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Author: Silvia Alboni Maria Vittoria Micioni di Bonaventura Cristina Benatti Maria Elena Giusepponi Nicoletta Brunello Carlo Cifani

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Hypothalamic expression of inflammatory mediators in an animal model of binge eating

Silvia Alboni[a], Maria Vittoria Micioni di Bonaventura[b], Cristina Benatti[a], Maria Elena Giusepponi[b], Nicoletta Brunello[a], Carlo Cifani[b,c]

[a] Department of Life Sciences, University of Modena and Reggio Emilia, 41125 Modena, Italy

[b] School of Pharmacy, Pharmacology Unit, University of Camerino, 62032 Camerino, Italy

[c]Intramural Research Program, National Institutes of Health/National Institute on Drug Abuse, Baltimore, Maryland 21224

Corresponding author:

Dr Silvia Alboni, PhD. University of Modena and Reggio Emilia, Department of Life Sciences, Via Campi, 287, 41125, Modena, Italy. e-mail address: silvia.alboni@unimore.it

Highlights

- Food-restricted rats stressed during non-estrus develop binge eating (BE) behavior
- BE is associated to changes in mRNA levels of inflammatory markers in hypothalamus
- iNOS mRNA increases in the anterior-tuberal hypothalamus of BE rats
- IL-18/IL-18R system is down-regulated in hypothalamus of BE rats

Abstract

Binge eating episodes are characterized by uncontrollable, distressing eating of a large amount of highly palatable food and represent a central feature of bingeing related eating disorders. Research suggests that inflammation plays a role in the onset and maintenance of eating-related maladaptive behavior. Markers of inflammation can be selectively altered in discrete brain region where they can directly or indirectly regulate food intake. In the present study we measured expression levels of different components of cytokine systems (IL-1, IL-6, IL-18, TNF-α and IFN-ɣ) and related molecules (iNOS and COX2) in the preoptic and anterior-tuberal parts of the hypothalamus of a validated animal model of binge eating. In this animal model, based on the exposure to both food restriction and frustration stress, binge-like eating behavior for highly palatable food is not shown when animals are exposed to the frustration stress during the estrus phase. We found a characteristic down-regulation of the IL-18/ IL-18 receptor system (with increased expression of the inhibitor of the pro-inflammatory cytokine IL18, IL-18BP, together with a decreased expression of the binding chain of the IL-18 receptor) and a three-fold increase in the expression of iNOS specifically in the anterior- tuberal region of the hypothalamus of animals that develop a binge-like eating behavior. Differently, when food restricted animals were stressed during the estrus phase IL-18 expression increased while iNOS expression was not significantly affected. Considering the role of this region of the hypothalamus in controlling feeding related behavior, this can be relevant in eating disorders and obesity. Our data suggest that by targeting centrally selected inflammatory markers, we may prevent that disordered eating turns into a full blown eating disorder.

Keywords: binge eating, stress, food restriction, cytokine systems, gene expression, hypothalamus.

1. Introduction

Eating disorders are characterized by a persistent disturbance of eating and eating-related behavior that results in the altered consumption of food and significantly impairs physical health and/or psychosocial functioning [1]. Binge eating is a proto-typical eating-related maladaptive behavior that represents a central feature of bulimia nervosa, binge-eating disorder, and binge-purge anorexia nervosa [1] and it contributes to aggravate obesity and its associated pathologies [2, 3, 4].

A large body of evidence suggests that dieting, stress, and negative affective states represent triggers of binge eating in patients suffering from binge-eating disorder or bulimia nervosa [2]. Several studies have examined the linkage between stress response, inflammation and eating disorders [5, 6]. Pro-inflammatory cytokines (soluble factors which promote inflammation) can regulate hypothalamic–pituitary–adrenal (HPA) axis functionality and glucocorticoids secretion [7] while conversely stress can evoke the activation of pro-inflammatory cytokines such as interleukin (IL)-1, IL-6, IL-18, and tumor necrosis factor (TNF)-α causing general fatigue, sleep disturbance, and also appetite loss [8, 9, 10].

Indeed, cytokines can affect appetite and food intake both in a direct and an indirect way in animals as well as in humans [11]. Different cytokines [IL-1, IL-6, IL-18, TNF-α, and interferon (IFN)-γ] suppress food intake, by direct action in the central nervous system [12]. IL-1, IFN-γ, and TNF-α affect those hypothalamic neurons implicated in the regulation of eating behavior [13]. Moreover IL-1 and TNF-α alter the firing rate of glucose-sensitive neurons in the lateral hypothalamus possibly affecting peripheral signals to the feeding centers [14].

Corcos and colleagues [7] also described the indirect actions of cytokines, such as IL-1β, that increase plasma levels of catecholamines thereby yielding food intake suppression [15]. It has been suggested that an elevation of cytokines promotes the cascade of biochemical events

culminating in a dysregulation of neurohormones, neuropeptides, and neurotransmitters which contributes to the development and persistence in either anorexia or bulimia nervosa [13].

However, while the role of cytokine systems in contributing to the development or perpetuation of eating disorders has been in some way explored [7], the involvement of these systems in the binge-eating behavior remains largely unknown. In particular no data are available so far on the regulation of cytokine systems in brain regions belonging to feeding circuits when binge-eating behavior is induced.

Here we measured expression levels of immune targets (including pro-inflammatory cytokines and related molecules) in the hypothalamus of an animal model of binge eating and relative controls. In this animal model, binge eating for a familiar highly palatable food is evoked in female rats by exposure to cyclic food restrictions combined to frustration stress [16, 17] according to the hypothesis that dieting and stress are key etiological determinants of binge eating.

