Year : 2017

ROLE OF CRTC1 IN THE NEUROBIOLOGICAL UNDERPINNINGS OF MOOD AND EATING DISORDERS

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ROSSETTI Clara, 2017, ROLE OF CRTC1 IN THE NEUROBIOLOGICAL UNDERPINNINGS OF MOOD AND EATING DISORDERS

Originally published at : Thesis, University of Lausanne

Posted at the University of Lausanne Open Archive http://serval.unil.ch
Document URN : urn:nbn:ch:serval-BIB_3917AEC953BD3

UNIL | Université de Lausanne
Faculté de biologie et de médecine

Centre de Neurosciences Psychiatriques, Département de Psychiatrie

ROLE OF CRTC1 IN THE NEUROBIOLOGICAL UNDERPINNINGS OF MOOD AND EATING DISORDERS

Thèse de doctorat en Neurosciences

présentée à la

Faculté de Biologie et de Médecine
de l'Université de Lausanne

par

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Lausanne, 2017

Programme doctoral interuniversitaire en Neurosciences des Universités de Lausanne et Genève

LEMANIC NEURO SCIENCE DOCTORAL SCHOOL

UNIL | Université de Lausanne

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UNIVERSITÉ DE GENÈVE

Programme doctoral interuniversitaire en Neurosciences des Universités de Lausanne et Genève

Imprimatur

Vu le rapport présenté par le jury d'examen, composé de

Président	Monsieur Prof. Bogdan **Draganski**
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le Conseil de Faculté autorise l'impression de la thèse de

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intitulée

ROLE OF CRTC1 IN THE NEUROBIOLOGICAL UNDERPINNINGS OF MOOD AND EATING DISORDERS

Lausanne, le 13 janvier 2017

pour Le Doyen
de la Faculté de Biologie et de Médecine

Prof. Bogdan Draganski

ACKNOWLEDGEMENTS

I wish to begin by expressing my sincere gratitude to my thesis director Jean-René Cardinaux and to my co-director Benjamin Boutrel for their excellent and constant supervision, for believing in me and giving me the motivation and the support I needed to progress in my project. I also thank them for all the precious suggestions they provided me along these years. I am really grateful to both for allowing me to work independently and reconcile working and family life.

In the second place, I would like to thank Prof Pierre Magistretti and Prof Olivier Halfon for accepting my registration in the Doctoral Lemanic Neuroscience School.

I also wish to thank Prof. Jean Pierre Hornung, Prof. Luc Tappy and Prof. Urs Albrecht for agreeing to be part of my thesis committee.

I don't know where I should start from in thanking my closest colleague Elsa Meylan…..thank you very much for collaborating with me, for helping me any time I was in troubles and for sharing with me all pleasant and hard moments of this thesis program, and….. your very useful and detailed lab protocols! Your kindness and friendship made the lab a nice place to stay.

I am very grateful to all master and pre-master students I supervised: Giuseppe Spena, Laura Barras, Aurélie Hubner, Daniel Sciarra and Laetitia Guerra. Your participation in this study has been of great utility.

Warm thanks go to all the CNP staff and in particular to Benjamin, Anthony, Kshitij, and Iréne for their kindness, sympathy and assistance.

A very special "Grazie" goes to Fulvio Magara, who was the first person encouraging me to continue my scientific formation. I also thank him for all pertinent scientific observations he always made about my project. I should like to express my particular thanks to all the CEC staff, Valdemar and Deborah, for their effort in taking care of animals without of which no result of this thesis would had been obtained.

I sincerely thank Dr. Jean-Marie Petit for all the equipment he lent me and for its important help in the planning and discussion of experiments concerning the circadian activity in mice.

Finally, I will endlessly thank all my relatives who have been supporting me during these last years. Many thanks go to Roberto, Giorgia and Erica for their patience and unconditional help, as well as, to Carmela, Rosalba, Gianni, Michele, and Lorena for their constant encouragement.

ABSTRACT

Nowadays, obesity and depression are a major health and social burden. Accumulating evidence suggests that these pathologies may have common etiology and share some neurobiological pathways. CREB-regulated transcription coactivator 1 (CRTC1) has been shown to participate in both mood and energy balance regulation. Consistently, CRTC1 deficient mice rapidly develop a depressive-like and obese phenotype in early adulthood, and are therefore a relevant animal model to explore possible biological mechanisms linking mood disorders and obesity.

In the first part of this work, the obese phenotype of male and female *Crtc1*$^{-/-}$ mice was characterized by investigating CRTC1's role in the homeostatic and hedonic regulation of food intake, as well as its influence on daily locomotor activity. We found a strong gender difference in energy homeostasis. Mutant males were hyperphagic and rapidly developed obesity on normal chow diet, whereas *Crtc1*$^{-/-}$ females exhibited mild late-onset obesity without hyperphagia. Overeating of mutant males was accompanied by alterations in the expression of several orexigenic and anorexigenic hypothalamic genes. Intriguingly, hyperphagia and higher locomotor activity occurred only during the resting phase of the light cycle indicating that the lack of CRTC1 may affect circadian rhythmicity.

Binge eating, which is an aberrant eating pattern characterized by loss of control over palatable food consumption, is frequently observed in obese patients with comorbid psychiatric disorders. In line with this clinical observation, in the second part of this study we reproduced a model of binge eating in the rat based on intermittent access to palatable food. We observed that compulsive overeating was accompanied by moderate anxiety, blunted corticosterone response to an acute stress and altered expression of genes in brain areas controlling emotional responding and cognitive functions. When tested on the same feeding procedure, *Crtc1*$^{-/-}$ mice showed high vulnerability to develop binge eating-like behavior.

Collectively, our findings highlight the male-specific involvement of CRTC1 in the central control of energy balance and circadian locomotor activity and identify *Crtc1*$^{-/-}$ mice as a suitable model for investigating obesity and compulsive overeating in association with depression.

RÉSUMÉ

L'obésité et la dépression comptent parmi les préoccupations majeures de la santé publique. De plus en plus d'études montrent qu'elles partagent des mécanismes étiologiques communs. CRTC1 (CREB-regulated transcription coactivator 1) est impliqué dans certaines voies neurobiologiques modulant aussi bien l'humeur que la balance énergétique. De ce fait, des souris déficientes en CRTC1 développent un phénotype obèse et dépressif déjà au stade de jeune adulte et constituent donc un modèle animal intéressant pour étudier les mécanismes communs aux troubles de l'humeur et à l'obésité. Premièrement, le phénotype d'obésité des souris *Crtc1*$^{-/-}$ mâle et femelle a été caractérisé en étudiant le rôle de CRTC1 dans la régulation homéostatique et hédonique de la consommation de nourriture, ainsi que son influence dans l'activité locomotrice quotidienne. Nous avons trouvé une grande différence entre les sexes concernant l'homéostasie de la balance énergétique. Effectivement, les mâles mutants sont hyperphagiques et développent une obésité avec de la nourriture standard, alors que les femelles *Crtc1*$^{-/-}$ deviennent obèses plus tardivement en absence d'hyperphagie. La suralimentation des mâles mutants est accompagnée d'une altération de l'expression de plusieurs gènes hypothalamiques orexigènes et anorexigènes. Etonnement, les mâles mutants manifestent un comportement hyperphagique uniquement lors de la phase de repos du cycle de lumière. Ce comportement alimentaire anormal est associé à une activité locomotrice plus élevée indiquant un effet sur le rythme circadien, dû à l'absence de CRTC1. Deuxièmement, nous avons reproduit un modèle de compulsion alimentaire pour le rat avec un accès alterné à une nourriture appétissante. La compulsion alimentaire, qui est un comportement alimentaire aberrant caractérisé par une perte de contrôle sur la prise de nourriture attrayante, est fréquemment observée chez les patients obèses présentant des troubles psychiatriques co-morbides. Nous avons observé que le développement d'une telle compulsion est accompagné d'un niveau modéré d'anxiété, d'une réponse atténuée de la corticostérone à un stress aigu et d'une expression altérée de gènes présents dans les régions cérébrales contrôlant les réactions émotionnelles et les fonctions cognitives. Lorsque les souris *Crtc1*$^{-/-}$ ont été testées avec un modèle identique à celui utilisé chez les rats, les résultats ont montré que ces animaux tendent vers une compulsion alimentaire.

