02**	**0.34 ± 0.07** *↑	**0.18 ± 0.03**	**0.34 ± 0.06** *↑
	DOPAC+HVA/DA	0.90 ± 0.11	0.79 ± 0.09	0.95 ± 0.08	0.73 ± 0.08
	NA	0.24 ± 0.02	0.28 ± 0.07	**0.20 ± 0.03**	**0.34 ± 0.03** *↑
	5HT	0.13 ± 0.02	0.16 ± 0.04	**0.11 ± 0.02**	**0.24 ± 0.04** **↑
	5HIAA/5HT	1.78 ± 0.59	1.92 ± 0.47	1.72 ± 0.41	1.43 ± 0.29
DR	DA	0.05 ± 0.01	0.09 ± 0.01	0.07 ± 0.01	0.09 ± 0.03
	DOPAC+HVA/DA	0.31 ± 0.04	0.17 ± 0.03	0.33 ± 0.08	0.33 ± 0.06
	NA	0.15 ± 0.02	0.21 ± 0.02	0.18 ± 0.05	0.19 ± 0.05
	5HT	0.04 ± 0.01	0.08 ± 0.01	0.11 ± 0.03	0.19 ± 0.06
	5HIAA/5HT	1.07 ± 0.47	1.30 ± 0.68	1.67 ± 0.74	1.13 ± 0.43
LC	DA	0.07 ± 0.01	0.08 ± 0.01	0.06 ± 0.01	0.10 ± 0.01
	DOPAC+HVA/DA	0.87 ± 0.14	0.71 ± 0.16	0.64 ± 0.06	0.70 ± 0.08
	NA	0.32 ± 0.06	0.29 ± 0.07	**0.22 ± 0.05**	**0.40 ± 0.05** *↑
	5HT	0.07 ± 0.03	0.10 ± 0.04	**0.06 ± 0.01**	**0.16 ± 0.04** *↑
	5HIAA/5HT	2.76 ± 0.95	2.41 ± 0.37	2.63 ± 0.34	2.98 ± 0.53

OEA treatment dampened AcbSh DA release induced by stress exposure or amphetamine challenge

In agreement with previous reports [120-123], in both rat groups treated with vehicle, dialysate DA levels significantly exceeded the basal values in response to stress exposure or to amphetamine challenge, with no change induced by HPF consumption (Fig. 9). The first increase in response to stress exposure was transitory (15 min) and reached 292 and 194% in NR + S veh and R + S vehicle rats, respectively; the second increase was long-lasting (about 90 min) and reached maximum values of 764% and 638%, in non-bingeing and bingeing vehicle-treated rats, respectively. OEA administration did not alter DA basal levels in either experimental groups, but significantly attenuated the increase in DA efflux evoked by *frustration stress* and by amphetamine challenge, independently from the history of caloric restriction (Fig. 9 a, b). The results obtained by the two-way ANOVA for repeated measures revealed a significant effect of time, treatment, and a significant interaction between the two factors (R + S: Ftime = 17.252, df = 18/234, $P < 0.001$, Ftreatment = 27.407, df = 1/13, $P < 0.001$, and Finteraction = 5.018, df = 18/234, $P < 0.01$; NR + S: Ftime = 15.216, df = 18/324, $P < 0.001$, Ftreatment = 6.154, df = 1/18, $P < 0.05$, and Finteraction = 3.142, df = 18/324, $P < 0.05$). The results obtained by post hoc tests are reported in Fig. 9 a, b.

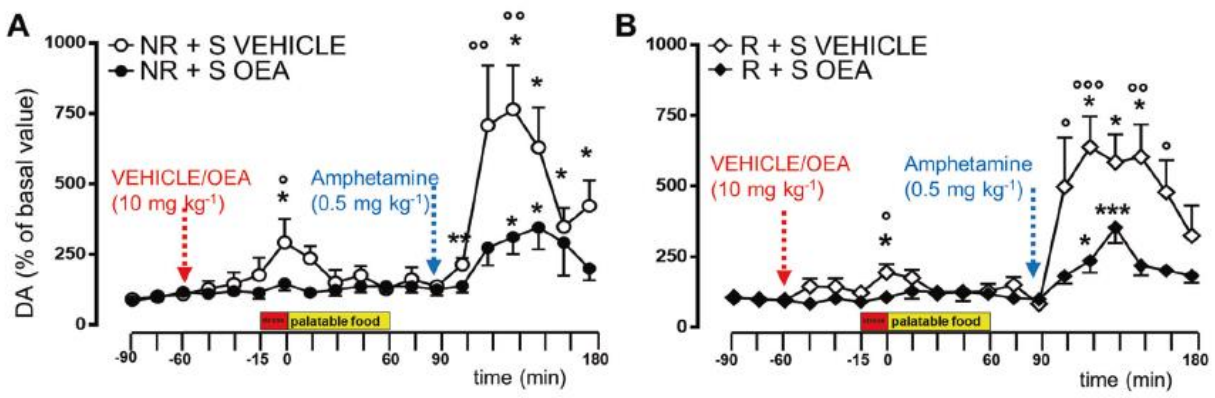

Figure 9. OEA treatment dampened AcbSh DA release induced by stress exposure or amphetamine challenge. Time course of extracellular DA levels (expressed as % of basal values) measured in the nucleus accumbens shell (AcbSh) of NR + S (non restricted + stressed, a, N = 9–11) and R + S (restricted + stressed, b, N = 6–9) rats during microdialysis experiment. The first three samples were collected before treating rats with vehicle (veh) or OEA (10 mg kg^{-1}, i.p.) and used as baseline (NR + S baseline = 225.5 ± 43.66; R + S baseline = 205.1 ± 21.07, no statistically significant difference); 45 min after treatment, rats were subjected to the stress procedure for 15 min and subsequently received the HPF for 60 min. Thirty minutes after the end of HPF exposure, rats were administered with amphetamine (0.5 mg kg^{-1}, subcutaneous injection (s.c.)). Data are expressed as mean ± SEM. *P < 0.05; **P < 0.01; ***P < 0.001 vs the mean of the first three samples (basal values) within the same group (Dunnett's multiple-comparison test). °P < 0.05; °°P < 0.01; °°°P < 0.001 vs OEA-treated rats in the same time point of the same diet regimen group (Bonferroni's test for betweengroup comparisons). Red arrow: veh or OEA (10 mg kg^{-1}, i.p.) administration; blue arrow: amphetamine administration (0.5 mg kg^{-1}, s.c.) [111].

OEA treatment affected CRF, oxytocin mRNA levels and oxytocin receptor expression in selected brain areas of bingeing rats

We previously demonstrated a crucial role of oxytocinergic neurotransmission in mediating the hypophagic effect of OEA [96], as well as the pivotal role played by CRF system in sustaining binge eating behavior in the experimental model used in the present study [36]. Since both oxytocin and CRF can be affected by stress and food intake, we assessed the "pure" effects of OEA on stress response without the potential impact of caloric consumption, to evaluate whether the anti-bingeing effects of OEA might be attributed to a reduced effect of stress exposure. To this aim, we measured both CRF and oxytocin mRNA levels by in situ hybridization in the brains of NR + S and R + S rats treated with either OEA or vehicle and sacrificed at the end of the stress exposure (Fig. 6 EXP.4). As shown in the representative autoradiography reported in Fig. 10 a, c, CRF mRNA signal was detected and measured in the CeA and the PVN. The results of the

densitometric analyses of CeA were statistically analyzed by two-way ANOVA that revealed no effect of caloric restriction and no effect of treatment, but a significant interaction between the two factors ($F_{interaction} = 9.491$, df = 1/19, $P < 0.01$). Post hoc analyses demonstrated that OEA treatment reduced CRF mRNA in the CeA of bingeing rats (Fig. 10 b), whereas the two-way ANOVA in the PVN revealed no significant effect (Fig. 10 d). As shown in the representative autoradiography reported in Figure 10 e oxytocin mRNA signal was detected and measured in the PVN. Two-way ANOVA analyses revealed a significant effect of food restriction ($F_{restriction} = 9.897$, df = 1/20, $P < 0.01$), no effect of treatment, and a significant interaction between the two factors ($F_{interaction} = 5.544$, df = 1/20, $P < 0.05$). The post hoc analyses demonstrated that oxytocin mRNA expression was significantly increased in bingeing rats treated with OEA (Fig. 10 f). Oxytocin receptors are abundantly expressed in the striatum [60], where they control, through different mechanisms, dopaminergic neurotransmission. Therefore, as the last step of our study, we investigated whether bingeing rats show different oxytocin receptor immunoreactivity in the CPu and Acb (Fig. 10 g, i), as compared with non-bingeing rats, and whether OEA treatment might affect such parameters. The results obtained by the semiquantitative densitometric analyses of optical densities revealed that binge eating behavior in R + S rats was associated with a reduced oxytocin receptor expression within both the dorsal (CPu) and the ventral (Acb) striatum, and that OEA treatment completely restored such decrease, reporting oxytocin receptor immunoreactivity to the level observed in NR + S rats (Fig.10 h, l). In particular, two-way ANOVA analyses of oxytocin receptor expression within the Acb revealed a significant effect of treatment ($F_{treatment} = 7.445$, df = 1/11, $P < 0.05$), no effect of food restriction, and significant interaction between the two factors ($F_{interaction} = 6.363$, df =

1/11, P < 0.05). The same effect was observed within the CPu (Finteraction = 8.479, df = 1/11, P < 0.05) (Fig. 10 h, l).

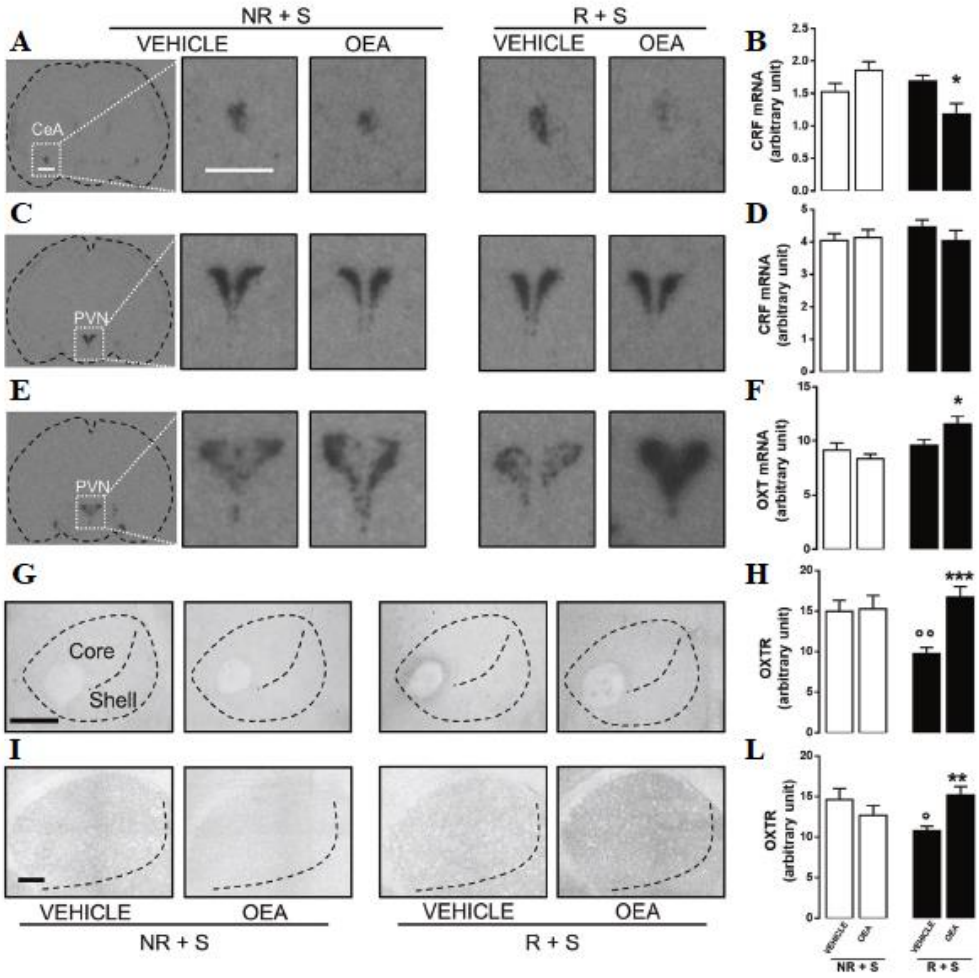

Figure 10. OEA treatment affected CRF, oxytocin mRNA levels, and oxytocin receptor expression in bingeing rats. Representative in situ hybridization images (scale bar = 1 mm) of CRF mRNA expression within the central amygdala (CeA, a), CRF, and oxytocin mRNA in the paraventricular nucleus (PVN, c, e) of NR+ S (non restricted + stressed) and R + S (restricted + stressed) rats treated with either vehicle (veh) or OEA (10 mg kg^{-1}, i.p.), and sacrificed 60 min after the treatment. Semiquantitative densitometric analyses of CRF mRNA in the CeA (b), and CRF and oxytocin mRNA in the PVN (d, f, respectively) of NR + S and R + S rats treated with either veh or OEA (10 mg kg^{-1}, i.p.), and sacrificed 60 min after treatment. Data are expressed as mean ± SEM. *P < 0.05 vs veh in the same diet regimen group (Tukey's post hoc test, N = 4–6). Representative photomicrographs (scale bar = 500 μm) showing oxytocin receptor