Although several brain areas (such as bed nucleus of the stria terminalis- BNST-,lateral habenula, or ventral tegmental area) are involved in regulating feeding, appetite, motivational behavior, and are sensitive to changes in cytokine systems [18, 19], here we have focused our attention on the hypothalamus. This area represents indeed the neuro-endocrine interface in the brain and it is a key station for central circuits to orchestrate the maintenance of body homeostasis or allostasis also because of its high responsivity to immune signals. Among the basic life functions controlled by the hypothalamus there are feeding, energy metabolism, and stress response [20]. The hypothalamus includes a number of nuclei that can be grouped along the antero-posterior axis in the preoptic, anterior, tuberal, and posterior (mammillary) regions. While the preoptic area contains nuclei participating in fluid homeostasis and electrolyte metabolism, reproduction and maternal behavior, and in the regulation of sleep and wakefulness, the anterior and tuberal regions contain centers controlling feeding, appetite and ingestive behavior, and stress responses (autonomic and endocrine responses) [21, 22].

First, we evaluated changes in the transcription of inflammatory genes in both the anterior-tuberal and in the preoptic hypothalamus of animals exposed to cyclic food restrictions and frustration

stress developing a binge-like eating behavior to look for the region specificity of the effects with respect to feeding and related behavior.

Secondly, the effect of the estrus phase was investigated in the same experimental conditions, given that food-restricted animals experiencing the frustration stress during estrus failed to develop binge-like eating behavior [23].

2. Material and Methods

2.1 Subjects and diet composition

Female Sprague-Dawley rats (Charles River, Calco, Italy), weighing 200-225 g at the beginning of the experiments were used. Rats were housed under a 12-h light/dark cycle (lights on at 08:00 am) with free access to food and water for 2 weeks prior to the experiments. They were kept in a room at constant temperature (20-22°C) and humidity (45-55%). Rats were housed individually in metal cages (30 x 30 x 30 cm). All experiments were carried out in accordance with the EC guidelines governing animal welfare and protection (EEC Council Directive 2010/63/UE), Italian legislation on animal experimentation (Decreto Legislativo n. 116, 27 January 1992) with the guidelines of the National Institutes of Health on the use and care of laboratory animals, and had the approval of the local Ethical Committee. All efforts were made to minimize animal suffering and to reduce the number of animals used in this study.

Rats were given standard rat food pellets and, during habituation and feeding test, highly palatable food as previously described [24, 25]. The highly palatable food (3.63 kcal/g) was a paste prepared by mixing Nutella (Ferrero, Alba, Torino, Italy) 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 weight/weight percent ratio: 52% Nutella, 33% food pellets, 15% water.

2.2 Binge eating experimental procedure

In the experiments described below (Fig. 1A) 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 palatable food for 2 h during the light cycle between 10:00 am and 12:00 pm (2 h after the onset of the light cycle) on days 5-6 and 13-14 of the first two cycles (total of 4 exposures). According to our previous experiments, where we also demonstrated that restricted and non-stressed or non-restricted and stressed animals do not show binge eating, in order to induce the impaired feeding behavior, food restricted animals were also exposed to a frustration stress manipulation (at 10:00 am) consisting of 15 min exposure to the coffee cup containing the palatable food that was placed inside a metallic grid container and hung up on the anterior wall of the cage. Rats could see and smell the palatable food but could not access it. During this 15 min period, the rats engaged in repeated movements of the forepaws, head, and trunk, suggesting that they were attempting to reach the palatable food. Thirty-six rats (n = 18 each group) were used for behavioral test (experiment 1). Twenty-six rats were used for molecular biology studies selected from a cohort of forty animals to obtain 13 animals in estrus and 13 in non-estrus phase (n = 6-7) (experiment 2).

2.2.1 Experiment 1: effect of history of food restriction and frustration stress on highly palatable food intake in female rats

On the test day, we assessed palatable food intake for 2 h immediately after exposure to frustration stress. After 15 min, the palatable food cup was placed inside the cage and food intake was determined for 2 h. During the test session, we measured food intake for 15 and 120 min in stressed animals and relative controls (Fig. 1A) [24, 26].

2.2.2 Experiment 2: effect of history of food restriction and frustration stress on cytokine systems components expression in preoptic and anterior- tuberal parts of rats hypothalamus

For molecular analyses, immediately after receiving stress, rats were sacrificed by decapitation and dissection was performed following the rat brain atlas [27].

The hypothalamus was removed and was immediately divided into preoptic (lying above the optic chiasm; Bregma level -0.26/-1.30) and anterior/ tuberal area (ending at the mammillary bodies;

Bregma level -1.30/-3.60). Anatomic subdivision of the hypothalamus was performed based on the organizational scheme of the structure of the hypothalamus reported by Simerly [22] and Saper [20]. The tissues were immediately frozen on dry ice and stored at -80°C until gene expression analyses.

2.3 Determination of the estrous cycle phases of rats

The day of the behavioral and molecular analyses we collected vaginal smears and analyzed them under a microscope to assess the ovarian phase. We determined the estrous cycle phase in a blind manner to the experimental conditions.

Vaginal secretions were collected with a moistened cotton swab, with warm physiological saline (NaCl 0.9%) by inserting the tip superficially into the rat vagina. A drop of vaginal fluid was smeared on a glass slide, and the unstained material was observed under a light microscope. Three types of cells were identified: round and nucleated epithelial cells, irregular cells without nuclei or cornified cells, and small round cells or leukocytes. The proportions of these types of cells in a sample determine the estrous cycle phase [28]: proestrus (primarily large, round, nucleated cells), estrus (large number of cornified cells), metestrus (large number of leukocytes, some cornified cells, and almost no nucleated cells), or diestrus (predominance of leukocytes, some nucleated and almost no cornified cells). The rats in diestrus, metestrus, and proestrus were all combined into non-estrus group.