Ainsi, nos résultats mettent en évidence une implication de CRTC1 dans le contrôle central de l'équilibre énergétique et dans l'activité locomotrice circadienne spécifiquement chez les mâles. De plus, nous avons identifié les souris *Crtc1*$^{-/-}$ comme étant un modèle adéquat pour l'étude de l'obésité et de la compulsion alimentaire associées à la dépression.

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LIST OF ABBREVIATIONS

ACTH	Adrenocorticotropic hormone
ADHD	Attention-deficit/hyperactivity disorder
AgRP	Agouti-related peptide
ARC	Arcuate nucleus
BBB	Blood brain barrier
BDNF	Brain-derived neurotrophic factor
BED	Binge Eating Disorder
BMAL1	Brain and muscle Arnt-like protein 1
BN	Bulimia Nervosa
CART	Cocaine-amphetamine regulated transcript
CLOCK	Circadian locomotor output cycles kaput
CREB	cAMP response element binding protein
CRH	Corticotropin-releasing hormone
CRTC	CREB-regulated transcription coactivator
CRY	Cryptochrome
DHA	Docosahexaenoic acid
DMH	Dorsomedial hypothalamus
DSM	Diagnostic and Statistical Manual of Mental Disorders
ED	Eating Disorders
ER	Estradiol
GABA	Gamma-amino-butyric acid
GCs	Glucocorticoids
GLP-1	Glucagon-like peptide 1
GH	Growth hormone
GRE	Glucocorticoid response element
GSH-R1a	Growth-hormone-secretagogue receptor 1a
HPA axis	Hypothalamus-pituitary-adrenal axis
HPG axis	Hypothalamus-pituitary gonadal axis
LH	Lateral hypothalamus
MCH	Melanin-concentrating hormone

MCR3	Melanocortin receptors 3
MCR4	Melanocortin receptors 4
α-MSH	α-Melanocyte stimulating hormone
NAc	Nucleus accumbens
NPY	Neuropeptide Y
NTS	Nucleus of the solitary tract
Ob-Rb	Leptin receptor b (long form)
OFC	Orbital frontal cortex
ORX	Orexin
OXM	Oxyntomodulin
PER	Period
PFC	Prefrontal cortex
POMC	Pro-opiomelanocortin
PP	Pancreatic polypeptide
PYY	Peptide YY
RHT	Retino-hypothalamic tract
SCN	Suprachiasmatic nucleus
SUD	Substance Use Disorders
TORC	Transducer of regulated CREB activity
TRH	Thyrotropin-releasing hormone
TrkB	Tropomyosin-related kinase B
VMH	Ventromedial hypothalamus
VP	Ventral pallidum
VTA	Ventral tegmental area

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1 INTRODUCTION

1.1 MENTAL HEALTH AND HEALTHY NUTRITION

Life expectancy has dramatically increased in the 20th century and represents one of the greatest achievements of the modern society. This rise in longevity is mainly the consequence of better living standards and health improvement. With regards to health, drastic reduction of life-threatening diseases has been attained with infectious diseases management, drug discovery and better nutrition [1]. Indeed, converging evidence now support the idea that a balanced diet capable to provide adequate energy and nutrients is very important for good health.

For a long time, healthy nutrition has been prevalently considered as a means to promote physical health and prevent metabolic and cardiovascular diseases. Nevertheless, in the last decades, several lines of evidence have begun to show that a balanced diet is also essential for brain functions and that cognitive abilities, in particular, are heavily affected by nutrients intake. In line with this, dietary deficiency in omega 3-polyunsaturated fatty acids, flavonols and folic acid, has been associated with cognitive impairments and mental disorders, including attention-deficit disorder, dementia, depression and schizophrenia [2].

Another example of an essential nutrient for the brain is the docosahexaenoic acid (DHA) that affects synaptic function providing membrane fluidity at synaptic regions. Although it is the most abundant omega 3-fatty acid in cell membranes, it is not efficiently synthesized by the human brain and has to be taken with the diet. Interestingly, during hominid evolution, the development of cognitive skills and the increase of the brain mass (encephalization) overlapped in time with the adaptation to consume fish, an aliment rich in DHA [3]. The growing interest for nutrients, as a means to promote mental health, is also attested by the recent development of a new scientific discipline, called Nutritional Psychiatry, that propose to use particular dietary supplements as a complementary therapy for mental disorders [4, 5].

The interaction between mental functions and nutrition is not limited to the effects of ingested aliments. Indeed, at the anatomical level, brain structures involved in cognitive functions are functionally interconnected with those that modulate eating behavior. Likewise, at the molecular level, it has been observed that several peripheral hormones implicated in central regulation of food intake also play a role in mental processes, such as learning and memory, reward appreciation and mood regulation. Leptin, ghrelin, insulin and brain-derived neurotrophic factor (BDNF) are only some examples of peptides hormones having this double activity.

Additionally, emerging literature is showing that immunoglobulins produced by the immune system upon stimulation of gut microbiota, may cross-react with neuropeptides acting in the brain and regulate their activity. As a consequence, stress-induced altered gut-microbiota, inappropriate nutrition or infections may have a significant impact on different brain functions. At the moment, evidence for such a mechanism has been found for some neuropeptides involved in food intake and motivated behavior [6].

Besides biological considerations, the interaction between mental health and healthy nutrition emerges also from clinical practice [7-9]. Mental disorders have serious consequences on eating behavior: some psychiatric patients, who experience distress, anxiety and depressed mood, can lose interest for food and consume insufficient calories or nutrients, while others can increase food intake and eat high-calorie food, also known as “comfort food”, in an attempt to improve their emotional state [10]. On the other hand, it is also evident that eating, which is for humans a social behavior, can heavily impact on psychological well-being. Excessive eating or excessive dieting are stigmatized behaviors in western societies and they are frequently accompanied by low self-esteem, sense of guilt and negative emotions that can lead, in more vulnerable subjects, to severe mood alterations. In agreement with these facts, an overlap between obesity and mental disorders often occurs. Indeed, clinical and preclinical studies of the last decades not only have highlighted that obesity and mental disorders are intertwined but have also suggested that they may share some anatomical and molecular pathways [11]. Considering the medical and social impact of obesity and mood disorders in our society, a better knowledge of these biological mechanisms, and their contribution to the etiology of both pathologies, is of higher importance. Although the scientific interest for this topic is growing, the study of the relationship between obesity and mental disorders is made difficult by the heterogeneity of mental disorders and by the problematic nosology of obesity. Hereafter, we will address this point and in the following paragraphs we will discuss the physiological regulation of food intake and its relevance in the development of obesity. Finally, in the last part of this section we will focus on these biological factors that contribute to the development of both pathologies.

1.2 THE PROBLEMATIC NOSOLOGY OF OBESITY IN RELATION WITH MENTAL DISORDERS

Mood Disorders and Eating Disorders are both classified in the Diagnostic and Statistical Manual of Mental Disorders (DSM) while obesity, *per se*, is not. Before the release of the last

version, the DSM-V, the Eating Disorders Work Group discussed on the relevance to include also obesity in the manual. Despite the growing evidence documenting a relation between obesity and numerous psychiatric disorders [12-14], defining obesity as a psychiatric disorder remains debatable [15, 16].

Many epidemiologists claim that most forms of obesity, considered as a long-term imbalance between energy intake and expenditure, are mainly the consequence of a sedentary lifestyle and a regular, but modest, increase in calorie intake [17]. On the other hand, clinical experience suggests that obesity is a heterogeneous pathology caused by the interplay of genetic, individual and environmental factors. The complex interaction of all these factors results in a diversified number of obese phenotypes [18].