(OXTR) immunostaining within the nucleus accumbens (core and shell, g) and the caudate putamen (i) in brain slices collected from both NR + S (non restricted + stressed) and R + S (restricted + stressed) rats treated with either vehicle (veh) or OEA (10 mg kg^{-1}, i.p.) and sacrificed 120 min after treatment. Semiquantitative densitometric analysis of oxytocin receptor expression within the nucleus accumbens (h) and caudate putamen (l) of NR+ S and R + S rats treated with either veh or OEA (10 mg kg^{-1}, i.p.) and sacrificed 120 min after treatment. Data are expressed as mean ± SEM. **P < 0.01; ***P < 0.001 vs veh in the same diet regimen group; °P < 0.05; °°P < 0.01 vs NR + S in the same treatment group (Tukey's post hoc test, N = 3) [111].

DISCUSSION

OEA prevents binge-like palatable food consumption

This study demonstrates that OEA prevents binge-like palatable food consumption induced by stress in female rats with a history of food restriction (R + S rats), supporting the hypothesis that this lipid signal might represent a potential target for the development of more efficacious and safer treatments for BED or for other eating disorders characterized by binge episodes. The effect of OEA was dose- and time-dependent, being long-lasting at the dose of 10 mg kg^{-1} i.p. According to previous reports from our laboratory and from other research groups [88, 96, 99, 101, 113], this dosage of OEA is able to induce satiety in both free-feeding and food-deprived rats, without causing motor impairment, malaise, pain, or hormonal and body temperature alterations. In the present study, we were unable to detect any significant effect of OEA on feeding behavior in the other three experimental groups, which did not show binge-like palatable food intake within the timeframe of the experiment. This observation suggests that the anti-binge effects of OEA, rather than the expression of satiety induction, might likely be the consequence of the selective inhibition of "hedonic hunger" [124, 125].

OEA modulates monoaminergic tone in key brain areas

Based on this evidence, to investigate the neurobiological counterparts of OEA effect on binge eating, we focused our attention on the so-called "DA motive system" controlling the reinforcement and motivational aspects of feeding, including compulsive eating [126]. Our choice is based on previous findings demonstrating the capability of OEA to counteract different addiction-related behaviors, by acting within this system [127-129]. The results obtained by analyzing c-Fos expression in brain areas that partake directly or indirectly to this system suggest that the anti-binge effects of OEA are associated with its ability to dampen the "trigger" effects of stress in R + S rats. This action is accomplished by "normalizing" the activity of areas responding to stress exposure (Acb, CPu, SN, and AMY), and by increasing the activation of areas involved in the control of food intake (VTA and PVN). The effect observed in the Acb was associated with a decrease of DA tissue levels induced by OEA administration to R + S rats. Expanding this latter result, data obtained from microdialysis of the AcbSh revealed that OEA dampened DA response to stress and to amphetamine challenge in both R + S and NR + S rats. Previous studies from Tellez and collaborators have demonstrated that OEA treatment restored a normal dopaminergic nigrostriatal response to fat intake in diet-induced obese mice [103]. The results of our experiments expand their observation, demonstrating that OEA is able to restore a normal dopaminergic response not only to food consumption but also to stress-induced appetitive motivation. However, in our study, the attenuation of AcbSh DA release evoked by OEA in response to stress exposure did not perfectly parallel the selective behavioral effects. In fact, although OEA induced the same effect on DA release in the Acb of both R + S and NR + S rats, it significantly inhibited HPF consumption only in the R + S group, thus suggesting the involvement of other possible systems. Based on

previous observations, we hypothesized that these systems might include 5-HT, NA, CRF, and oxytocin. In support of this hypothesis, we found that in R + S rats, OEA selectively enhanced NA levels in the CPu, VTA, and LC, as well as it increased 5-HT tissue levels in most of the brain areas analyzed (mPFC, Acb, HIPP, VTA, and LC). These results are in accordance with previous studies showing that OEA exerts anti-depressant-like effects in different animal models [108], by regulating 5-HT and NA levels [105], and suggest that the anti-binge effects of OEA might occur, at least in part, by promoting a high serotonergic/ noradrenergic tone.

OEA affects central CRF and oxytocinergic systems in bingeing rats

The results obtained within the AMY, where OEA significantly decreased stress-induced c-Fos activation in bingeing rats, prompted us to investigate whether OEA might influence the CRF system, known to coordinate the *frustration stress* response in a rat model [110]. In agreement with this notion, and overlapping with the trend of c-Fos induction in the AMY, we found that OEA decreased CRF mRNA level in the CeA of R + S rats, without producing any effect on the PVN, and without affecting the same parameters in NR + S rats. The results confirm previous findings demonstrating that hypothalamic CRF system is not sufficient to account for binge-like HPF consumption in our BED model [36, 110], and that CRF in the CeA plays a key role in other models of excessive HPF consumption [130-132]. These latter observations were further supported by the findings that treatments with CRF antagonists can prevent binge eating by interacting with CRF receptors in bed nucleus of the stria terminalis [11, 110, 133] and CeA [130-132], rather than at hypothalamic levels. Furthermore, we hypothesized that the ability of OEA to increase c-Fos expression within the PVN of R + S rats might be

linked to the activation of oxytocinergic neurons [101]. In agreement with our previous studies [96, 102], we found that OEA treatment increased oxytocin mRNA levels in the PVN of R + S rats, without producing any effect on NR + S rats. We expanded this notion by also analyzing oxytocin receptor expression. We observed a reduced immunoreactivity for oxytocin receptors within both the CPu and the Acb of R + S rats, as compared with NR + S rats. This result suggests a hypofunctionality of the oxytocinergic system at the level of these two brain regions that might be associated with the compulsive eating in response to stress. In fact, it is well demonstrated that oxytocin transmission has a key role in attenuating stress responses by exerting inhibitory actions on the HPA axis, sympathetic activity, and anxiety-related behavior during exposure to stressful stimuli [134-136]. Our functional hypothesis is that cycles of food restriction might attenuate oxytocin sensitivity in R + S rats; OEA treatment might be able to rescue this alteration by normalizing oxytocin receptor density and stimulating oxytocin release from the PVN, thus overall increasing oxytocin transmission in bingeing rats. Such effect might contribute, in turn, to the reduced CRF synthesis in the CeA, as supported by several findings demonstrating a genomic effect of oxytocin on CRF gene expression [137].

REGULATION OF ADENOSINE A_{2A} RECEPTOR GENE EXPRESSION IN A MODEL OF BINGE EATING IN THE AMYGDALOID COMPLEX OF FEMALE RATS

INTRODUCTION

Adenosine is a neuromodulator that controls several brain functions under both physiological and pathophysiological conditions, via the activation of four G-protein-coupled receptors (GPCR), the A_1, A_{2A}, A_{2B} and A_3 receptors [138, 139]. In the brain, there is a dynamic regulation of adenosine levels, whose extracellular concentration are determined by intracellular and extracellular enzymes involved in adenosine metabolism and by its transport across cell membrane [140]. An important source of adenosine is metabolic stress, which triggers the dephosphorylation of adenosine triphosphate (ATP), producing adenosine monophosphate (AMP), then converted by the 5'-nucleotidase into adenosine. Subsequently, the release of adenosine occurs by facilitated diffusion, via the the equilibrative nucleoside transporters (ENT) [140, 141]. Alternatively, adenosine can be produced by the extracellular conversion of ATP, through the action of ectonucleotidases [140, 142]. As shown in Figure 11, adenosine is primarly metabolized by the adenosine kinase, which is predominantly expressed in the astrocytes, where it controls the astrocyte-based adenosine cycle [138, 141, 143].

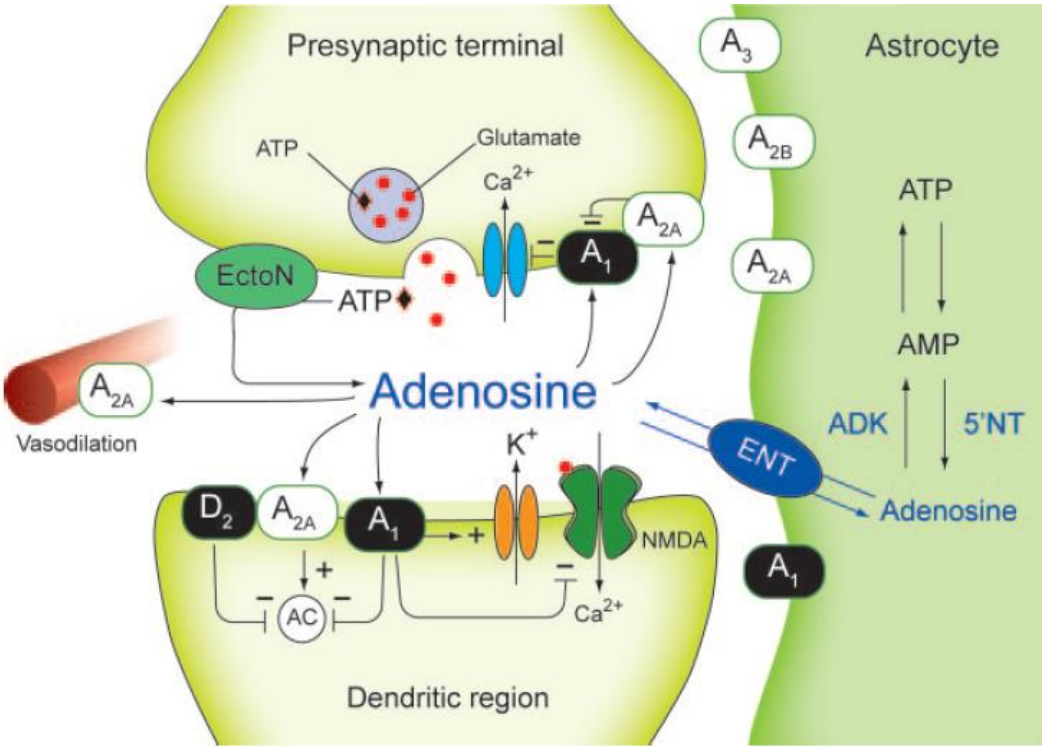

Figure 11. Schematic representation of adenosine signalling and regulation in the CNS. 5'NT: 5'-nucleotidase; AC: adenylate cyclase; ADK: adenosine kinase; AMP: adenosine monophosphate; ATP: adenosine triphosphate; EctoN: ectonucleotidases; Ca^{2+}: calcium; K^{+}: potassium; ENT: equilibrative nucleoside transporters; NMDA: N-methyl-D-aspartate [140].