2.4 Total RNA extraction, reverse transcription, and real time polymerase chain reaction

RNA extraction and DNAse treatment were performed as previously described [29] using GenElute™ Mammalian Total RNA Miniprep Kit and DNASE70-On-Column DNase I Digestion Set (Sigma Aldrich ®, Milan, Italy). Two µg of total RNA were reverse transcribed with High Capacity cDNA Reverse Transcription Kit (Life Technologies Corporation, Carlsbad, CA, USA). Furthermore Real Time PCR was performed, as previously described [30], in ABI PRISM 7900 HT (Life Technologies Corporation, Carlsbad, CA, USA) using Power SYBR Green mix (Life Technologies Corporation, Carlsbad, CA, USA) and specific forward and reverse primers at a final concentration

of 150 nM (see Supp. table 1). Ct (cycle threshold) value was determined by the SDS software 2.2.2 (Life Technologies Corporation, Carlsbad, CA, USA), mRNA expression was calculated with the ΔΔCt method with glyceraldehydes-3-phosphate dehydrogenase (GAPDH) as endogenous control.

2.5 Statistical analysis

In experiment 1: main effects on palatable food intake of the estrus phase (estrus, non-estrus), of the experimental procedure (history of food restriction + frustration stress: no, yes) or the interaction between the two terms were evaluated with a two-way (estrous cycle x experimental procedure) ANOVA. Post hoc analysis, when necessary, were performed using Bonferroni tests to follow-up on significant interaction or main effects using Systat version 10.0 (Systat Software Inc.).
In experiment 2, when only two means were to be compared, the data were expressed using the average levels of expression of non-food restricted, non-stressed control animals at a matching estrus phase as calibrator and analyzed by an independent Student's t-test using SPSS for Windows® v. 22 (SPSS Inc., Chicago, USA). Main effects were analyzed via two-way ANOVA using as calibrator the average levels of expression of not food restricted, not stressed, non-estrus group. Data were presented as mean ± standard error (S.E.M.) and $p < 0.05$ was considered statistically significant.

3. Results

3.1 Females rats with a history of food restrictions consumed more highly palatable food following exposure to an acute frustration stress when tested in non-estrus (experiment 1)

As in our previous studies [16], we found that while rats lost weight during the 4-day food restriction period they regained it during the subsequent 4-day *ad libitum* food period. On the 25th day (day of the frustration stress and feeding test or sacrifice; experiment 1 and 2 respectively) the body weight of the rats in the food restricted and non-food restricted groups was not significantly different (Fig. 1B).

The statistical analysis, which included the between-subjects factors of history of intermittent food restriction and stress during testing (no, yes), and the estrus phase (estrus, non-estrus) showed that palatable food intake was significantly affected by history of food restriction and stress [$F(1, 32) = 39.9$, $p < 0.01$], by estrus phase [$F(1, 32) = 66.9$, $p < 0.01$] with a significant interaction between the two factors [$F(1, 32)= 11.02$, $p < 0.01$]. Post-hoc tests revealed a significant ($p < 0.01$) increase in food consumption at 0-15 min time point in rats with a history of food restriction followed by frustration stress not in estrus in comparison to control non-estrous group. In contrast, estrous rats did not show binge-eating behavior; in other words, the palatable food intake of the stressed and restricted estrous group was not significantly different from the estrous control group ($p > 0.05$) (Fig. 2A). In addition, estrous rats ate significantly less in each group during 15 min compared with the corresponding non-estrous group ($p < 0.01$ in restricted and stressed group, $p < 0.05$ in control group). ANOVA of the cumulative 2 h palatable food intake showed a significant effect of the history of food restriction and stress [$F(1, 32) = 7.9$, $p = 0.01$] and of the estrus phase [$F(1,32) = 60.0$, $p < 0.01$] with a significant interaction between the two factors [$F (1,32) = 24.20$, $p < 0.01$] (Fig. 2B). Post-hoc tests showed that non-estrous restricted and stressed rats ate significantly more palatable food in comparison to non-estrous non-restricted and non-stressed group ($p < 0.01$) and to restricted and stressed rats in estrous phase ($p < 0.01$).

3.2 A history of intermitted food restriction combined with an acute frustration stress induced c-fos mRNA expression in the hypothalamus immediately after the stress exposure (experiment 2)

Induction of c-fos, an anatomic landmark of activated neurons, is known to be evoked by different stimuli including stress or inflammatory mediators [31].

History of food restriction combined to frustration stress resulted in a general significant increase in expression of the immediate early gene c-fos in the hypothalamus suggesting an activation of this brain area. In the anterior-tuberal region, food restricted and stressed animals showed an increase in c-fos mRNA with respect to their non-restricted and non-stressed counterparts both at estrus

and non-estrus phase (t = 5.961; $p < 0.0001$ and t = 5.343; $p < 0.0001$ for non-estrus and estrus rats respectively) (Fig. 3A). Also in the preoptic area, animals experiencing restricted access to food and exposed to stress showed higher levels of c-fos than unstressed control animals with ad libitum access to food, irrespective of the estrous phase (t = 4.892; $p = 0.008$ and t= 2.551; $p = 0.027$ for non-estrus and estrus rats respectively) (Fig. 3B).

3.3 The expression of inflammatory related markers was differently affected by food restrictions and acute frustration stress depending of the hypothalamic region evaluated (experiment 2)

In non-estrus animals, we demonstrated different patterns of inflammatory related markers expression, following food-restriction and stress, between the two regions of the hypothalamus evaluated, highlighting specific transcriptional effects in the anterior- tuberal region of animals developing binge eating.

Exposure to food restriction plus frustration stress significantly decreased the expression levels of IL-1 receptor antagonist (**IL-1Ra**) in both the hypothalamic regions evaluated (t = -7.877; $p < 0.0001$ and t = -3.273; $p = 0.007$ in the anterior- tuberal and preoptic hypothalamus respectively) (Fig. 4A and 5A), whereas specific changes in the IL-18 system and iNOS expression were observed following food restriction and stress in the hypothalamic anterior- tuberal region of animals developing binge-like eating behavior (Fig. 4B- C and 5B- C).