One of the most frequent altered eating phenotypes observed in obesity, is binge eating that consists of recurrent episodes of uncontrollable eating. Binge eating is a constant feature of Binge Eating Disorder (BED) but is often observed in patients suffering of Bulimia Nervosa (BN). Both BED and BN are classified in the DSM-V as mental disorders under the class of Eating Disorders (ED) and one possibility, which has been debated, would be to include obesity in this section. Nevertheless, it has to be said that, even though there is a positive correlation between severity of binge eating and severity of overweight [19], not all individuals that manifest binge-eating are obese, and conversely, many obese individuals do not show binge-eating.

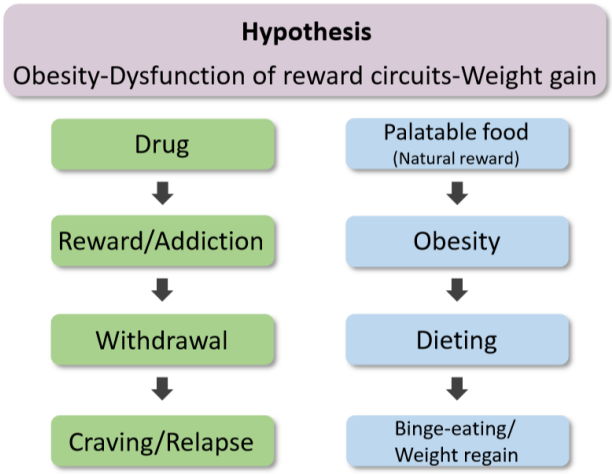

Figure 1. Hypothesis of obesity as an analogy of drug addiction. Addictive drugs are both rewarding and reinforcing. Repeated use of addictive drugs produces multiple changes in the brain that may lead to addiction. Withdrawal occurs when drug-taking stops. Withdrawal symptoms drive one to reuse the drug. Excessive consumption of palatable foods might parallel to drug addiction. Repeated taking of palatable food produces multiple changes in the brain that may lead to obesity. Dieting could trigger binge-eating of palatable food and weight regain. (Adapted from Yamada-Goto et al.,2013, http://dx.doi.org/10.5772/56228).

A different way to classify obesity may be to consider the large consumption of calorie-dense food as a behavioral phenotype similar to that found in drug addiction (Figure 1). As a result of this assumption, obesity might be classified under the Substance Use Disorders (SUD) section. However, considering obesity as a SUD is challenging because food, unlike drugs of abuse, is necessary for survival. Moreover, not all obese individuals have a pattern of behavior resembling to substance abusers. A more convincing approach might be to consider as "food addicted" only the obese individuals that lose control over food, i.e. binge eaters. Although some animal and human studies have supported this concept, it remains a largely debated issue.

The relation between obesity and psychiatric disorders is not limited to ED and SUD. A growing number of epidemiological studies has documented an association between obesity and other psychiatric disorders, including mood and anxiety disorders [20], personality disorder [21], attention-deficit/hyperactivity disorder (ADHD) [13] and posttraumatic stress disorders [14]. This ubiquity of the obesity-psychiatric disorders interaction highlights both the heterogeneity of obesity and, indirectly the difficulty to include it in a specific diagnostic category. The exact cause of comorbidity between obesity and psychiatric disorders, and in particular with depression, remains unknown. However, obesity shares a number of symptomatic features with depression, including increased appetite (especially observed in atypical depression), decreased physical activity, stress hyperactivity and sleep disturbances, which have led to the hypothesis that obesity and depression may be influenced by common biological risk factors.

1.3 REGULATION OF FOOD INTAKE

The regulation of energy balance is a crucial biological function, whose alteration is a direct cause of obesity. The maintenance of an appropriate and stable body weigh requires equality between calorie intake and calorie expenditure. Whereas energy expenditure depends on multiple processes, namely the thermal effect of feeding, the physical activity and the resting metabolic rate, energy intake is merely the result of the amount of food a person eats.[22] Two factors drive eating: the first is the "metabolic" hunger, originating from energy need, and the second is the "hedonic" hunger, that orientates toward the consumption of high calorie-dense foods.

Metabolic and hedonic hunger are controlled by two separated, but intimately connected brain systems, which are respectively the homeostatic and the hedonic system[23].

1.3.1 Homeostatic regulation of food intake

1.3.1.1 Hypothalamus: the main central structure in feeding regulation

Control of appetite has long been considered to be triggered by the stomach, but progressively a consensus defined the brain, and in particular the hypothalamus, as the main regulator of energy balance [24]. Indeed, early studies on animals showed that lesions of mediobasal hypothalamic nuclei (notably, the arcuate nucleus (ARC), the ventromedial hypothalamus (VMH) and the dorsomedial hypothalamus (DMH)) produced hyperphagia and obesity [25], whereas lesions of the lateral hypothalamus (LH) led to starvation and eventually death [26]. Later works developed these findings pointing out that hypothalamic nuclei maintain energy homeostasis through reciprocal and complex neuronal connections.

The hypothalamic regulation of energy balance is controlled by peripheral signals via two different mechanisms. The first is the indirect stimulation by the nucleus of the solitary tract (NTS), which receives parasympathetic afferent fibers of the vagus nerve conveying information about the physical quality of food, chemical nature of nutrients and mechanical changes of the gut. The second mechanism is the direct effect of several peptide hormones that are produced in peripheral tissues, secreted in the bloodstream and that reach the brain by crossing the blood brain barrier (BBB) [27].

1.3.1.2 Peripheral regulators of energy balance

The relevance for the body of an accurate control over the energetic flow is attested by the large number of factors regulating food intake and energy stores that are produced in peripheral tissues. Most of them, released by the adipose tissue, the pancreas and the gastrointestinal tract are described in Figure 2.

Depending on their effect on feeding and energy balance, these peripheral factors can be divided in short-term signals, which act in the brain to control meal size and satiety, and long-term signals that fluctuate in proportion to changes in basal energy stores. Since a detailed description of all these factors, is beyond the scope of this manuscript, only leptin and ghrelin will be shortly described. Leptin and ghrelin, a long-term and short-term factor respectively, are functionally antagonistic hormones. Both have a major role in homeostatic hunger but they are also involved in hedonic hunger (see paragraph 1.2.2.3).