Among adenosine receptors, the high-affinity A_1 and A_{2A} are the most abundantly found in the CNS. The A_1 receptors are coupled to G_i proteins, inhibit adenylate cyclase, activate phospholipase C, increase potassium (K^{+}) conductance via inward rectifying K^{+} channels, and inhibit presynaptic N- and P/Q type calcium (Ca^{2+}) channels, resulting in a global inhibitory modulation of synaptic transmission, while the A_{2A}ARs are coupled to G_s and G_{olf} proteins, stimulate adenylate cyclase, and can behave as homodimers or functional heteromeric receptor complexes with other GPCRs [138, 140, 141]. Moreover, it has been established the ability of A_{2A}AR synaptic activation to downregulate the A_1R and to reduce the affinity of its agonists [144]. Generally, through the activation of A_1R and A_{2A}AR, which results in opposing functions, adenosine behaves as an upstream

modulator for the fine-tuning and integration of excitatory and inhibitory neurotransmission in the CNS [138, 142], and alterations in the adenosine signalling are associated with several neuropsychiatric and neurological disorders including epilepsy [145], cerebral ischemia [146], pain [147], Parkinson's disease [148], schizophrenia [149], and addiction [150].

Adenosine was demonstrated to profoundly influence eating behavior, and it was proposed as a feedback regulatory signal between the adipose tissue and the hypothalamic feeding centers [151]. Accordingly, intracerebroventricular administration of adenosine was reported to suppress food intake in rats [152] and peripheral injections were able to attenuate food intake induced by opioid receptors agonists, suggesting an interaction between opioids and purines in the control of feeding [153]. Additionally, the A_{2A}AR agonist CGS 21680, i.p. injected in male rats, was revealed to suppress both food-reinforced lever pressing, under a fixed ratio 5 schedule of reinforcement, as well as consumption of laboratory chow, even though at doses that produced signs of sedation and drowsiness [154]. On the other hand, administration of the A_{2A}AR antagonist KW6002 reversed the decreased lever pressing and increased chow intake observed after injection of haloperidol, a D2R antagonist, in a concurrent choice lever-pressing/chow-feeding task [155]. In line with these findings, a subsequent study by Pritchett et al. reported that intra-accumbens administration of another A_{2A}AR antagonist, MSX-3, increased the consumption of a high fat diet, an effect that is fully blocked by prior treatment with the opioid antagonist naltrexone, supporting a role for striatal A_{2A}ARs in mediating the intake of highly palatable and reinforcing foods [156].

It is well known that the consumption of HPF increases dopaminergic transmission in the mesolimbic system, in particular by triggering DA release from the VTA into the Acb

[157-159], and this was proposed as a common neurobiological mechanism shared by both drugs of abuse and palatable foods, which correlates with their reinforcing properties [126, 160-162]. Data from human and animal studies have revealed that alterations in dopaminergic neurotransmission are also critically involved in the pathophysiology of binge eating [30]. Indeed, an enhanced striatal DA release has been reported during food stimulation in individuals with BED [163], and genetic studies showed that an increased frequency of DA transporter and polymorphisms of the D2R might be potential markers in the binge eating pathology [30]. Interestingly, several lines of evidence reported an interaction of adenosine and the mesolimbic DA system, with a critical role in the context of food motivation [164]. Indeed, the A_{2A}AR is highly expressed in striatal areas [165-167], and, more specifically, on the GABAergic striatopallidal medium spiny neurons [168], where it shows an high degree of colocalization with the D2R [165, 169, 170]. It has been demonstrated an antagonistic functional interaction for these two receptors, with A_{2A}AR activation being able to reduce the affinity of DA at D2R, through a direct intramembrane interaction, and also to antagonize the neurochemical and behavioral effects induced by D2R stimulation [171, 172]. Generally, it is conceivable that the A_{2A}AR-D2R heteromer strongly modulates the excitability of the striato-pallidal GABAergic neurons, counteracting D2R signaling, and this led to the potential use of A_{2A}ARs antagonists in Parkinson's disease, while A_{2A}ARs agonists revealed efficacy in the treatment of drug addiction [173, 174]. Given the discussed interaction of adenosine and DA neurotransmission, in our previous study we have highlighted the ability of A_{2A}AR agonists to attenuate voluntary ethanol intake and self-administration, in genetically selected alcohol-preferring rats [175]. Moreover, to determine whether these compounds might influence the compulsive consumption of HPF observed during the

episodes of binge eating, we examined the effects of two A_{2A}AR agonists, CGS 21680, a classical reference compound for A_{2A}AR agonists, and VT 7, an A_{2A}AR agonist with similar affinity for this receptor, but lacking the acidic function found in CGS 21680 [176], in a preclinical model of binge eating, in which the binge eating episode is elicited in female rats, combining an acute stress procedure with cycles of food restriction and refeeding [87]. We found that the A_{2A}AR agonists exerted a suppressive effect on binge eating behavior, probably mediated by the influence on dopaminergic neurotransmission, supporting a potential role for these compounds as pharmacological agents to control bingeing-related eating disorders [177].

Considering the results obtained by our previous studies, the aim of the present work is to investigate, in the same animal model of binge eating, the trascription of A_{2A}AR and D2R gene, and the potential effects of epigenetic mechanisms in their regulation, particularly in brain regions associated with food-seeking behavior and stress, such as the NAc, CPu and the amigdaloid complex.

MATERIAL AND METHODS

Subjects, diet composition and binge eating experimental procedure

Female Sprague–Dawley rats (Charles River, Como, Italy) were used. Their body weight was 225–250 g at the beginning of the experiments. Rats were housed individually in metal cages (30 × 30 × 30 cm) under a 12-h light/dark cycle (light on at 08:00 a.m.), with free access to chow and water for 2 weeks before the experiments. They were kept in a room at a constant temperature (20–22°C) and humidity (45–55%). All the procedures were conducted in adherence with the European Community Council Directive for Care

and Use of Laboratory Animals and with the Italian Legislative Decree 116 of 27 January 1992, and they were approved by the Ethics Committee of the University of Camerino (Protocol n. 7/2012). Rats were offered standard rat food pellets, 4RF18 (2.6 kcal/g). The HPF was offered as a paste, prepared by mixing Nutella (Ferrero, Torino, Italy) chocolate cream (5.33 kcal/g; 56, 31 and 7% from carbohydrate, fat and protein, respectively), ground food pellets (4RF18) and water in the following weight/weight per cent ratio: 52% Nutella, 33% food pellets and 15% water. The HPF diet had a caloric content of 3.63 kcal/g.

Binge-eating experimental procedure

Four groups of female rats were exposed (or not exposed) for 24 days to three 8-day cycles of food restriction (66% of chow intake on days 1–4 and free feeding on days 5–8 of each cycle) during which they were given access to HPF for 2 h during the light cycle between 10:00 a.m. and 12:00 p.m. (2 h after the onset of the light cycle) on days 5–6 and 13–14 of the first two cycles (total of four exposures). On day 25 (binge test day), the rats were exposed (or not exposed) to the *frustration stress* manipulation between 10:00 a.m. and 12:00 p.m. as described in Table 3.

Table 3. The experimental procedure to induce binge-like eating in female rats [178].

Group	Days 1–4	Days 5 and 6	Days 7 and 8	Days 9–12	Days 13 and 14	Days 15 and 16	Days 17–20	Days 21–24	Day 25
NR+NS	*Ad lib* chow	*Ad lib* chow+HPF (2 h)	*Ad lib* chow	*Ad lib* chow	*Ad lib* chow+HPF (2 h)	*Ad lib* chow	*Ad lib* chow	*Ad lib* chow	No stress+*ad lib* chow+HPF (2 h)
NR+S	*Ad lib* chow	*Ad lib* chow+HPF (2 h)	*Ad lib* chow	*Ad lib* chow	*Ad lib* chow+HPF (2 h)	*Ad lib* chow	*Ad lib* chow	*Ad lib* chow	Stress+*ad lib* chow+HPF (2 h)
R+NS	Restricted chow to 66%	*Ad lib* chow+HPF (2 h)	*Ad lib* chow	Restricted chow to 66%	*Ad lib* chow+HPF (2 h)	*Ad lib* chow	Restricted chow to 66%	*Ad lib* chow	No stress+*ad lib* chow+HPF (2 h)
R+S	Restricted chow to 66%	*Ad lib* chow+HPF (2 h)	*Ad lib* chow	Restricted chow to 66%	*Ad lib* chow+HPF (2 h)	*Ad lib* chow	Restricted chow to 66%	*Ad lib* chow	Stress+*ad lib* chow+ HPF (2 h)

During the stress manipulation the rats could see and smell the HPF inside a cup, but they could not access it for 15 min, leading to increased plasma corticosterone levels [179-181]. After 15 min, finally the cup was placed inside the cage, and chow and HPF intake were assessed for 2 h, after *frustration stress* or no stress exposure. We operationally define "binge eating episode" in our model as significantly higher HPF consumption during the 2 h test in the repeated restriction plus *frustration stress* condition than in the other experimental conditions. Immediately after testing, we determined the oestrous cycle phase in a blind manner to the experimental conditions. We found previously that stress-induced binge eating in our model is not observed during the oestrous phase [11, 112]; therefore, we excluded rats that were in this phase from the statistical analysis.

Drugs

The A_{2A}AR agonist VT 7 (5'-N-ethylcarboxamido-2-(2-phenethylthio)adenosine or 2-phenylethylthioNECA; [176]) and the A_{2A}AR antagonist ANR 94 (8-ethoxy-9-ethyladenine) [182] were synthesized by the co-authors of the University of Camerino. VT 7 was dissolved by adding dimethylsulfoxide (DMSO), polyethylene glycol (PEG 400) and water in the ratio (50:150:800) and vortexing vigorously. VT 7 was injected i.p. (2 ml kg^{-1}) at doses of 0.05 and 0.1 mg kg^{-1} selected on previous studies [175, 177] and showed in Table 4 or injected bilaterally into the CeA at doses of 300 or 900 ng/side.

Table 4. Effect of VT 7 (0.05 and 0.1 mg kg^{-1} or vehicle) administered by an i.p. injection on binge eating in female rats. Values are expressed as mean ± SEM of 6 rats. **P < 0.01; *P < 0.05 vs Vehicle; $^{\#\#}$P < 0.01 vs non-restricted and non-stressed Vehicle [183].

	Palatable Food Intake (kcal kg^{-1})		**Chow Intake (kcal kg^{-1})**
Group	**15 min**	**120 min**	**120 min**
Non-restricted and non-stressed			
Vehicle	88.4 ± 6.7	125.5 ± 9.3	1.0 ± 1.0
VT 7 0.05 mg kg^{-1}	92.0 ± 9.0	128.9 ± 2.6	1.6 ± 1.3
VT 7 0.1 mg kg^{-1}	59.7 ± 8.5*	112.0 ± 7.8	0.8 ± 0.6
Non-restricted and stressed			
Vehicle	84.1 ± 5.6	117.8 ± 8.4	0.8 ± 0.8
VT 7 0.05 mg kg^{-1}	82.2 ± 4.6	115.5 ± 12.3	0.9 ± 0.6
VT 7 0.1 mg kg^{-1}	54.4 ± 4.9*	93.6 ± 7.0	1.3 ± 1.0
Restricted and non-stressed			
Vehicle	84.7 ± 5.4	121.7 ± 9.8	0.5 ± 0.5
VT 7 0.05 mg kg^{-1}	73.5 ± 9.8	112.4 ± 10.9	0.7 ± 0.7
VT 7 0,1 mg kg^{-1}	53.0 ± 4.7*	120.6 ± 16.6	1.1 ± 1.1
Restricted and stressed			
Vehicle	142.3 ± 17.2$^{\#\#}$	191.7 ± 11.3$^{\#\#}$	1.1 ± 0.7
VT 7 0,05 mg kg^{-1}	132.4 ± 13.4	184.1 ± 11.3	1.1 ± 1.1
VT 7 0.1 mg kg^{-1}	62.0 ± 8.3**	109.2 ± 8.8**	1.0 ± 0.8

ANR 94 was dissolved by adding DMSO, PEG 400 and water in the ratio 50:350:600 and vortexing vigorously; the clear solution was injected i.p. in a volume of 2 ml kg^{-1} at dose 1 mg kg^{-1}. The dose of the A_{2A}AR antagonist, ANR 94 (1 mg kg^{-1}) was chosen from the experiment reported in the Table 5.