Both in the anterior- tuberal and preoptic regions of the hypothalamus we observed a down-regulation of the IL-18\ IL-18R system following food restriction and stress in non-estrus animals. However, while in the anterior- tuberal region the experimental procedure increased the expression of the IL-18 binding protein (IL-18BP) of about the 44% (t = 3.744; $p = 0.003$) and decreased the expression of the IL-18R binding chain (IL-18Rα- t = -2.683; $p = 0.027$); in the preoptic region levels of mRNA coding for IL-18 were decreased (t = -2.581; $p = 0.027$) when compared to their respective control group (Fig. 4B and 5B). The expression levels of the receptor chain involved in signal transduction (IL-18Rβ) were not affected by food restriction plus frustration stress with

respect to control animals in both anterior- tuberal and preoptic nuclei of the hypothalamus irrespective of the estrous stage of the animals.

Similarly, no effects were observed for the other possible negative regulators of IL-18 activity: IL-18Rα type II and IL-18Rβ short [32, 33], when comparing food restricted and stressed animals with their controls at a matching estrous stage in both hypothalamic region considered (*data not shown*).

In the anterior- tuberal hypothalamus, expression levels of **iNOS** were strongly increased in the food restricted and stressed group at non-estrus with respect to their matching non-food restricted/non-stressed counterparts ($t = 3.254$; $p = 0.014$) (Fig. 4C). As for the preoptic region of the hypothalamus, no alteration in iNOS mRNA levels were associated with exposure to cyclic food restriction and frustration stress while we found reduced cyclooxygenase (COX-2) mRNA levels ($t = -2.491$; $p = 0.030$) (Fig. 5C). On the contrary, no effect was observed in COX-2 mRNA levels in the anterior- tuberal hypothalamus (Fig. 4C).

History of food restriction and frustration stress had no effect on expression levels of **IL-1**, **IL-6,** IL-6 signal-transducing receptor (**gp130**), and **IFN-γ** in both the hypothalamic regions considered while **TNF-α** mRNA levels were decreased following cyclic food restriction and frustration stress only in the preoptic hypothalamus ($t = -2.816$; $p = 0.017$) (Fig. 4A and 5A).

However, because exposure to both food restriction and frustration stress is needed to induce a binge-like eating behavior and many of the investigated targets may be stress-responsive genes [34, 35], to discriminate between the effect of the stressful procedure itself and that of the combination of food restriction plus frustration stress, we evaluated the transcriptional effects on selected targets in the anterior-tuberal and preoptic regions of the hypothalamus of a group of rats that were not exposed to food restriction (while receiving chow and highly palatable food as for non-stressed and non-restricted controls), but on day 25 were exposed to frustration stress, and from their vaginal smears were considered at non-estrus (Supp. Fig. 1A).

The overall increase in c-fos expression observed in animals developing binge-like eating behavior in both preoptic and anterior-tuberal hypothalamus, was blunted in non-estrus stressed animals that were feed regularly. In particular, a significant increase in c-fos mRNA was relegated to the

preoptic region of stressed animals with respect to their controls (t = 2.640; p = 0.022). No effect was observed in the anterior-tuberal hypothalamus (Supp. Fig. 1B).

As observed for food restricted and stressed non-estrus animals presenting a binge-like alteration in food intake, a down-regulation of the IL-18\ IL-18R system in the anterior-tuberal hypothalamus was present also in animals without a history of food restriction but exposed only to frustration stress. As for iNOS, stress alone was unable to affect its expression in the hypothalamus (Supp. Fig. 1)

In particular, frustration stress caused an upregulation of **IL-18BP** mRNA levels (t = 3.179; p = 0.008) with respect to their non-estrous unstressed counterparts (Supp. Fig. 2). This effect is in accordance with the proposed involvement of IL-18 in stress response [34]. However, IL-18Rα mRNA levels were not different between non-estrus animals exposed only to the frustration stress and their unstressed counterparts. The downregulation of the binding chain of IL-18 mRNA was present only when the stress procedure was coupled with a history of food restriction, suggesting that this effect may be specific for animals that developed binge-like eating behavior which ends up with a more compromised functionality of the IL-18\ IL-18R system.

As observed for the main negative regulator of IL-18, also the decrease in expression levels of IL-1Ra observed in stressed-restricted non-estrus rats seemed to be stress-mediated: a significant decrease of **IL-1Ra** mRNA was observed in the anterior- tuberal region of the hypothalamus of non-estrus stressed animals with respect to their not stressed counterparts (t = -2.643; p = 0.021) (Supp. Fig. 2).

3.4 In food restricted animals exposed to frustration stress during estrus phase IL-18 expression was increased in the anterior- tuberal part of the hypothalamus (experiment 2)

To further evaluate the specificity of the transcriptional effects observed in the hypothalamus of animals developing binge eating we measured the mRNAs expression levels of the same markers of inflammation in the hypothalamus following exposure to cycles of food restriction plus frustration stress when the latter was experienced in the estrus phase.

In general, transcriptional effects observed in the hypothalamus of animals in estrus were different than those observed in non-estrus, thus demonstrating a role for the estrous cycle in affecting stimulus dependent regulation of the transcription of inflammatory mediators.

In particular, history of food restriction and frustration stress lead to an upregulation of IL-18\ IL-18R system in estrus animals that did not develop binge-like eating behavior, as opposed to what observed in non-estrus animals developing an alteration in feeding behavior. The expression levels of anorexigenic cytokine **IL-18** in the anterior-tuberal region ($t = 2.413$; $p = 0.050$) were increased in food-restricted and stressed rats at estrus with respect to their matching controls (Fig. 6B), while mRNA levels of **TNF-α** were significantly decreased ($t = -3.016$; $p = 0.013$) (Fig. 6A). As for the preoptic region, exposure to food restriction and frustration stress led to a decrease in mRNA levels of **IL-1β** in estrus rats versus their matching control group ($t = -4.569$; $p = 0.002$) (Fig. 7A). No other effect was observed for the evaluated targets in the hypothalamus of estrus animals that did not develop binge-like eating behavior (Fig. 6-7).