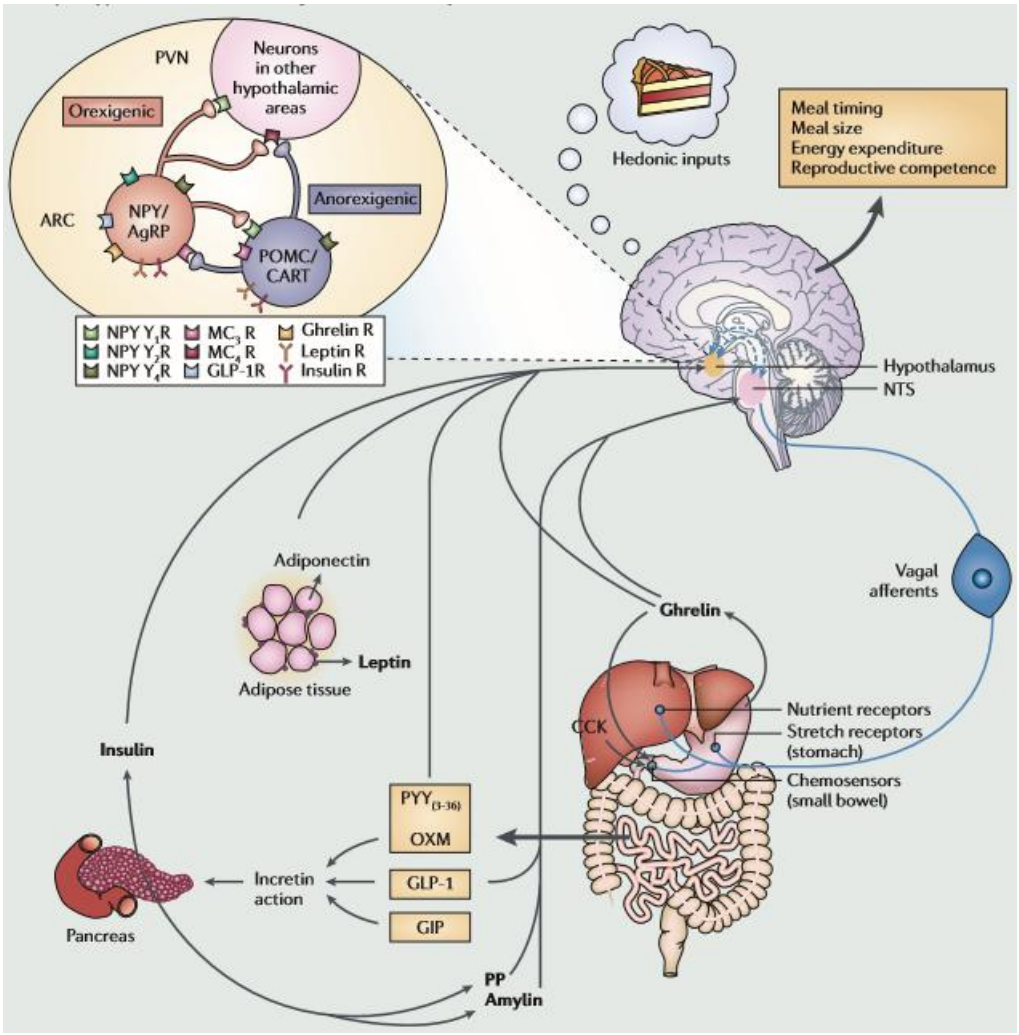

Figure 2. Central regulation of appetite and energy homeostasis

The brain integrates multiple peripheral and neural signals to control the regulation of energy homeostasis, maintaining a balance between food intake and energy expenditure. Peripheral factors indicative of long-term energy status are produced by adipose tissue (leptin, adiponectin) and the pancreas (insulin), whereas the acute hunger signal ghrelin (produced in the stomach), and satiety signals such as the gut hormones peptide $YY_{3–36}$ ($PYY_{3–36}$), pancreatic polypeptide (PP), amylin and oxyntomodulin (OXM) indicate near-term energy status. The incretin hormones glucagon-like peptide-1 (GLP-1), glucose-dependent insulinotropic peptide (GIP), and potentially OXM improve the response of the endocrine pancreas to absorbed nutrients. Further feedback is provided by nutrient receptors in the upper small bowel, and neural signals indicating distention of the stomach's stretch receptors, which are primarily conveyed by the vagal afferent and sympathetic nerves to the nucleus of the solitary tract (NTS) in the brain stem. (From D. Cook and S. Bloom, Nature Reviews, 2006 [27]).

LEPTIN

The hypothesis that a peripheral factor could regulate body weight was postulated for the first time in the 1970s when Coleman showed that recessive mutations in the mouse *ob* gene led to obesity and diabetes [28]. More than 20 years later, Friedman's team identified and characterized the *ob* gene and its product, which was named leptin from the Greek word *leptos* = thin [29]. Leptin,

whose structure is similar to cytokines, is mainly but not exclusively synthesized by white adipose tissue in proportion with stored fats.

Mutations of the *ob* gene in mice cause leptin deficiency, hyperphagia, hypothermia, and morbid obesity accompanied by several metabolic and neuroendocrine abnormalities [30]. Human *ob* gene mutations, coding for a leptin protein incapable of being secreted, are rare and were first reported in two Pakistani children and in three members of a Turkish family [31]. Intriguingly, unlike ob/ob mice, hyperinsulinemia, hyperglycemia, hypercorticism and hypothermia have not yet been reported in obese leptin-deficient patients. The reasons for these species differences are not known but they might reflect distinct contribution of leptin in the adaptation to changes in energy balance.

Multiple factors seem to regulate leptin gene expression because regulatory elements have been identified within the *ob* gene promoter, including a c-AMP response element-binding (CREB) binding site and a glucocorticoid response element (GRE) [32]. Glucocorticoids (GCs) have been shown to enhance leptin release, and leptin regulates in turn the hypothalamus-pituitary-adrenal (HPA) axis by reducing the adrenal production of GCs [33, 34]. There is also strong evidence of a positive correlation between insulin and leptin, with leptin expression increasing after insulin infusion, whereas during fasting, the fall in insulin level is accompanied by a decline in plasma leptin [35].

To date, six different isoforms of leptin receptor have been identified. Leptin receptors originate from the alternate splicing of the diabetic gene *db*. All isoforms share an identical extracellular ligand-binding domain, typical for class I cytokine receptors, but differ at the intracellular carboxy-terminus [36]. Depending on the length of the intracellular domain, the six isoforms can be classified into three classes: soluble (Ob-R_e), short (Ob-R_a, Ob-R_c, Ob-R_d and Ob-R_f) and long (Ob-R_b). Although initially only the long form of the receptor has been thought to be the functional receptor, subsequent researches have demonstrate that also the other isoforms have physiological relevance. The short forms are highly expressed in non-neural cells of the choroid plexus and microvessels where they seem to regulate leptin transit across the blood-brain barrier [37], whereas, the soluble form acts as a buffering system for free circulating leptin [38]. Long leptin receptors (Ob-Rb) are expressed both centrally and peripherally and this wide distribution in neural and extra-neural tissues, associated with the activation of several signaling pathways [39-41], reflects the extreme functional pleiotropy of leptin.

The first physiological function attributed to leptin, after the discovery of its receptors in the hypothalamus, was the ability to regulate energy homeostasis and reduce adiposity. This activity is

the result of food intake reduction, stimulation of energy expenditure through brown-adipose tissue fat oxidation, and secretion of other hormones influencing food intake and fat deposition, notably the thyrotropin-releasing hormone (TRH), the growth hormone (GH), the corticotropin-releasing hormone (CRH) the adrenocorticotropic hormone (ACTH) and glucocorticoids [42]. Since, leptin receptor immunoreactivity and mRNA are also expressed in many brain areas that are not directly associated with the regulation of energy balance [43], other functional roles for this adipokine have been suggested. Now there is strong evidence that leptin, besides energy homeostasis, is also implicated in reward motivation, learning and memory, stress responsivity, reproduction and brain development [44].

A common feature of obese rodents and humans is an increase of the blood free-circulating leptin accompanied by a loss of sensitivity to this hormone in the brain. As with insulin, obese subjects develop leptin resistance [45]. Although several studies have been conducted to elucidate the mechanisms underlying this resistance, this phenomenon remains to be fully understood. It is worth to note that leptin resistance is not a specific characteristic of obesity because it can also occur in the frame of the normal physiology of leptin. Indeed, loss of sensitivity to leptin appears in rodents and humans during pregnancy and lactation but also in seasonal animals that regulate their body fat deposition depending on the external environment [46].

Different mechanisms have been suggested to explain the reduced activity of leptin in the brain during obesity. They include: a defective transport across the blood brain barrier [47, 48], an alteration of the number of receptors expressed on cell membranes [49] and finally, an impairment of leptin signaling due to inhibition of its intracellular cascade pathway [40].

GHRELIN

Ghrelin, a potent orexigenic factor, is mainly produced by the entero-endocrine cells of the oxyntic mucosa of the fundus of the stomach, but smaller amounts are also released by the gut and other peripheral organs. Centrally, ghrelin immunoreactive neurons are found in the medial hypothalamus, closed to the third ventricle [50]. Ghrelin released by these cells and that produced peripherally regulates the activity of ARC and PVH neurons stimulating food intake upon binding on its receptor (GSH-R1a) [51].