Table 5. Effect of ANR 94 (1, 3 and 5 mg kg^{-1} or Vehicle) administered by i.p. injection on binge eating in female rats. Values are expressed as mean ± SEM of 7-8 rats. *P < 0.05 vs Vehicle [183].

	Palatable Food Intake (kcal kg^{-1})	
Treatment	**15 min**	**120 min**
Vehicle	111.5 ± 5.5	147.3 ± 7.3
ANR 94 1 mg kg^{-1}	118.6 ± 7.2	164.7 ± 8.9
ANR 94 3 mg kg^{-1}	139.3 ± 5.2 *	191.5 ± 18.1
ANR 94 5 mg kg^{-1}	147.0 ± 8.5	197.8 ± 13.0 *

Drug or vehicle was administered by i.p. injection 30 min before access to HPF.

HPF intake was expressed as the mean ± SEM kcal/kg ingested and it was measured at 15 and 120 min of access.

Bilateral cannulas (22 gauge; Unimed) were stereotaxically implanted and cemented to the skull with jeweler's screws and dental cement as previously reported [110]. CeA coordinates, taken according to Paxinos and Watson rat brain atlas (2005) [184] were as follows: CeA, anteroposterior (AP) -2.0 mm from bregma; lateral (L) 4.0 mm from the sagittal suture; ventral (V) 7.0 mm from the skull surface. Intracranial injections were made as previously reported [110]. At the end of the experiments, rats were euthanized, their brains were removed, snap frozen in -40°C isopentane, and stored at -80°C for subsequent verification. Brains were sliced into coronal sections (30 μm) to examine the cannula placements under a microscope. After one week of recovery, twenty-seven rats were subjected to the same binge eating schedule described above for restricted and stressed rats. On day 25, rats were divided into three subgroups (n = 9) that received the following treatment: Vehicle; VT 7 300 ng/side and VT 7 900 ng/side. VT 7 was injected 30 min before the 2 h HPF access (15 min before the beginning of the *frustration stress* for the stressed groups).

Experiment 1: Regulation of A_{2A}AR and D2R gene transcription in a model of binge eating

To evaluate whether an acute stress procedure after cycles of food restriction/refeeding in female rats determines changes in the regulation of A_{2A}AR and D2R gene transcription, we analyzed mRNA levels on day 25 (binge test day). Female rats were divided into four groups in a 2 (history of intermittent food restriction: no, yes) × 2 (stress during testing: no, yes) factorial design. Thirty-six rats (n = 9/group) and 48 rats (n = 12/group) were used respectively for behavioural tests and biology studies. On the binge intake test day, we exposed or did not expose the rats to 15 min of *frustration stress*, and we measured the HPF consumption for 2 h. For the molecular biology experiments, after the stress manipulation, the rats were sacrificed by decapitation, brains were quickly removed, placed in an ice-cold matrix and sliced into coronal sections containing the Acb, CPu and amygdaloid complex. Samplepunches of the Acb, CPu and amygdaloid complex (thus including basolateral complex and central nucleus) were dissected under stereomicroscope in accordance with a rat brain atlas [184], frozen immediately on dry ice and stored at −80°C until analysis.

Real-time qPCR (RT-qPCR)

Total RNA was isolated from the brain regions according to the method of Chomczynski and Sacchi (1987). RT-PCR reactions were performed using the RevertAid H Minus First Strand cDNA Synthesis Kit (Thermo Scientific, Waltham, MA, USA). The relative abundance of each mRNA species was assessed by quantitative real-time RT-PCR (qRTPCR), using SensiFAST SYBR No-ROX Kit (Bioline) on a DNA Engine Opticon 2

Continuous Fluorescence Detection System (MJ Research, Waltham, MA, USA). All data were normalized to the endogenous reference genes β-actin and GAPDH. The primers used for PCR amplification are reported in Table 6. One microlitre of the first strand cDNA product was used for amplification in triplicate in a 20-μL reaction solution, containing 10 μL of SensiFAST SYBR No-ROX Kit and 10 pmol of each primer. The following PCR programme was used: 95°C for 10 min, followed by 50 amplification cycles of 95°C for 10 s, 57°C for 30 s and 72°C for 30 s.

Table 6. List of Primers used for quantitative real-time RT–PCR and DNA methylation [183].

Gene	**Primers (5' – 3')**	**Assay**
β-ACT	Fwd: AGATCAAGATCATTGCTCCTCCT Rev: ACGCAGCTCAGTAACAGTCC	Gene expression
GAPDH	Fwd: AGACAGCCGCATCTTCTTGT Rev: CTTGCCGTGGGTAGAGTCAT	Gene expression
A_{2A}AR	Fwd: TTCGCCTGTTTTGTCCTGGT Rev: AAGCCATTGTACCGGAGTGG	Gene expression
	Fwd (Biotin): ATTAGGGTGGGGGTGGGA Rev: AAACCCCCAACAAAACACCCTT Seq: AAACACCCTTCTCCC	DNA Methylation
D2R	Fwd: TACGTGCCCTTCATCGTCAC Rev: GTGGGTACAGTTGCCCTTGA	Gene expression
	Included in the assay	DNA Methylation

Experiment 2: Effect of frustration stress on Fos expression in CeA and basolateral amygdala (BLA)

In Experiment 2 we determined whether *frustration stress* increases Fos expression in CeA and BLA. We used a new cohort of 36 rats (n = 9 per group) in a 2 (history of

intermittent food restriction: no, yes) × 2 (*frustration stress* during testing: no, yes) factorial design. On day 25, we exposed or did not expose the rats to the 15-min *frustration stress* manipulation and extracted their brains (after anaesthesia and perfusion) 90 min later for subsequent Fos immunohistochemistry assays. The Fos-IR procedure is based on our previous studies [185, 186].

Experiment 3: DNA methylation status and protein levels of A_{2A}AR and D2R in amygdaloid complex

In order to evaluate whether acute stress procedure after cycles of food restriction/refeeding determines changes in the regulation of A_{2A}AR and D2R, we analyzed the percentage of methylation on the gene promoter and the protein levels in the amygdaloid complex from brains of Experiment 1.

Analysis of DNA methylation

Methylation status of the A_{2A}AR and D2R promoter regions was determined using pyrosequencing of bisulfite converted DNA. After DNA extraction, 0.5 μg of DNA from each sample was treated with bisulfite using a DNA methylation kit (Zymo Research, Orange, CA, USA). Bisulfitetreated DNA was amplified by PyroMark PCR Kit (Qiagen) according to the manufacturer's protocol. PCR 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. PCR products were verified by agarose electrophoresis. Pyrosequencing methylation analysis was conducted using the Pyro-Mark Q24 (Qiagen). The level of methylation was analyzed using PyroMark Q24 Software (Qiagen), which calculates the methylation percentage (mC/(mC+C)) for each CpG site, allowing quantitative

comparisons (mC is methylated cytosine, C is unmethylated cytosine). Details of pyrosequencing assays used, including primer sequences and QIAGEN (Hilden, Germany) assay names, are provided in Table 6.

Western blotting

Tissue was homogenized using a lysis buffer [pH 8.0, 50 mM Tris–HCl; 1% triton–X100; 150 mM NaCl; 2 mM EDTA; 100 mM NaF, 10% glycerol; 1 mM MgCl; 1% protease inhibitor cocktail (Sigma Aldrich)] using a blue polypropylene pestle. The homogenates were centrifuged for 15 min at 14000 rpm at 4 °C, and the resulting supernatant fractions were assayed for protein concentration using Bio-Rad Protein assay (Bio-Rad Laboratories, Hercules, CA, USA). The protein samples (20 µg/lane for A_{2A}AR and GAPDH) were boiled for 5 min in sodium dodecyl sulfate (SDS) sample buffer, separated, and transferred to a polyvinylidene difluoride (PVDF) membrane. The membrane was blocked with 5% skim milk for 20 min and 5% BSA for 40 min, and incubated 2 h at room temperature with the primary rabbit polyclonal A_{2A}AR antibody (diluted 1:1000 Thermo Fisher Scientific) and rabbit polyclonal GAPDH antibody (diluted 1:2000, Cell Signaling Technology, Inc. Danvers, MA), in 5% BSA. For D2R, we followed the procedure described by Bagalkot et al. (2015) with modifications. The protein samples (20 µg/lane) were kept at room temperature without boiling for 1 h in sample buffer, separated and transferred to a PVDF membrane. The membrane was blocked with 2% BSA for 1 h and then incubated for 36 h at 4 °C with the primary rabbit polyclonal D2R antibody diluted 1:350 (Elabscience Biotechnology Co., Ltd). The next days, the primary antibody was detected using a secondary horseradish peroxidase

(HRP)-conjugated goat anti-rabbit IgG antibody (diluted 1:10000, Santa Cruz Biotechnology Inc.) (60 min, 25 °C).

Experiment 4: Effect of systemic administration of the A_{2A}AR compounds on binge eating

The purpose of this experiment was to evaluate the effects of systemically administered A_{2A}AR agonist, antagonist or their co-administration on gene transcription regulation as well as on binge eating behaviour. The dose of the A_{2A}AR antagonist, ANR 94 (1 mg kg^{-1}), was chosen from the experiment reported Table 5, and the VT 7 dose (0.1 mg kg^{-1}) was based on our previous data [175, 177] (Table 4). ANR 94 was injected i.p. 15 min before VT 7, which was given 30 min before access to HPF. A new cohort of 36 rats was subjected to the same binge eating schedule described in Experiment 1 for restricted and stressed rats. On day 25, rats were divided into four subgroups (n = 9):

1. Vehicle + Vehicle
2. ANR 94 1 mg kg^{-1} + Vehicle
3. Vehicle + VT 7 0.1 mg kg^{-1}
4. ANR 94 1mg kg^{-1} + VT 7 0.1 mg kg^{-1}

After 1 day off at the end of behavioural test, the same animals received an additional 8-day cycle as reported in Cifani et al. (2009) [87]. The animals were sacrificed by decapitation immediately after the stress procedure. Brains were quickly removed and the amygdaloid complex dissected for real-time qPCR (RT-qPCR) and analysis of DNA methylation as described in Experiments 1 and 3.

Statistical analysis

The results are reported as mean ± SEM. The data were analyzed with factorial analysis of variances (ANOVA) (Systat Software 10.0) using the factors described in Results. We

used Bonferroni's post hoc tests to follow up on significant interaction or main effects ($P < 0.05$) from the factorial ANOVAs. Data reported in Figure 18 were analyzed by one-way ANOVA, followed by post hoc (Bonferroni's) tests when appropriate. In Figure 16 (c) and (d), data are compared by Spearman's rank correlation coefficient.

RESULTS

Experiment 1: Regulation of A_{2A}AR and D2R gene transcription in a model of binge eating

As in our previous studies [110, 133, 178, 187], body weight of rats was reduced during the 4 days of food restriction (in restricted rats), but immediately afterwards rats increased their food intake and rapidly recovered their body weight to levels of controls (non-restricted rats) by the end of each cycle (Fig. 12 a).