Indeed, two-way ANOVA [estrus phase (estrus, non-estrus) x the experimental procedure (history of food restriction + frustration stress: no, yes)] revealed a specific interaction between the two terms for IL-1Ra expression in both the anterior- tuberal and the preoptic region [$F(1,19) = 22.320$; $p < 0.0001$ and $F(1,24) = 7.772$; $p = 0.011$ for the anterior- tuberal and preoptic region respectively] and for IL-1β and IL-18 only in the preoptic region [$F(1,21) = 9.221$; $p = 0.007$ for IL-1β; $F(1,23) = 6.147$; $p = 0.022$ for IL-18]. Moreover, a main effect of the estrus phase was observed for IL-1Ra and COX-2 in the anterior- tuberal area [$F(1,19) = 7.952$; $p = 0.012$ and $F(1,23) = 4.758$; $p = 0.0041$ respectively], IL-1β and iNOS in the preoptic hypothalamus [$F(1,21) = 22.762$; $p < 0.0001$ and $F(1,21) = 10.268$; $p = 0.005$ respectively], and IL-18 mRNA in both regions [$F(1,23) = 22.459$; $p < 0.0001$ and $F(1,23) = 12.268$; $p = 0.002$ for the anterior- tuberal and preoptic region respectively].

Discussion

The main finding of the present study is that, while cycles of food restriction combined with frustration stress caused a general induction in c-fos expression, markers of inflammation are differentially affected, at the transcriptional level, in a validated animal model of binge-like eating behavior depending both on the hypothalamic region evaluated and the estrous cycle phases at the moment of analyses. This is especially relevant considering the different role in regulating feeding behavior of the hypothalamic regions evaluated and the influence of estrogen in binge eating and eating disorders [36, 37]. Although additional behavioral and nuclei specific analyses will be needed, our results may inform future research aimed to define the specific role of these molecules in regulating homeostasis and these behaviors. Our data suggest that the transcriptional changes observed in the anterior-tuberal region of the hypothalamus of food restricted animals stressed at non-estrus phase may be associated to behavioral feeding impairment since comparable effect has been observed neither in an area not directly involved in feeding control nor in animals that did not develop binge eating (i.e. animals food restricted exposed to frustration stress during estrus or only stressed at non-estrus phase).

While the body weight at the day of behavioral and molecular tests was not different between free feed and food restricted animals, only animals exposed to both food restriction and frustration stress ate a larger amount of familiar highly palatable food [16, 26, 38, 39-41]. Moreover, we observed that when animals were maintained under an *ad libitum* feeding for six more weeks after stress, the body weight of rats exposed to food restriction and frustration stress was significantly higher than those of control rats, thus supporting a role for the observed change in triggering maladaptive responses and eventually leading to diseases and related symptoms (i.e. obesity and insulin resistance).

The role of pro-inflammatory cytokines in eating disorders has gained increasing attention, considering that they are known to regulate feeding behavior [7], and are extremely responsive to

stressful stimuli and to glucocorticoids [6, 42]. Indeed, inflammatory mediators are known to be affected by both feeding schedule and stress [34, 35]. Studies evaluating either circulating levels of cytokines or cytokine secretion from peripheral blood mononuclear cells (PBMCs) report significant alterations of these pro-inflammatory mediators in eating disorders [3, 7, 43]. Moreover, a low but chronic inflammatory state has been associated to overweight and obesity, two conditions that are strongly associated with binge-eating behavior: over 35% of those who regularly binge are overweight or obese, and body mass index (BMI) correlates with prevalence of bingeing [44, 45]. However, pro-inflammatory cytokines can be locally produced also within the central nervous system where they can act directly on hypothalamic neurons involved in regulation of food intake and eating behavior, thus possibly contribute to excessive weight gain and in some instance obesity associated to the impaired behavior. Notwithstanding the knowledge of these mechanisms may contribute to prevent the development and progression of eating disorders and/or related complications, there is still insufficient awareness of the central immune setting in models of binge-eating behavior. Here we were specifically interested in evaluating the early transcriptional effects on markers of inflammation in the hypothalamus of a validated model of binge-like eating behavior. This area, because of its localization in the body and the plethora of receptors it expresses, is a neuro-endocrine interface in the brain [46]. Moreover, limbic structures that receive environmental information able to influence feeding behavior are known to project to the hypothalamus [19, 20].

In the anterior- tuberal part of the hypothalamus of animals that develop the binge-eating behavior we demonstrated a downregulation of the IL-18/ IL-18R system and a strong induction of iNOS expression.

IL-18 is an anorexigenic cytokine that can affect food intake, metabolism, and adiposity during adulthood through central actions: in fact IL-18/ IL-18R system was shown to be constitutively expressed in all the regions of the hypothalamus and also to be regulated by peripheral immune challenge, proposing for IL-18 a role in mediating the anorectic and metabolic component of the sickness behavior [32, 47]. Impairment of IL-18 functionality, by knocking out the cytokine itself or its receptor [48, 49], or by overexpressing its natural antagonist, IL-18BP [50], results in genetically modified mice that present abnormalities characteristic of the metabolic syndrome like insulin