Plasma ghrelin levels, in humans and rodents, fluctuate according with food intake [52]. Indeed, they rise during fasting and immediately before meals, whereas they fall one hour after food consumption, suggesting a role of ghrelin in meal anticipation [53]. In line with this, GSH-R1a

expression is up-regulated during fasting or protracted food restriction. Circulating ghrelin is also under the control of energy stores as attested by the high plasma concentration found in anorexic patients and by the reduced levels observed in obese subjects [54, 55]. Besides appetite stimulation, ghrelin also mediates additional biological activities that promote positive energy balance, including the release of growth hormone and the metabolism of glucose and lipid [56].

Like leptin, the abundant central expression of ghrelin receptor outside the hypothalamus reveals the involvement of this hormone in other functions. Accordingly, ghrelin is involved in reproduction, learning and memory and motivational processes [57], in particular in the modulation of dopamine cortico-mesolimbic circuitry.

1.3.1.3 Neural circuits controlling homeostatic food intake

The arcuate nucleus (ARC) of the hypothalamus is situated between the third ventricle and the median eminence, a region where the BBB is relatively permeable. Consequently, this nucleus can receive and integrate several peripheral hormone signals, which act on two functionally different types of neurons that promote or inhibit food intake (Figure 2). The first population of neurons, found in the medial part of the ARC, is orexigenic and releases neuropeptide Y (NPY), agouti-related peptide (AgRP) and gamma-amino-butyric acid (GABA). The second class of neurons, located in the lateral portion of the ARC, is anorexigenic and expresses POMC (pro-opiomelanocortin) and CART (cocaine-amphetamine regulated transcript). These neurons reduce food intake by releasing the melanocortin α-MSH (α-melanocyte stimulating hormone). NPY neurons can also actively block the firing of POMC/CART neurons through direct inhibitory GABAergic projections.

After release, NPY exerts its orexigenic activity by binding Y1 and Y5 receptors, whereas α-MSH and AgRP behave respectively as an agonist and an antagonist of melanocortin receptors (MCR3 and MCR4), whose activation lead to food intake suppression and energy expenditure elevation.

Leptin and insulin are the most important hormones informing the brain about the energy stored. Both reduce food intake by stimulating, in the ARC, POMC/CART neurons and blocking the firing of NPY/AgRP neurons. The anorexigenic activity of other satiety peptides secreted by the gut after a meal, including peptide YY (PYY), pancreatic polypeptide (PP), and the incretin hormones glucagon-like peptide 1 (GLP-1) and oxyntomodulin (OXM), depends on their inhibitory effect on

NPY/AgRP neurons. According to its orexigenic property, ghrelin has opposite action by enhancing the release of NPY and AgRP.

As shown in Figure 3, arcuate neurons exert their effect on food intake by modulating the activity of second order neurons located in other hypothalamic nuclei. In the PVH, NPY/AgRP and melanocortin projections terminate on thyrotropin-releasing hormone (TRH) neurons, which regulate the pituitary-thyroid axis and influence metabolic rate and appetite. TRH neurons also express LepRb and, are thus directly affected by leptin [58]. A similar mechanism, in the PVH, exists also for corticotropin releasing hormone (CRH) neurons that are simultaneously regulated by arcuate neuronal projections and by leptin. Like PVH, VMH contains second order neurons that are functionally connected with ARC cells. The most important class of second order neurons of the VMH is represented by brain-derived neurotrophic factor (BDNF)-releasing neurons that co-express melanocortin and leptin receptors. Once activated by α-MSH and/or leptin, these neurons help to stop food consumption and to enhance thermogenesis [59].

Finally, reciprocal connections exist between the ARC and the LH. On the one hand, ARC neurons regulate orexin (ORX) and melanin-concentrating hormone (MCH) neurons located in the LH, whilst, on the other hand orexin neurons send their projections back to the arcuate nucleus stimulating the activity of NPY/AgRP neurons and promoting food intake. It is worth to note that LH is also a bridge structure linking the hypothalamus with brain areas of the reward system, notably the ventral tegmental area, the nucleus accumbens and the amygdala. The connections between the LH and these areas are ensured by both ORX and MCH neurons [60, 61]. The release of orexin and MCH into the ventral tegmental area (VTA) and the nucleus accumbens (NAc) has been shown to modulate the mesolimbic dopamine release and increase motivation for food intake [62, 63].

This short description of hypothalamic circuits regulating food intake is not exhaustive, but highlights the intricate connections existing inside the hypothalamus. Over the past 20 years, animal studies have largely contributed to improve our understanding of these circuits and genetic approaches have allowed to identifying specific factors that have a critical part in these processes regulating energy homeostasis. However, in humans, monogenic forms of obesity due to mutations in genes coding, for instance, for leptin, POMC or MCR4 are extremely rare [64].

Although obese patients present alterations in plasmatic levels of many peripheral hormones (such as leptin, insulin and ghrelin), as well as concomitant leptin and insulin resistance, these changes are rather the result of body weight gain, than the cause of obesity. Recently genome-wide association studies have revealed that more than 30 genes, whose function is unknown, are

expressed in the brain and correlate with obesity [65]. It is therefore possible that future studies will allow to identify other factors and biological mechanisms involved in the regulation of energy balance and responsible for obesity vulnerability.

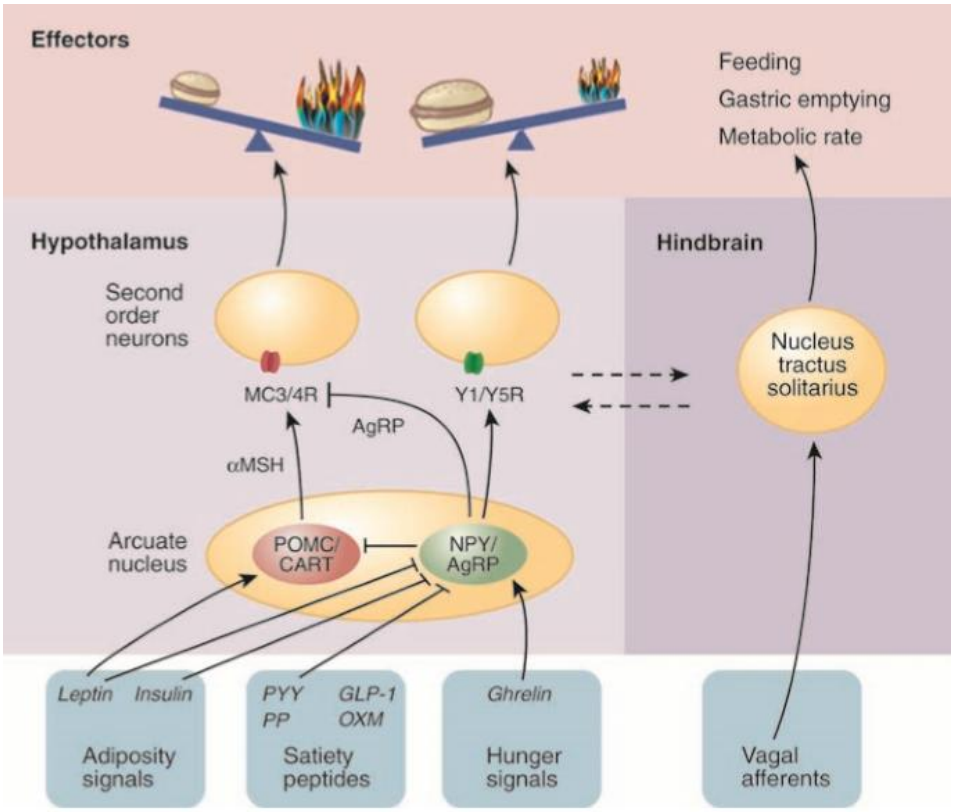

Figure 3. Simplified representation of gut peptides acting on the hypothalamus.
Access of circulating agents into the arcuate nucleus of the hypothalamus is facilitated by a relaxed blood-brain barrier. Primary neurons in the arcuate nucleus contain multiple peptide neuromodulators. Appetite-inhibiting neurons (red) contain pro-opiomelanocortin (POMC) peptides such as a melanocyte-stimulating hormone (αMSH), which acts on melanocortin receptors (MC3 and MC4) and cocaine- and amphetamine-stimulated transcript peptide (CART), whose receptor is unknown. Appetite-stimulating neurons (green) contain neuropeptide Y (NPY), which acts on Y receptors (Y1 and Y5), and agouti-related peptide (AgRP), which is an antagonist of MC3/4 receptor activity. Integration of peripheral signals within the brain involves interplay between the hypothalamus and hindbrain structures including the NTS, which receives vagal afferent inputs. Inputs from the cortex, amygdala, and brainstem nuclei are integrated as well, with resultant effects on meal size and frequency, gut handling of ingested food, and energy expenditure. ⟶ direct stimulatory; ⟊ direct inhibitory; – –► indirect pathways. (From: Badman MK and Flier JS, Science, 2005[66]).