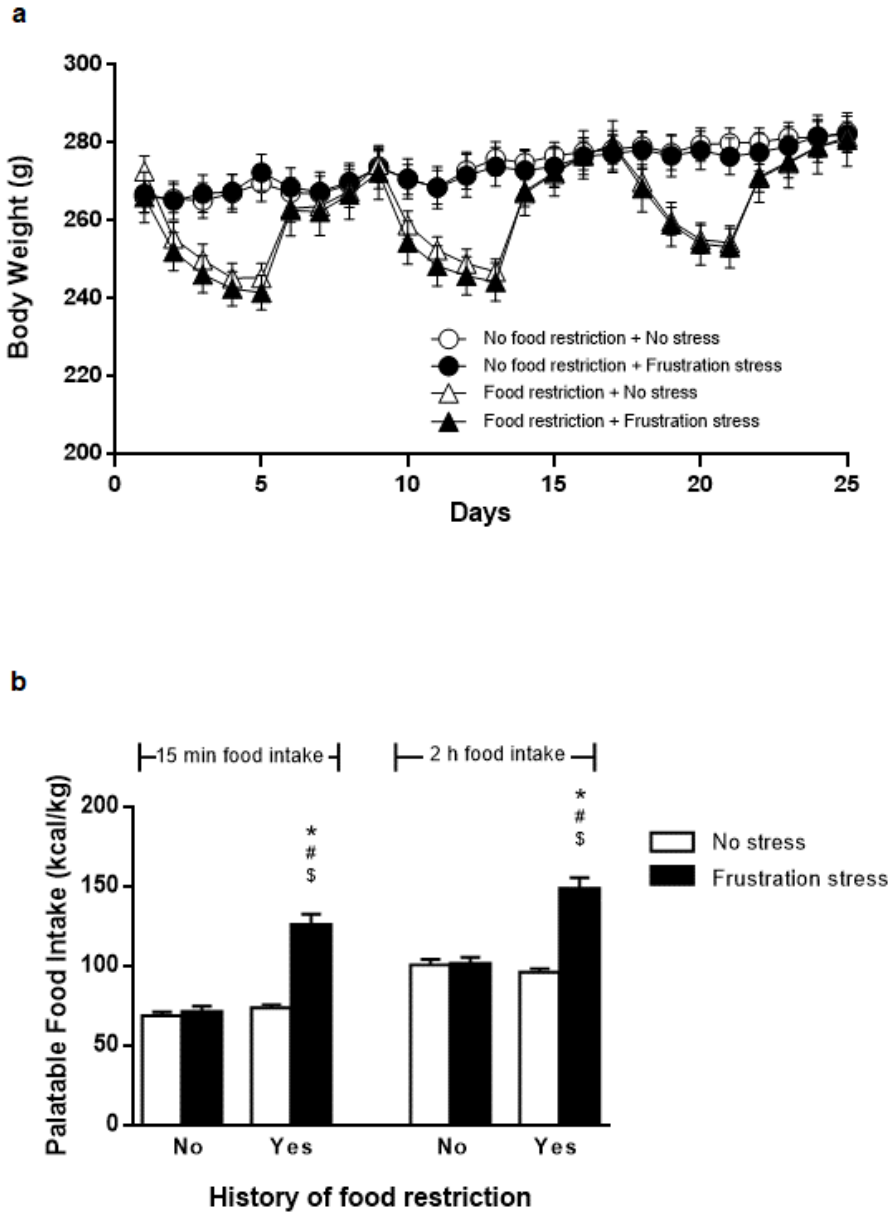

Figure 12. (a) Body weight of female rats during the three 8-day cycles of food restriction/refeeding. Values are means ± SEM. (b) Palatable food intake (kcal kg^{-1}) on the test day in female rats exposed to either cycles of food restriction or stress or a combination of both. Data are means ± SEM of nine rats. Two-way ANOVA and Bonferroni's test: *P < 0.05 versus no food restriction and no stress. #P < 0.05 vs no food restriction and *frustration stress*, $P < 0.05 vs food restriction and no stress [183].

At 15 min, two-way ANOVA showed a significant interaction between the two factors (food restriction and stress) [$F_{(1,34)} = 44.8$, $P < 0.05$]. As shown in Figure 12 (b), post hoc comparisons indicated that HPF intake of the restricted and stressed group was markedly higher than in the other groups ($P < 0.05$). ANOVA of the 2 h cumulative HPF showed a two-way interaction (food restriction, stress) [$F_{(1,34)} = 35.8$, $P < 0.05$]. Post hoc tests are shown in Figure 12 (b).

To evaluate whether acute stress after cycles of food restriction/ refeeding in female rats determined changes in the regulation of gene transcription, $A_{2A}AR$ and D2R mRNA levels were analyzed in the amygdaloid complex, CPu and Acb (Fig. 13).

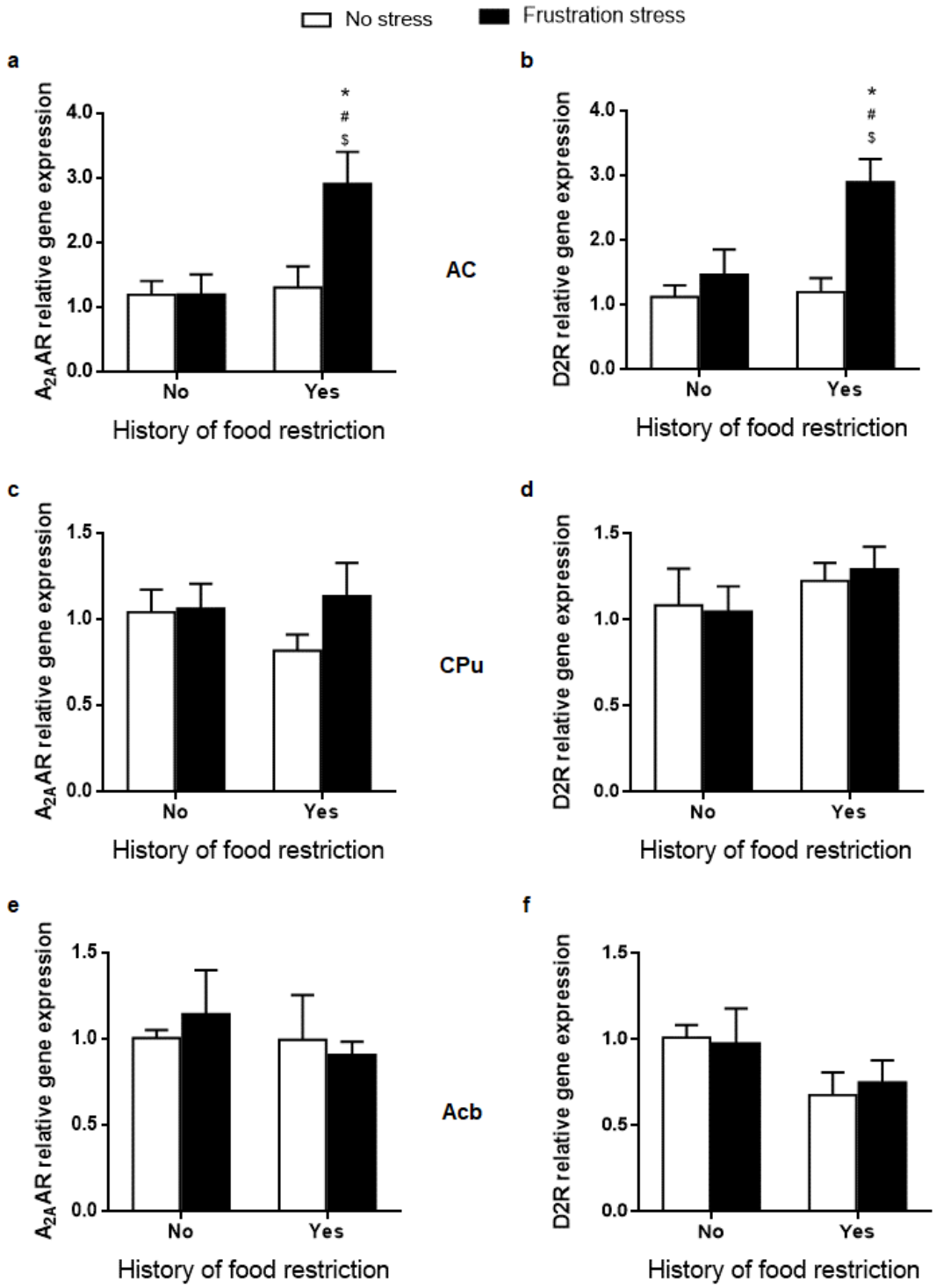

Figure 13. RT-qPCR analysis of A_{2A}AR and D2R gene expression. Level of A_{2A}AR (left panels) and D2R (right panels) mRNA in (a and b) amygdaloid complex (AC), (c and d) caudate putamen (CPu) and (e and f) nucleus accumbens (Acb). Two-way ANOVA and Bonferroni's test: *P < 0.05 versus no food restriction

and no stress. #P < 0.05 vs no food restriction and *frustration stress*, $P < 0.05 vs food restriction and no stress [183].

Significant changes in gene expression were detected for A_{2A}AR and D2R in the amygdaloid complex (respectively Fig. 13 (a) and (b)). Statistical analysis by two-way ANOVA showed that alterations in A_{2A}AR mRNA levels were affected by history of food restriction ($F_{(1,33)} = 5.63$; $P < 0.05$) and *frustration stress* ($F_{(1,33)} = 4.37$; $P < 0.05$) with a significant interaction between these two factors ($F_{(1,33)} = 4.33$; $P < 0.05$). In regard to the D2R expression levels (Fig. 13 (b)), the two-way ANOVA revealed a significant effect of food restriction ($F_{(1,33)} = 5.74$; $P < 0.05$), *frustration stress* ($F_{(1,33)} = 10.50$; $P < 0.05$) and interaction between these two factors ($F_{(1,33)} = 4.62$; $P < 0.05$). Post hoc group differences are indicated in Figure 13 (a) and (b). No changes in the A_{2A}AR e D2R gene expression were detected in other analyzed brain regions (Fig. 13 (c) to (f)).

Experiment 2: Effect of frustration stress on Fos expression in CeA and basolateral amygdala (BLA)

We found increased Fos immunoreactivity in the CeA of rats with a history of intermittent food restriction and *frustration stress* (Fig. 14 b). The statistical analysis showed a significant interaction between food restriction history and stress during testing ($F_{(1,22)} = 22.7$; $P < 0.05$). Post hoc group differences are indicated in Figure14 b. The statistical analysis of Fos immunoreactivity in BLA did not show a significant interaction between the two factors (food restriction and stress) ($F_{(1,22)} = 2.6$; $P > 0.05$) (Fig. 14 c).