resistance, hyperglycemia, and obesity. IL-18 deficiency resulted in hyperphagia before the onset of overweight in male and female mice, while central (but not peripheral) administration of IL-18 suppressed food intake in both knockout and WT animals [50]. Current evidence from clinical studies demonstrates impaired functionality of IL-18/ IL-18R system in eating disorders: peripheral circulating levels of IL-18 are positively correlated with BMI in obese women [51] and are elevated in overweight adolescent [52], while PBMCs from obese patients have been shown to produce significantly less IFN-γ in response to IL-18 stimulation under stimulatory condition. Moreover, a decreased expression of IL-18R chains was observed in human leukocytes in presence of metabolic disorders with respect to lean healthy individuals, suggesting an altered expression and improper assembly of a functional IL-18 receptor [53]. Our results are in line with these observations. In fact, here we showed that in animals that developed binge-like eating on palatable, the regulation of the expression of the IL-18/ IL-18R system may lead to a general impairment of this cytokine functionality thorough decreased expression of the alpha chain and increased mRNA levels of IL-18BP in the anterior hypothalamus, while IL-18 was decreased in the preoptic area. Interestingly, exposure to frustration stress alone was unable to affect the expression of IL-18Rα in the anterior- tuberal hypothalamus suggesting that the combination of food restriction and stress is crucial to obtain the downregulation of the canonical isoform of the ligand binding chain of the IL-18R. On the other hand, in estrus animals that did not develop binge-like eating behavior following a history of repeated cycles of food restriction and frustration stress, IL-18 expression was increased in the anterior-tuberal hypothalamus.

Nitric oxide (NO) is another neuromodulator that participates in the interaction between neuroendocrine and neuroimmune systems and that is induced by pro-inflammatory cytokines. NO is also involved in feeding control [54] and is a central component in neuropeptide regulation of appetite [55]. Clinical data suggest that NO production is impaired in eating disorders: an altered NO production was found in plasma of patients affected by anorexia nervosa or bulimia nervosa [56]. Consistently with these data, enhanced NO production in platelets and elevated levels of exhaled nitric oxide were found in patients suffering from anorexia nervosa [43] and impaired NO functionality has also been linked to obesity [57].

We found a significant increase in the expression levels of iNOS in the anterior-tuberal hypothalamus of non-estrus rats with a history of food restriction and frustration stress.

Our data are consistent with several preclinical evidence suggesting an orexigenic effect for the NO system, in fact a general inhibition of NOS led to hypophagia in both healthy animals and in genetically obese mice and NO is also involved in mediating hyperphagia induced by food deprivation [55]. The NO system seems to be involved in the control of satiation and hunger especially under a stressful situation [55], however the majority of the studies were focused on the role of neuronal NOS, while the role of iNOS in eating disorders remains to be fully elucidated. The inducible isoform of NO synthase is known to be upregulated in the hypothalamus by stress exposure [35], but in our experimental conditions, this effect was not observed in animals exposed only to the frustration stress, suggesting a specific effect on iNOS transcription in this region of the hypothalamus for the combination of history of food restriction and frustration stress.

A similar pattern was observed for the notoriously stress-responsive gene, c-fos, that was induced in the anterior hypothalamus only when animals experienced both cyclic food restriction and frustration stress, and not frustration stress alone.

Also for other cytokines, including IL-1 and IL-6, a role in feeding and food motivated behavior has been described [11]. Members of IL-1 superfamily have been reported to regulate food intake, energy metabolism, and glucose homeostasis by acting on hypothalamic neurons implicated in the regulation of eating and appetite [58]. IL-1Ra deficient mice showed a leaner phenotype with respect to wild-type mice, which may reflect aberrant lipid metabolism [59]. IL-1Ra systemic levels were found to be altered in metabolic disorders and eating disorders, circulating IL-1Ra was elevated in obese adults, and correlated with lean BMI and leptin levels. Interestingly, it has been demonstrated that leptin, an adipokine that functions as a peripheral signal to the brain to regulate food intake, is able to regulate hypothalamic IL-1β function and to induce IL-1Ra in the brain [60].

Here we showed that the effects observed on the transcription of members of the IL-1 superfamily, mainly the lowering of IL-1Ra mRNA, were not specific for the anterior- tuberal part of the hypothalamus of animal developing the binge-like eating behavior suggesting that these alterations may be involved in mediating the general response to the experimental procedure, rather than only

food intake. In fact, by replicating the human environment and mimicking the interaction between history of dieting and stress, it is very likely that in this model the animals will develop also other behavioral sequelae, not only concerning altered food consumption or metabolic alterations associated with eating disorders [44].

Hypothalamic IL-6 and its signal transducer mRNAs were not affected by a history of food restriction and frustration stress irrespective of the estrus phase. However, we cannot exclude a role for the IL-6 system in the development and/or sustenance of binge-like eating behavior. IL-6-mediated effects may require other intermediate mediators or take place elsewhere. For example, it has been demonstrated that circulating IL-6 can increase the peripheral secretion and production of glucagon-like peptide 1 (GLP-1), which in turn may act centrally to affect food intake and body weight [61, 62].

Conclusions

Our data support the hypothesis that changes in inflammatory mediators, in specific brain regions (induced by dieting and food associated stressors), can contribute to the onset of eating-related maladaptive behavior and that sex hormones may play a role in the interplay between HPA axis activity and immune regulation. These findings are relevant to the development of pharmacological strategies for the prevention and treatment of the notoriously difficult to treat eating disorders.

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Declaration of interest:

All authors declare that there are no actual or potential conflicts of interest including any financial, personal or other relationships with other people or organizations that could inappropriately influence, or be perceived to influence, our work.

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Figure captures:

Fig. 1: Study design and body weight. (A) Timeline of the experimental procedures for the non-food restricted (top) and food restricted (bottom) rats. (B) Mean ± S.E.M. body weight (g) of female rats exposed or not to repeated intermittent cycles of food restriction/refeeding.

Fig. 2: Effect on palatable food intake. Mean ± S.E.M. of palatable food intake at 15 min time point during testing (A) and total 2-h food intake (B) in experiment 1. Comparisons were made by two-way ANOVA followed by Bonferroni post-hoc test; ** $p < 0.01$ significant difference from the control animals not experiencing a history of intermittent food restriction and stress; # $p < 0.05$, ## $p < 0.01$ significant difference from the non-estrus respective counterparts.