1.3.2 Hedonic regulation of food intake

1.3.2.1 Evolutionary relevance of hedonic regulation

Energy need is not the only driver of food intake. Everybody has, at least once, experienced the desire to eat a delicious dessert after a satiating meal. This temptation to eat, in absence of caloric need, is the result of the rewarding properties of food. From an evolutionary perspective, the

importance of hedonic processes makes sense. The association between positive hedonic experiences with caloric (enriched) food has evolved to provide the necessary motivation to engage in carbohydrate- and fat-enriched foods that represents the caloric load necessary for survival [67]. Eating behavior is not limited to food ingestion but consists of preparatory, consummatory and post-consummatory phases, all of which depend on the activation of the reward system [68]. In the *preparatory phase*, before any oral contact with food, reward expectancy plays a pivotal role. This phase requires a decision-making process that allows switching attention from other activities toward food seeking. Research suggests that, in order to make this choice, the brain uses representations of reward-expectancy and effort/risk requirements, both acquired from prior experiences [69, 70]. Later, during the *consummatory phase*, direct pleasure derived from gustatory and olfactory sensations, stimulates food consumption throughout the meal until satiation signals dominate [71]. At the end of the meal, during the *post-consummatory phase*, associations between the pleasure deriving from food ingestion and food-related cues, are learned and stored in brain areas controlling memory and executive processes [72]. Associative memories between food and environmental cues allow the creation of mental representations of reward-expectancy that stimulate again food seeking when needed.

When food is scarce, reward expectancy and the pleasure deriving from food ingestion represent the main drivers for food seeking. Given the relevance of the reward system in hedonic hunger a short description of this system will be provided in the next session.

1.3.2.2 Neural circuits controlling hedonic food intake

The brain reward system consists in a large group of brain structures, including midbrain nuclei, basal ganglia, limbic and cortical regions [73, 74] (Figure 4). Reward expectancy, which requires the assignment of incentive salience to rewards and the evaluation of the probability to get them, depends on the integrated function of mesolimbic and cortical structures. Among neural pathways that are activated by rewarding stimuli, the best characterized is the mesolimbic dopamine pathway. This neural circuit is constituted by VTA dopaminergic fibers that project to GABAergic neurons of the nucleus accumbens (NAc). [75]. VTA-dopaminergic neurons also innervate others brain regions, including the prefrontal cortex (PFC), the amygdala and the hippocampus, forming the so-called cortico-limbic dopamine pathway. Limbic and cortical regions are reciprocally connected by glutamatergic fibers and send their projections back to the NAc [76].

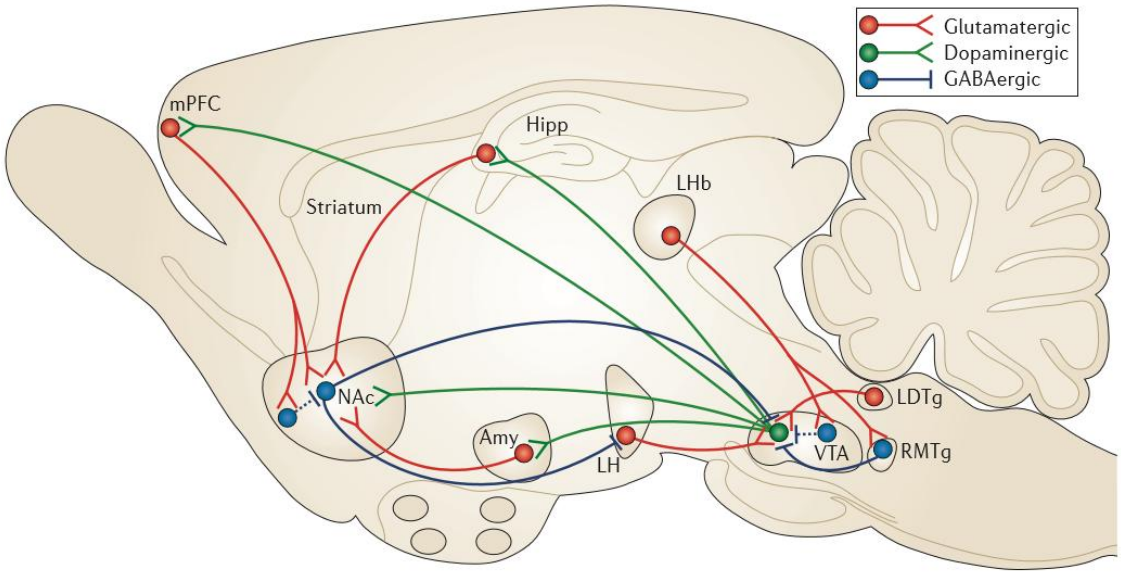

Figure 4. Reward circuits. A simplified schematic of the major dopaminergic, glutamatergic and GABAergic connections to and from the ventral tegmental area (VTA) and nucleus accumbens (NAc) in the rodent brain. The primary reward circuit includes dopaminergic projections from the VTA to the NAc, which release dopamine in response to reward-related stimuli. There are also GABAergic projections from the NAc to the VTA. The NAc receives dense innervation from glutamatergic monosynaptic circuits from the medial prefrontal cortex (mPFC), hippocampus (Hipp) and amygdala (Amy), as well as other regions. The VTA receives such inputs from the lateral dorsal tegmentum (LDTg), lateral habenula (LHb) and lateral hypothalamus (LH), as well as both GABAergic and glutamatergic connections from the extended amygdala (not shown). These various glutamatergic inputs control aspects of reward-related perception and memory. The dashed lines indicate internal inhibitory projections. The glutamatergic circuit from the LH to the VTA is also mediated by orexin (not shown). From S.J. Russo and E.J. Nestler, Nature Reviews - Neuroscience, 2013 [77]).

Even though dopamine was initially thought to act as a hedonic signal, it is now believed that dopamine transmission mediates the assignment of incentive salience to rewards and reward-related cues. In order to assign incentive salience to a reward, the brain has to learn under which circumstances reward occurs and make a prediction about the probability to get it. It has been proposed that the way in which dopamine is released in the brain may reflect the accuracy of this prediction [78]. This hypothesis is supported by the observation that no prediction error (no deviation from expectation) is associated with a tonic release of dopamine, a positive reward prediction error (better than expected) is associated with phasic burst of dopamine and a negative prediction error (worse than expected) results in pauses in dopamine release [79].

Palatable food, like other kind of rewards, stimulates dopamine release in the NAc. This ability was first documented in a rat study in which increased levels of dopamine were detected in the NAc in response to eating and drinking using *in-vivo* microdialysis [80]. Other human brain imaging studies have confirmed this finding showing that food and food-related cues can activate corticolimbic and

mesoaccumbens dopamine circuits [81, 82]. Food-induced dopamine release has also been found to activate different brain regions in the human brain, which were comparable to those structures activated by palatable food consumption in the rat brain. In rodents, brain activation was attested by increased expression of immediate early genes, such as *c-fos*, *arc* or *zif268* mainly in corticolimbic structures [83, 84].