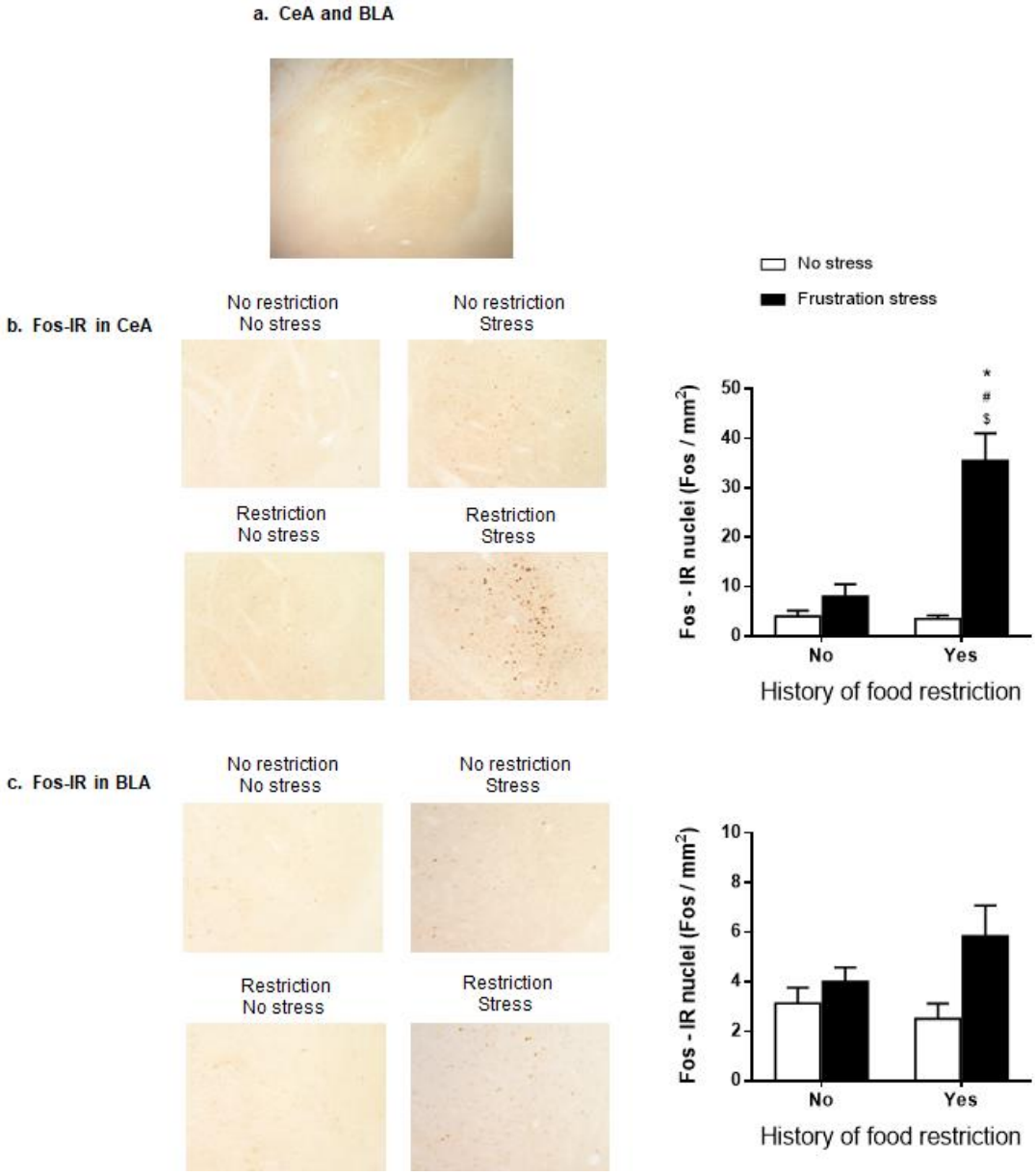

Figure 14. *Frustration stress* during testing increased neuronal activity in CeA in rats with a history of intermittent food restriction. (a), Representative photomicrograph of CeA (5X); black squares indicate the area of Fos quantification for CeA and BLA. (b) Mean ± SEM number of Fos-IR nuclei per square millimeter in CeA (left) and representative photomicrographs of Fos-IR nuclei in CeA (right; 10X) 90 min after *frustration stress* or no stress exposure. (c) Mean ± SEM number of Fos-IR nuclei per square millimeter in BLA (left) and representative photomicrographs of Fos-IR nuclei in BLA (right; 10X) 90 min after *frustration stress* or no stress exposure. Two-way ANOVA and Bonferroni's test: *P < 0.05 versus no

food restriction and no stress. #P < 0.05 vs No food restriction (No) and *frustration stress* (Yes), $P < 0.05 vs Food restriction (Yes) and no stress (No). Scale bar, 100 μm [183].

Experiment 3: DNA methylation status and protein levels of A2AAR and D2R in amygdaloid complex

In order to evaluate a relationship between gene expression and epigenetic regulation, we analyzed the DNA methylation of six CpGs site in the A_{2A}AR and D2R promoter region (Fig. 15) in the amygdaloid complex.

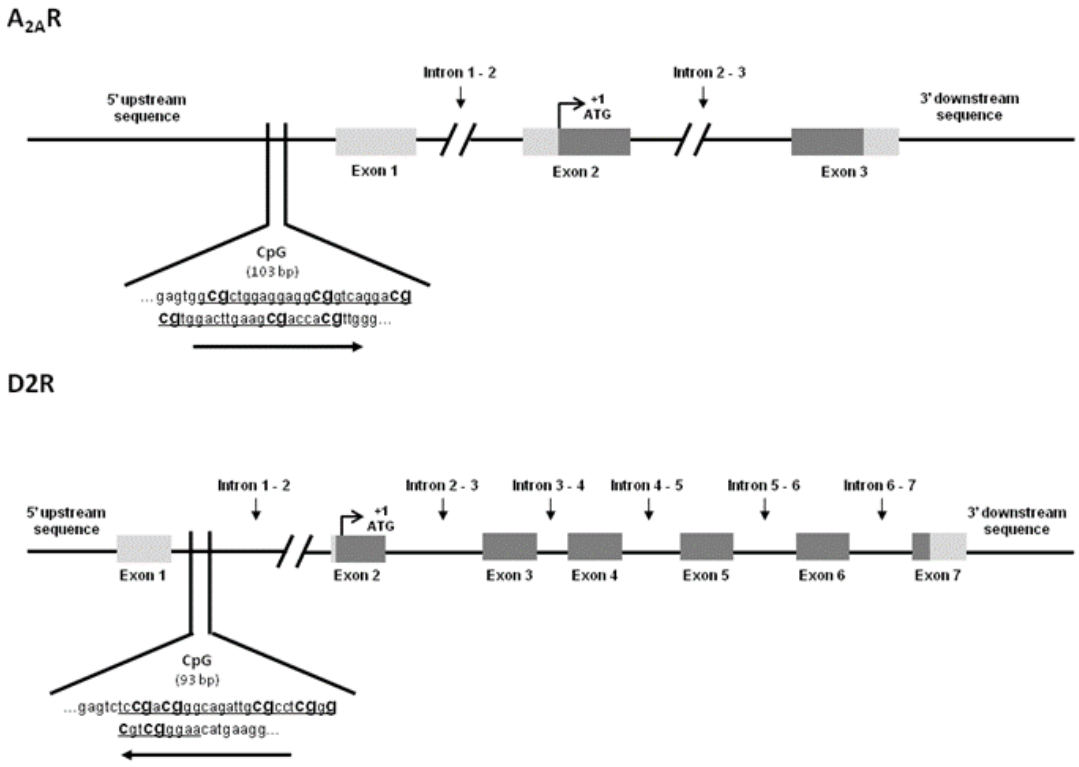

Figure 15. Schematic representation of A_{2A}AR and D2R gene. Position of transcription start site, translation start code (ATG), exons and introns are depicted. (A) CpG islands in the 5'upstream sequence of A_{2A}AR. (B) CpG islands in the Intron 1-2 sequence of D2R [183].

Methylation of the combined CpG sites examined is shown in Figure 16 a.

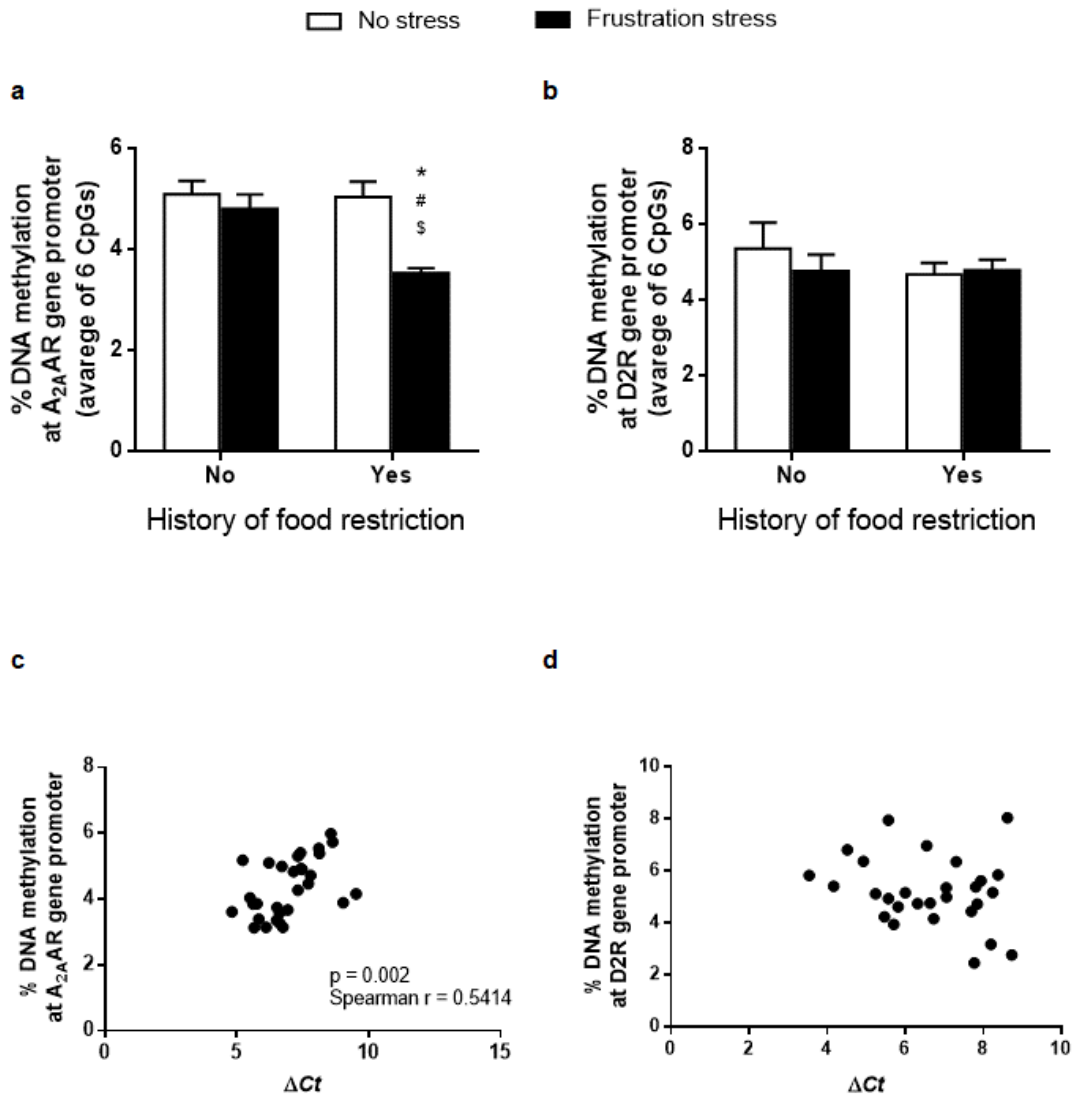

Figure 16. Amount of methylated DNA in the promoter region of $A_{2A}AR$ (a) and D2R (b) in the amygdaloid complex. Values are expressed as mean ± SEM of 7–11 rats. Two-way ANOVA and Bonferroni's test: *P < 0.05 versus no food restriction and no stress. $^{\#}P < 0.05$ vs no food restriction and *frustration stress*, $^{\$}P < 0.05$ vs food restriction and no stress. Correlation between gene expression and percentage change in DNA methylation at gene promoters. (c and d) Data for gene expression are expressed as Delta Ct (ΔCt) values calculated as the difference in the number of cycles required for the PCR reaction of the target gene and of the reference genes to enter the logarithmic phase. Low values of DCt correspond to high gene expression. Data are compared by Spearman's rank correlation coefficient. (c) $A_{2A}AR$ and (d) D2R [183].

As shown in Figure 16 (a), the two-way ANOVA revealed that the difference in DNA methylation in $A_{2A}AR$ promoters was significantly affected by food restriction ($F_{(1, 24)}$ = 7.43; $P < 0.05$) and by *frustration stress* ($F_{(1, 32)}$ = 13.50; $P < 0.05$) with a significant interaction between these two factors ($F_{(1, 24)}$ = 6.24; $P < 0.05$). Post hoc comparisons indicated there was a significant decrease of per cent DNA methylation at the $A_{2A}AR$ promoter region in rats with a history of food restriction and exposed to stress when compared to the other groups Figure 16 (a)). No significant change in per cent of DNA methylation at the D2R promoter region was observed (Fig. 16 (b)).