Fig. 3: Repeated food restrictions and acute frustration stress increased c-fos expression in the anterior- tuberal and preoptic hypothalamus. Female rats from experiment 2 with a history of food restriction and frustration stress (food restricted + stressed), were sacrificed immediately after the end of the stress procedure (experiment 2) with a matching control group (non-food restricted + non-stressed). c-fos mRNA expression, with GAPDH as an endogenous control, was calculated using the ΔΔCt method. Data are expressed as fold changes of non-food restricted + non-stressed animals at a matching estrus phase ± S.E.M.; ** $p < 0.01$, *** $p < 0.0001$ significant difference with respect to non-food restricted + non-stressed group.

Fig. 4: Effect of repeated food restrictions and acute frustration stress on gene expression in anterior- tuberal hypothalamus of non-estrus animals. Female rats from experiment 2 with a history of food restriction and frustration stress (food restricted + stressed), were sacrificed immediately after the stress procedure (experiment 2) with a matching control group (non-food restricted + non-stressed). Estrus phase at day 25 was assessed and only animals in non-estrus (n=6-7) were included in the analyses. Interleukin (IL-) 1β, IL-1 receptor (R)a, IL-6, gp130, Tumour Necrosis Factor (TNF) α, Interferon (IFN) γ, IL-18, IL-18 binding protein (bp), IL-18Rα, IL-18Rβ,

cyclooxygenase (COX)-2, and inducible nitric oxide synthase (iNOS) mRNA expression, with GAPDH as endogenous control, was calculated using the ΔΔCt method. Data are expressed as fold changes of non-food restricted + non-stressed animals ± S.E.M.; * $p < 0.05$, *** $p < 0.0001$ significant difference with respect to non-food restricted + non-stressed group.

Fig. 5: Effect of repeated food restrictions and acute frustration stress on gene expression in preoptic hypothalamus of non-estrus animals. Female rats from experiment 2 with a history of food restriction and frustration stress (food restricted + stressed), were sacrificed immediately after the stress procedure (experiment 2) with a matching control group (non-food restricted + non-stressed). Estrus phase at day 25 was assessed and only animals in non-estrus (n=6-7) were included in the analyses. Interleukin (IL-) 1β, IL-1 receptor (R)a, IL-6, gp130, Tumour Necrosis Factor (TNF) α, Interferon (IFN) γ, IL-18, IL-18 binding protein (bp), IL-18Rα, IL-18Rβ, cyclooxygenase (COX)-2, and inducible nitric oxide synthase (iNOS) mRNA expression, with GAPDH as endogenous control, was calculated using the ΔΔCt method. Data are expressed as fold changes of non-food restricted + non- stressed animals ± S.E.M.; * $p < 0.05$, *** $p < 0.0001$ significant difference with respect to non-food restricted + non-stressed group.

Fig. 6: Effect of repeated food restrictions and acute frustration stress on gene expression in anterior- tuberal hypothalamus of estrus animals. Female rats from experiment 2 with a history of food restriction and frustration stress (food restricted + stressed), were sacrificed immediately after the stress procedure (experiment 2) with a matching control group (non-food restricted + non-stressed). Estrus phase at day 25 was assessed and only animals in estrus (n=6-7) were included in the analyses. Interleukin (IL-) 1β, IL-1 receptor (R)a, IL-6, gp130, Tumour Necrosis Factor (TNF) α, Interferon (IFN) γ, IL-18, IL-18 binding protein (bp), IL-18Rα, IL-18Rβ, cyclooxygenase (COX)-2, and inducible nitric oxide synthase (iNOS) mRNA expression, with GAPDH as endogenous control, was calculated using the ΔΔCt method. Data are expressed as fold changes of non-food restricted + non-stressed animals ± S.E.M.; * $p < 0.05$ significant difference with respect to non-food restricted + non-stressed group.

Fig. 7: Effect of repeated food restrictions and acute frustration stress on gene expression in preoptic hypothalamus of estrus animals. Female rats from experiment 2 with a history of food restriction and frustration stress (food restricted + stressed), were sacrificed immediately after the stress procedure (experiment 2) with a matching control group (non-food restricted + non-stressed). Estrus phase at day 25 was assessed and only animals in estrus (n=6-7) were included in the analyses. Interleukin (IL-) 1β, IL-1 receptor (R)a, IL-6, gp130, Tumour Necrosis Factor (TNF) α, Interferon (IFN) γ, IL-18, IL-18 binding protein (bp), IL-18Rα, IL-18Rβ, cyclooxygenase (COX)-2, and inducible nitric oxide synthase (iNOS) mRNA expression, with GAPDH as endogenous control, was calculated using the ΔΔCt method. Data are expressed as fold changes of non-food restricted + non-stressed animals ± S.E.M.; ** $p < 0.01$ significant difference with respect to non-food restricted + non-stressed group.