Beside the motivational response ("wanting response"), which reflects how much the reward is desired [85], food elicits a hedonic response ("liking response"), which originates from the sensorial pleasurable properties of the reward. Even though pleasure and desire for food are often associated, extensive investigations of Kent Berridge and collaborators have shown that "liking" and "wanting" are distinct brain responses [86] Many brain regions are activated by pleasure, such as neocortical structures (PFC, orbital frontal cortex (OFC) and insular cortex) and subcortical forebrain structures (ventral pallidum (VP), NAc and amygdala) [87-90]. However, it is worth noting that not all brain structures activated by pleasure generate pleasure. In particular, two brain structures have this peculiarity: the NAc and the VP. Inside these structures, small regions called hedonic hot spots (about $1mm^3$ in the rat and $1cm^3$ in human brain) are responsible for generating the subconscious feeling of pleasure. The activation of these hot spots is subjected to a complex regulation exerted by different neurotransmitters, such as opioids, endocannabinoids, GABA and orexin, which can amplify the hedonic impact of palatable foods [91, 92].

In addition to the "wanting" and "liking" response some of the brain structures belonging to the reward system, notably the hippocampus, the amygdala and the PFC, mediate another type of response: the "learning" response, which assures the formation and the storage of associative memories linking reward pleasurable effect to paired external stimuli. The formation of these associative memories is crucial because it allows the recognition of cues in the environment, signals food availability, and triggers food seeking [93-95]. In rodents, four principal regions have been identified to support cue-induced feeding [96]. Whereas, amygdala, hippocampus, and prefrontal cortex are crucial for associative learning, and memory storage, the LH plays a role in motivated behavior and integrates hedonic information with metabolic signals [95]. In line with this role, animal studies have shown connections between the LH and brain reward areas [97, 98] and the electric stimulation of LH was found highly reinforcing [99, 100]. A bidirectional regulation exists between LH and brain reward structures. Orexin and MCH-producing neurons have been found to regulate the firing of dopaminergic neurons in the VTA [101, 102]. On the other hand, using a circuit tracing procedure, it has also been observed that the LH receives prominent projections from the NAc,

whose inhibitory effect on orexin and MCH neurons inhibit food consumption [103]. The LH is also functionally connected with other cortical and limbic sites implicated in the recognition of rewarding properties of palatable food, notably the OFC, insula, habenular complex and amygdala [104] In particular an amygdalo-hypothalamic circuit that links the basolateral amygdala to the LH is considered of high importance for mediating the ability of food-related cues to engage in food seeking in absence of energy requirements [95].

In physiological conditions, the impulsion to eat, induced by the reinforcing value of rewards (pleasure and motivation) and the conditioned learning, is inhibited by cortical top-down networks. Cortical regions, notably the PFC, the OFC and the insular cortex send projections to VTA/Nac and assure that the choice to eat is the consequence of a more complex decision making process taking into account both the energetic need and the hedonic drive [105]. The undermining of this cortical top-down control might result in impulsive and compulsive overeating (Figure 5).

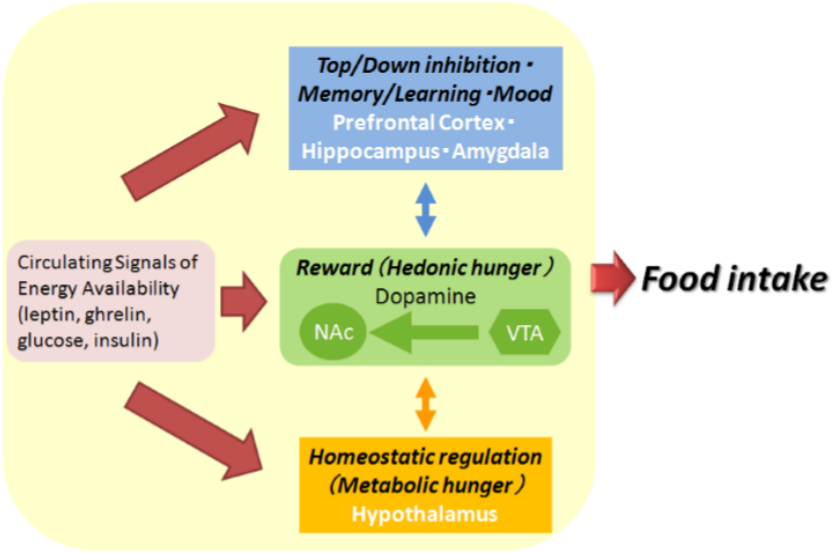

Figure 5. Schematic diagram of potential interactions between metabolic hunger and hedonic hunger which regulate food intake. Metabolic hunger regulated by homeostatic metabolic status is designed to preserve energy balance and protect minimal levels of adiposity. The hypothalamus plays crucial roles in the metabolic hunger. Reward circuit which is mainly regulated by the midbrain dopamine system from the VTA to NAc, is the main pathway of hedonic hunger. Memory and learning and mood interact with reward circuits. Circulating signals of energy availability, leptin, ghrelin, glucose, and insulin are thought to regulate food intake mainly via the hypothalamus, but recent studies show that they also regulate food intake via many extra-hypothalamic regions. VTA: ventral tegmental area, NAc nucleus accumbens. (From Yamada-Goto et al., 2013; http://dx.doi.org/10.5772/56228)

Given the strong implication of the reward system in promoting hedonic feeding, it is logic to hypothesize that impairments of its functions may have heavy consequence on normal eating behavior. Accordingly, it has been suggested that inefficient satiety signals combined with

alterations in the reward system or in the top-down inhibition exerted by cortical structures may lead to a prevarication of the hedonic over the homeostatic hunger.

1.3.2.3 Peripheral regulators of hedonic food intake

The pleasure and the motivation to eat change in relation to the nutritional (energetic) state of an organism. These changes are visible when brain responding to palatable food of hungry humans is compared with that of well-fed subjects. The hungry subjects showed stronger activation of NAc, amygdala, insula and OFC [106]. Conversely, overfeeding attenuated neuronal responses to palatable food, particularly in the insular cortex and hypothalamus [107-109].

LEPTIN

The observation that hunger and satiety can alter the rewarding properties of food has led to the hypothesis that peripheral energy balance modulators might affect hedonic hunger, as well. Evidence of such an effect has been observed for several peptide hormones (Figure 6). Growing literature on this topic is shading light on their involvement in hedonic, motivational and learned responses elicited by food and food-related cues.

The discovery of leptin receptors on VTA-dopaminergic neurons was the first evidence of a possible modulation of midbrain dopamine by this adipokine [110-112]. In following animal studies, it has been observed that direct injection of leptin in the brain decreased dopamine release and suppressed food intake [113], whereas adenoviral knockdown of the leptin receptor in VTA increased preference for palatable food and enhanced motivational properties of food [112, 114]. It has been argued that this effect of leptin on food motivation may be due to its ability to directly inhibit the firing of dopamine VTA neurons projecting to amygdala, and therefore, to its ability to suppress emotional cue-induced feeding [115]. A second postulated mechanism is the inhibition of ORX neurons that from the LH project to VTA-dopaminergic neurons. By doing so, leptin would reduce dopamine release indirectly suppressing the firing of ORX neurons [114, 116].

There is also evidence that leptin may affect the “liking” component of palatable food. Accordingly, congenital leptin deficiency in humans was found to increase striatal activation in response to images of food, while leptin replacement in the same subjects, attenuated the self-reported liking for food [117]. Similar investigations, in rodent models of leptin deficiency, have shown enhanced taste and olfactory sensitivity for sweet solutions [118-120]. All these observations suggest that the anorexigenic effect of leptin does not depend only on its regulation of homeostatic hunger

but, as well as, on its capacity to attenuate the hedonic and motivational components of food reward.