Methylation status of each single site was reported in Tables 7 and 8.

Table 7. DNA methylation at D2R gene promoter [183].

D2R		**History of food restriction**											
		No						**Yes**					
Stress	**No**	3.44 ± 0.37	4.95 ± 0.88	4.55 ± 0.57	8.55 ± 1.28	6.40 ± 0.76	4.21 ± 0.34	3.30 ± 0.21	4.20 ± 0.65	3.72 ± 0.38	7.35 ± 0.66	5.80 ± 0.34	3.63 ± 0.29
	Yes	2.93 ± 0.26	4.15 ± 0.67	3.60 ± 0.61	7.87 ± 0.96	5.27 ± 0.69	4.77 ± 0.84	2.98 ± 0.16	3.82 ± 0.44	3.98 ± 0.30	7.78 ± 0.54	5.97 ± 0.40	4.13 ± 0.34
CpG sites		**1**	**2**	**3**	**4**	**5**	**6**	**1**	**2**	**3**	**4**	**5**	**6**

Table 8. DNA methylation at $A_{2A}AR$ gene promoter [183].

$A_{2A}AR$		**History of food restriction**											
		No						**Yes**					
Stress	**No**	7.00 ± 0.41	8.82 ± 0.57	8.00 ± 0.50	2.00 ± 0.22	2.38 ± 0.21	2.39 ± 0.14	6.68 ± 0.59	7.74 ± 0.61	6.97 ± 0.51	1.95 ± 0.19	2.67 ± 0.55	2.65 ± 0.34
	Yes	6.57 ± 0.45	8.37 ± 0.53	7.77 ± 0.55	1.93 ± 0.22	2.20 ± 0.24	2.49 ± 0.19	5.36 ± 0.55	5.74 ± 0.19 **	5.14 ± 0.34 **	1.73 ± 0.12	2.30 ± 0.17	2.35 ± 0.20
CpG sites		**1**	**2**	**3**	**4**	**5**	**6**	**1**	**2**	**3**	**4**	**5**	**6**

Correlation analysis between gene expression and percentage change in DNA methylation at gene promoters were reported in Figure 16 (c) and (d). Data, compared by Spearman's rank correlation coefficient, show a significant correlation in $A_{2A}AR$ ($p < 0.05$, Spearman's $r = 0.5414$, Fig. 16 (c)). Finally, analysis of $A_{2A}AR$ and D2R protein levels in the amygdaloid complex were reported in Figure 17. Two-way ANOVA showed that the $A_{2A}AR$ protein levels were significantly affected by food restriction ($F_{(1, 20)} = 7.14$; $P < 0.05$) and by *frustration stress* ($F_{(1, 20)} = 7.02$; $P < 0.05$), and there was a significant interaction between these two factors ($F_{(1, 20)} = 4.55$; $P < 0.05$). Post hoc group differences are indicated in Figure 17. In regard to D2R, no change in protein levels was observed (Fig. 17).

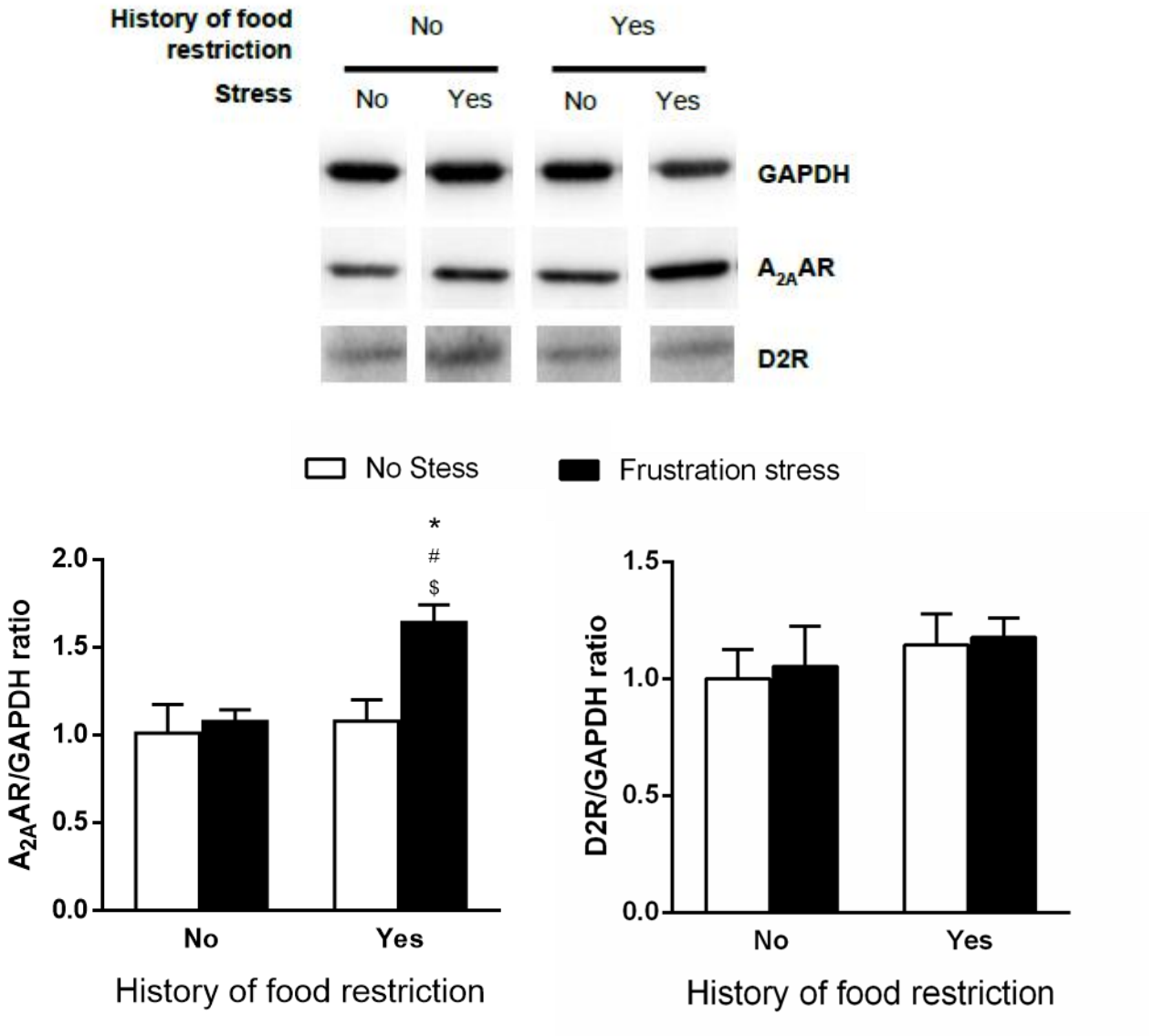

Figure 17. Analysis of $A_{2A}AR$ (48 kDa) and D2R (50 kDa) protein levels in Amygdala complex. Values represent mean ± SEM. The optical density was normalized to GAPDH (37 kDa). Two-way ANOVA and Bonferroni's test: *P < 0.05 versus no food restriction and no stress. #P < 0.05 vs No food restriction and *frustration stress*, $P < 0.05 vs Food restriction and no stress [183].

Experiment 4: Effect of systemic administration of the A_{2A}AR compounds on binge eating

The effect of the A_{2A}AR agonist VT 7 alone or in combination with ANR 94 on the restricted and stressed group is shown in Figure 18 (a).

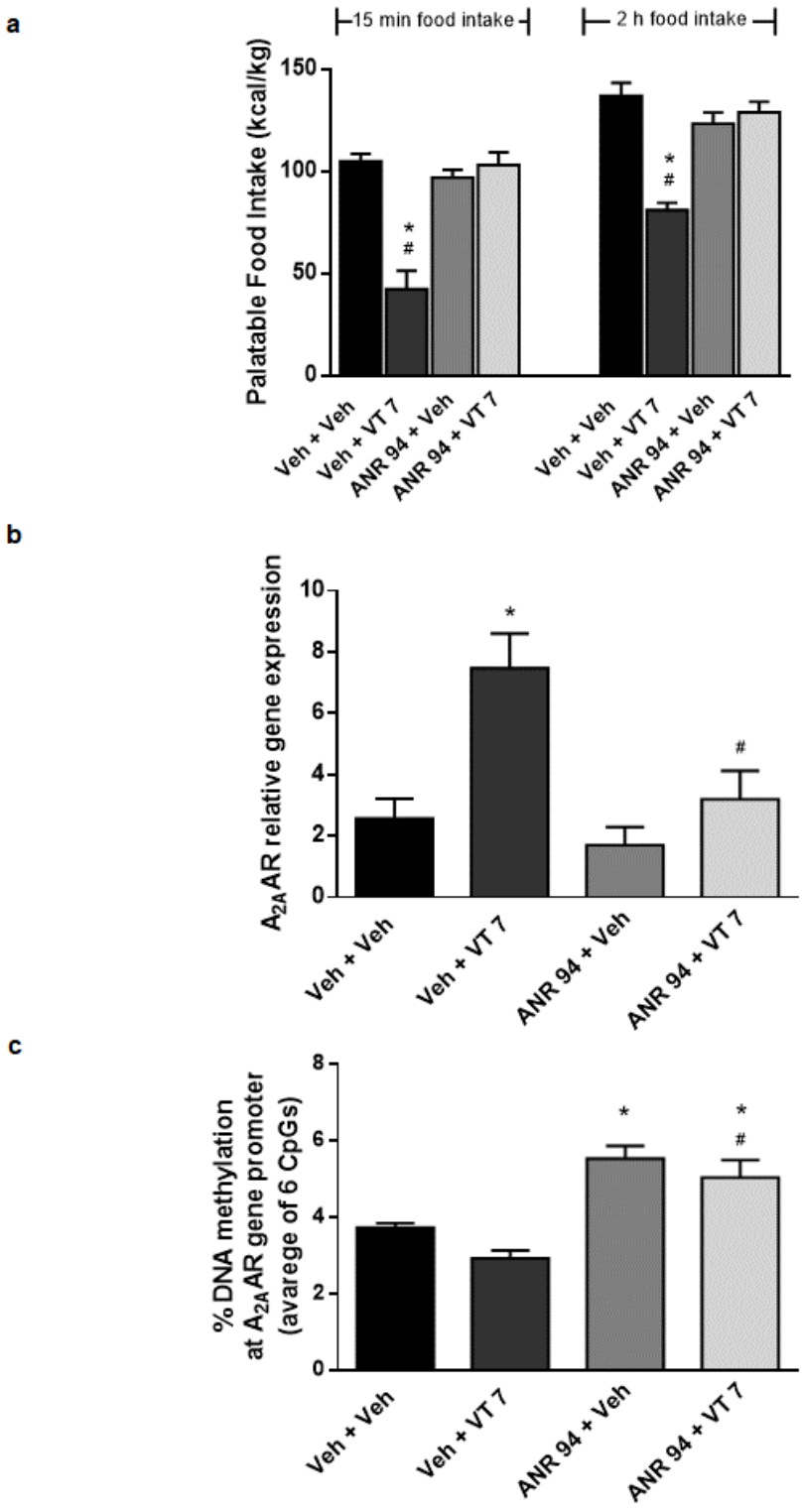

Figure 18. Effects of systemic administration of VT 7 (0.1 mg kg^{-1}) after pre-treatment with ANR 94 (1 mg kg^{-1}) or its vehicle on binge eating. (a) Food intake, (b) gene expression and (c) DNA methylation. Two-way ANOVA and Bonferroni's test: *P < 0.05 versus Veh + Veh; $^{\#}$P < 0.05 vs ANR 94 + VT 7; $^{\$}$P < 0.05 vs Veh + VT 7 [183].