Figure 1

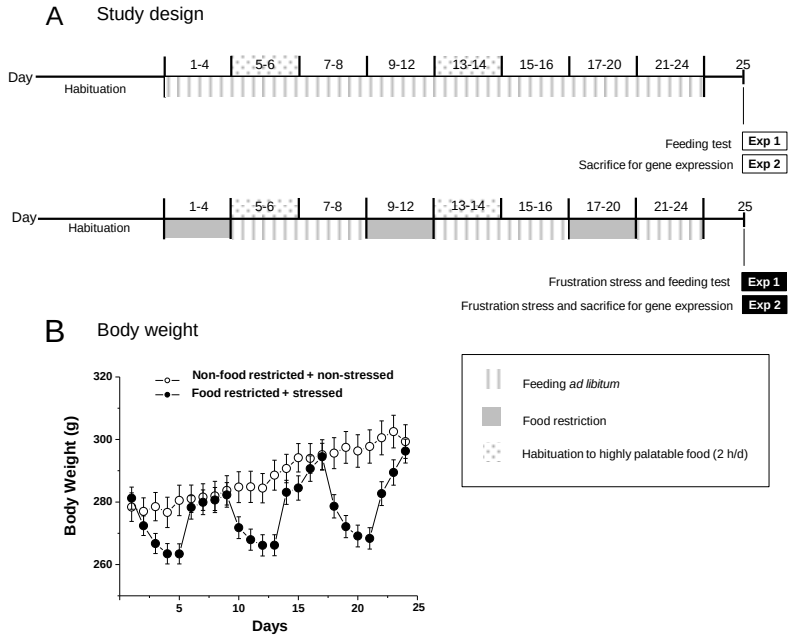

Figure 2

Figure 3

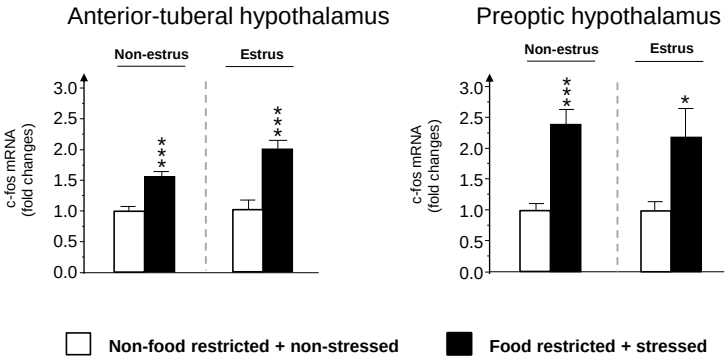

Figure 4

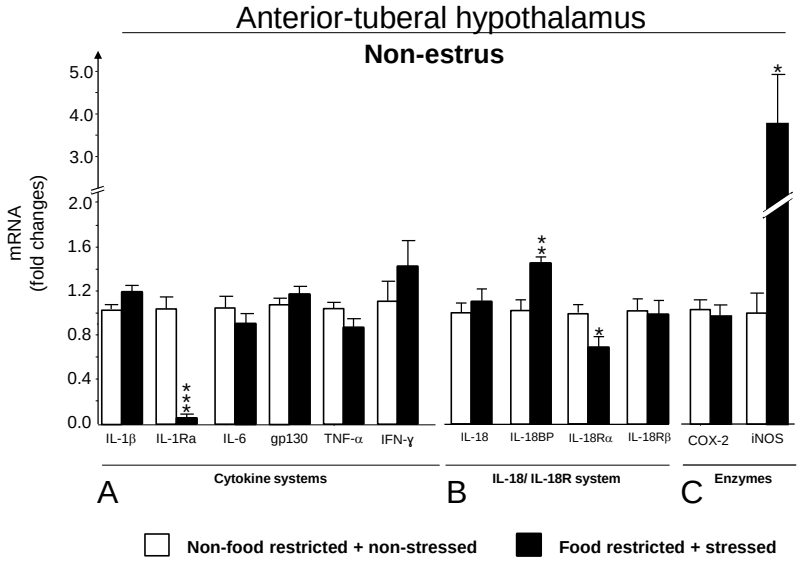

Figure 5

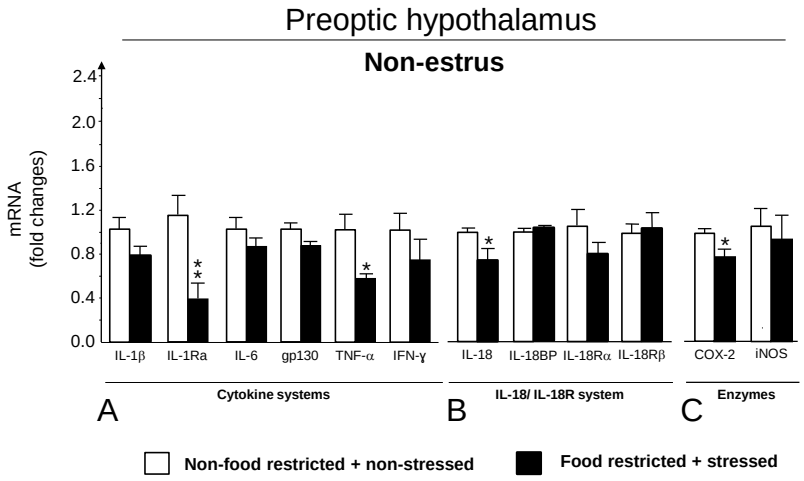

Figure 6

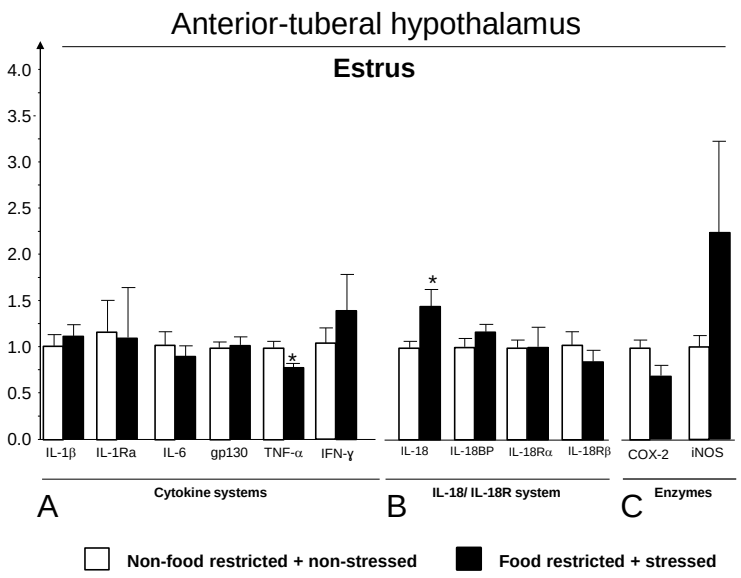

Figure 7