At the 15 min time point, ANOVA showed that the results were significantly affected by treatment ($F_{(3,27)} = 24.1$; $P < 0.05$). Post hoc comparisons showed that VT 7 at the dose of 0.1 mg kg^{-1} significantly reduced HPF intake when compared to the control group (Veh + Veh) ($P < 0.05$). This effect was completely abolished by pre-treatment with ANR 94 at a dose of 1 mg kg^{-1}, which was inactive per sé ($P < 0.05$ vs ANR 94 + VT 7) (Fig. 18 (a)). ANOVA of the 2 h cumulative HPF showed a significant effect of the treatment ($F_{(3,27)} = 20.3$, $P < 0.05$). Post hoc tests are shown in Figure 18 (a). The administration of the A_{2A}AR agonist and antagonist on restricted and stressed rats induced changes in the A_{2A}AR gene expression ($F_{(3,24)} = 10.4$; $P < 0.05$). The agonist VT 7 induced a significant increase of mRNA levels when compared to the control group, whereas no significant change in rats treated with the A_{2A}AR antagonist (ANR 94 + Veh) was observed. The effect of VT 7 was reduced by pre-treatment with ANR 94 at the dose of 1 mg kg^{-1} (Fig. 18 (b)). Pyrosequencing analysis revealed a relationship between gene expression and epigenetic regulation ($F_{(3,26)} = 21.55$; $P < 0.05$) (Fig. 18 (c)). Post hoc comparisons indicated a significant increase of per cent DNA methylation at the A_{2A}AR promoter region in restricted and stressed rats after administration of ANR 94 alone or in combination with VT 7. Post hoc tests are shown in Figure 18 (c). The methylation status of each single site was reported in Table 9.

Table 9. Effect of $A_{2A}AR$ compounds on DNA methylation level [183].

$A_{2A}AR$	History of food restriction						Treatment
Stress	5.36 ± 0.44	5.97 ± 0.28	5.53 ± 0.36	1.76 ± 0.11	2.32 ± 0.14	2.45 ± 0.17	**Veh + Veh**
	8.17 ± 0.66	9.75 ± 0.65 ***	9.22 ± 0.60 ***	2.06 ± 0.19	1.49 ± 0.34	2.41 ± 0.41	**ANR 94 + Veh**
	4.85 ± 0.59	5.43 ± 0.13	4.93 ± 0.43	1.55 ± 0.16	1.88 ± 0.20	2.01 ± 0.21	**Veh + VT 7**
	6.40 ± 0.90	8.58 ± 0.80	8.23 ± 0.81	2.60 ± 0.18 ##	1.96 ± 0.07	2.34 ± 0.22	**ANR 94 + VT 7**
CpG sites	**1**	**2**	**3**	**4**	**5**	**6**	

***P < 0.001 vs Veh + Veh; ## P < 0.01 vs Veh + VT 7

DISCUSSION

The combination of dieting and stress plays an important role in the development of binge eating in our preclinical model [87], in agreement with clinical data showing that binge eating episodes may be caused and maintained by the interaction between dieting and stress [188]. Indeed, as we have already reported, binge eating is elicited in female rats by yo–yo dieting and stressful exposure to HPF [36, 87]. We have previously observed that $A_{2A}AR$ agonists exert a rather pronounced inhibition of HPF intake [177]. In the present work, we show that the $A_{2A}AR$ selective antagonist ANR 94 completely reverts at a dose of 1 mg kg^{-1} (inactive per sé) the effect of the $A_{2A}AR$ agonist VT 7, confirming its effects are mediated by $A_{2A}ARs$. Moreover, ANR 94 injected at higher doses, 3 and 5 mg kg^{-1}, significantly increased HPF consumption in the restricted and stressed group (Table 5). To better evaluate the role of the adenosine system in the development of these compulsive-like eating behaviours, gene expression changes in $A_{2A}AR$ as well as in D2R in our binge eating model and also in response to adenosine compounds were analyzed.

The food restriction per sé did not induce any changes in target gene expression, in keeping with a previous study reporting unchanged levels of A_{2A}AR mRNA in the Acb of rats subjected to a restricted feeding protocol for 10 days [189]. However, we observed a consistent significant increase of A_{2A}AR gene expression selectively in the amygdaloid complex of rats restricted and stressed, whereas no changes have been observed in the Acb and CPu. A previous study suggested that excessive consumption of palatable energy-dense food induces a profound state of reward hyposensitivity and the development of compulsive-like eating arising from diet-induced deficits in D2R signalling [54]. In this frame, our data appear of relevance showing up-regulation of D2R mRNA following A_{2A}AR activation and subsequent reduction of HPF intake. The involvement of the amygdaloid complex is also supported by a selective increase in Fos immunoreactivity expression (a neuronal activity marker) in CeA only in binge-like-eating rats. Considering these results, the A_{2A}AR agonist VT 7 was injected into the CeA and completely blocked binge eating in restricted and stressed rats (Table 10), suggesting a crucial role for this brain area in the effect of the adenosine compound.

Table 10. Effect of VT 7 (300 and 900 ng/rat or Vehicle) administered by CeA injection on binge eating in female rats. Values are expressed as mean ± SEM of 5-7 rats. * $P < 0.05$ vs Vehicle [183].

	Palatable Food Intake (kcal kg^{-1})		**Chow Intake (kcal kg^{-1})**
Treatment	**15 min**	**120 min**	**120 min**
Vehicle	121.8 ± 8.7	162.0 ± 8.1	1.4 ± 0.5
VT 7 300 ng/side	114.7 ± 9.8	154.7 ± 6.4	1.1 ± 0.6
VT 7 900 ng/side	86.8 ± 2.4 *	125.9 ± 11.1	0.9 ± 0.5

It is known that this brain structure plays a key role in emotional reactivity, food-related behaviour and excessive eating of HPF [131, 190], whereas little has been explored on the involvement of A_{2A}Rs in the amygdala. Recently Cunha and co-workers demonstrated the importance of A_{2A}ARs to control fear memory in this brain area [191], and in other studies A_{2A} knockout mice showed an enhanced c-Fos immunoreactivity in the amygdaloid complex compared to wild-type mice [192]. Moreover, Rau et al. showed that postsynaptic adenosine A_{2A}Rs modulated intrinsic excitability of pyramidal cells in the rat basolateral amygdala [193]. It has been proposed that stress associated with caloric restriction could reprogramme orexigenic pathways [194, 195]. and alter the reward circuitry in the brain by affecting epigenetic mechanisms [194, 196]. We thus explored the behavioural impact of epigenetic modifications on the regulation of target gene expression. The human A_{2A}AR gene is localized to chromosome 22 [197] and consists of a non-coding exon (exon 1) located at 5' upstream exon 2 [105], and two coding exons (exon 2 and 3) separated by a single intron of nearly 7 kb, encoding alternative transcripts, whose expression is driven by at least four independent promoters. The regulation of these promoters is now under intense investigation, and it is becoming increasingly clear that A_{2A}AR gene expression is highly responsive to alterations in the extracellular environment [198].

Comparison of the rat and human A_{2A}AR genomic sequences (accession numbers AF107208 and ap000355.gb_pr5, respectively) reveals 65% identity in the 4.3-kb 5′-flanking region, supporting the interspecies importance of this 5′-flanking region in the regulation of A_{2A}AR expression [199]. Moreover, differential expression of A_{2A}AR isoforms has been reported indicating that 5'UTR plays an important regulatory role in A_{2A}AR expression [200]. It should also be recalled that rat and human 5′-UTRs also share

a high degree of sequence homology as well as complementarity (60% overall) with the 28S ribosomal RNA, and previous studies have indicated 5′-UTR regions that are complementary to ribosomal RNA can affect mRNA translation [201].

Thus, we decided to further examine A_{2A}AR epigenetic regulation as well as its levels in the rat amygdaloid complex. Our data clearly show a reduction in DNA methylation at the gene promoter site and an increase in the protein levels and gene expression in binge-like-eating rats. Other reports showed that DNA methylation plays a role in the endogenous expression of A_{2A}AR [202]. Here, we analyzed six CpG sites in the human 5′ UTR A_{2A}AR surrounding exon 1 which is included in the largest CpGI, already described [105]. However, as there is only a 30-bp gap between CpG sites, these could be seen as a unique site. Alterations of DNA methylation at the 5′ UTR of A_{2A}AR has already been observed in other diseases such as schizophrenia [203], Huntington's disease [204], and cardiomyopathies [205]. We here demonstrated for the first time the epigenetic regulation of A_{2A}AR in an animal model of binge eating. Even if the observed group differences as to mean DNA methylation levels are small (approximately 1–3%), it is plausible that these differences could have significant effects over gene transcription as already proposed by others [206, 207]. Unexpectedly, we did not find any difference in D2R DNA methylation among different groups. However, it cannot be ruled out that other epigenetic mechanisms (i.e., chromatin modification and miRNA) could be responsible for the observed alterations in gene expression. Moreover, in another eating disorder, anorexia nervosa, an association with D2R methylation has been documented [208]. Moreover, we assessed the role of the selective A_{2A}AR agonist VT 7 [176] and antagonist 8-ethoxy-9-ethyladenine (ANR 94) [182] on binge eating, and we observed that receptor up-regulation is even more pronounced after the treatment with the A_{2A}AR agonist VT 7,

which, as we confirmed here, reduces HPF intake. The increases in gene expression observed in rats restricted and stressed treated with the A_{2A}AR agonist, could be due to a compensatory mechanism developed by the system to counteract the episode of binge eating. A_{2A}AR antagonist ANR 94 administration completely reversed the alterations in receptor gene expression, confirming that the activation of this system is relevant for the effects on HPF intake. We also observed a reduction in DNA methylation at the A_{2A}AR gene promoter in the restricted and stressed group even if not significant following A_{2A}AR agonist VT 7 treatment in the same group. Again, the treatment with the A_{2A}AR antagonist ANR 94 induced an increase in DNA methylation at the gene promoter, which returned to control levels. Overall, our findings suggest that stress associated with food restriction promotes alterations in critical genes for feeding and reward circuitry that influence food intake and stress-related behaviours. These changes seem to be partially driven by epigenetic mechanisms promoting increased sensitivity of the A_{2A} pathway that alters reward circuitry. It is possible that the brain develops strategies (namely, A_{2A}AR DNA methylation) to guard against the likelihood that stress and restriction experiences would promote subsequent binge eating behaviours. Further studies will be necessary to confirm the potential use of VT 7 as a pharmacological agent to reduce binge eating episodes modulating A_{2A}AR gene expression.

CONCLUSION

BED is still unrecognized and underestimated, despite the high prevalence found globally. The only drug, for now, approved for the treatment of this eating disorder is LDX, which presents several mild and severe side effects. A large body of evidence suggests that the neurobiological mechanisms of BED converge, among other, on the activation of the mesocorticolimbic DA system, and on 5-HT and NA signaling.

In this work, alternatives and new perspectives have been proposed on possible treatments of BED that can be increasingly studied and evaluated for future clinical uses.

OEA and A_{2A}AR have been shown to significantly reduce the amount of HPF consumed during the binge eating episode in a preclinical model of binge eating. In addition to the behavioral aspect, genetic aspects such as receptors expressions and mRNA levels of several neurochemical endpoints have been evaluated, confirming initial hypotheses of contribution of OEA and adenosine to this altered dietary behavior and the possibility to restore and normalize neuronal alterations highlighted in the analyses carried out.

Specifically, OEA treatment might be able to normalize dopaminergic response, oxytocin receptor density and stimulating oxytocin release from the PVN, increasing oxytocin transmission in bingeing rats, and reducing CRF synthesis in the CeA. Moreover, OEA enhanced NA and 5-HT levels tissue in most of the brain areas analyzed, supporting an antidepressant-like effects of OEA.

Furthermore, a significant increase of A_{2A}AR mRNA levels was found in restricted and stressed rats with a reduction of the percentage of DNA methylation at the A_{2A}AR promoter region, possibly due to a compensatory mechanism to counteract the effect of binge eating. However, further studies should investigate the causative link between these

observations, and broaden the current knowledge of the role played by OEA and adenosine systems.

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