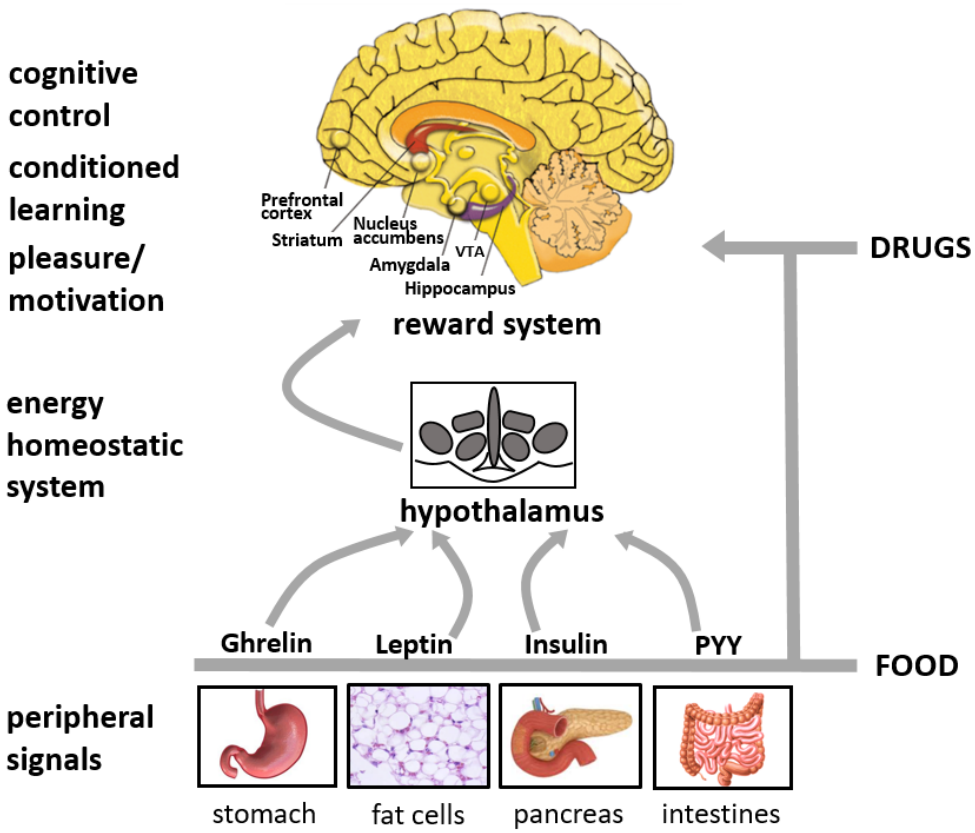

Figure 6. Effect of food and drugs on the brain reward-pathways. In contrast to drugs whose actions are triggered by their direct pharmacologic effects in the brain's reward pathways, the regulation of eating behaviors and hence the responses to food are modulated by multiple peripheral and central mechanisms that directly or indirectly convey into the brain's reward pathways, including those involved in pleasure and cognitive control. PYY, peptide YY; (Adapted from: Volkow et al., Biol Psychiatry 2013 [121])

INSULIN

Insulin is another hormonal regulator of energy balance that can influence hedonic food intake modulating striatal feeding circuits and midbrain dopamine input onto these circuits [122]. Accordingly, insulin receptors are expressed in the striatum [123] and on midbrain dopamine neurons [110]. The action of insulin is comparable to that of leptin because infusion of insulin in the VTA decreased food intake in rats [113, 124], whereas selective deletion of insulin receptors in mouse midbrain dopamine neurons resulted in hyperphagia and increased weight gain [125]. Likewise, diabetic rats have strongly diminished levels of dopamine in midbrain, yet demonstrating that insulin signaling is necessary to maintain appropriate dopamine transmission. Noteworthy, leptin and insulin exert a similar control on homeostatic and hedonic feeding and their actions are

reciprocally stimulated. A proposed explanation for this mutual activation is the convergence of their intracellular signaling pathways. Indeed, although leptin signaling depends mainly on the activation of the JACK/STAT3 pathway, this adipokine has also been found to trigger the insulin receptor substrate (IRS)/phosphatidylinositol 3-kinase (PI3K) intracellular cascade activated by insulin [35].

GHRELIN

Ghrelin, like leptin and insulin, controls hedonic feeding but in the opposite way. In rodents, VTA-dopamine neurons were found to express ghrelin receptors [126], and central or peripheral administration of ghrelin increased locomotion, dopamine release in the NAc and food intake [126-129]. On the other hand, intra-VTA administration of a GSH-R1a antagonist blocked ghrelin-induced food intake [130]. These and other similar observations have led to the hypothesis that ghrelin would stimulate dopamine release, and consequently, would promote food seeking behavior increasing the incentive value of food and food-related cues. According to this idea, ghrelin has been shown to drastically increase cue-related feeding in satiated rats [131, 132]. These rodent studies seem to be relevant in humans as well because, in a fMRI study, intravenous injection of ghrelin in healthy subjects induced amygdala, ventral striatum, insular and orbitofrontal cortex activation in response to food pictures [133].

More recently glucagon-like peptide (GLP-1) and peptide YY (PYY), two gut hormones acting as satiety signals, have been added to the list of peripheral factors that modulate hedonic hunger. Both have been found to affect key reward circuits in the VTA and NAc, and to suppress motivation for food rewards [134-136]. In sum, it is evident that the concept viewing peripheral signals as factors regulating only homeostatic hunger is no more valid. Therefore, further studies on obesity and eating disorders have to take into account the capacity of these factors to interact with both systems.

1.3.2.4 Reward system dysfunctions as possible cause of obesity and eating disorders

Hedonic regulation of food intake, so crucial in periods of food shortage, has become maladaptive in modern society because of the larger availability of highly rewarding palatable food (i.e. food enriched in sugar, fat and salt). Palatable foods increase food intake and promote obesity as demonstrated by the copious literature about the development of diet-induced obesity in humans and rodents [137-139]. These findings have led to the hypothesis that repeated consumption of

high-fat or high-sugar food may affect reward system functions (“wanting”, “liking” and “learning” responses) and, in the most severe situations, lead to a loss of control over food intake.

Hence, in line with this hypothesis, which implicitly considers that palatable food has similar properties than other abused substances, many studies have attempted to show abnormalities in the reward system of obese subjects. As expected, alterations between obese and lean subjects have been reported in motivational, hedonic and cognitive behavioral responses, as well as, in the activation of brain areas involved in these responses.

Considering the peculiar role of the striatal region in motivational aspect of food, several studies have been focused on this brain structure showing, in obese humans and rats, increased striatal activation in response to palatable food and visual food cues [140-145]. Moreover, a human PET study, targeting specifically dopamine neurotransmission found lower striatal expression of D_2 dopamine receptors in obese patients relative to normal-weight controls [146]. Likewise, decreased D_2 receptor levels were also detected in a rat model of obesity [147]. This reduction of dopamine D_2 receptors in striatal neurons is of particular interest because a similar observation was previously made in drug addicted subjects [148] and suggests that the repeated consumption of palatable food may modify brain dopamine transmission. Additionally, using a lentiviral approach, Johnson and Kenny demonstrated that knocking down striatal D_2 receptors in rats exposed to high caloric diet, exacerbated compulsive eating [149].

Other abnormalities between obese and lean subjects have been identified in the OFC. Dysfunction in this latter are associated with an impaired ability to modify the rewarding properties of food as a function of the context (i.e. to attribute less rewarding properties to food when satiated) and can result in compulsive consumption. Accordingly, damage of the OFC was found to induce binge-eating of palatable food in humans [150, 151] and lesions of the OFC in animals increased the preference for immediate small reward over delayed larger rewards [152, 153].

The insular cortex is mainly involved in the integration of gustatory properties of food but is also activated by other physical features such as texture, temperature, olfactory and visual properties. In two imaging studies, obese individuals and young adults at risk of developing obesity showed enhanced activation of this structure in response to palatable food [154, 155]. The insular cortex has been involved in a phenomenon called “negative contrast”, which consist in the rejection of standard food by humans and rodents habituated to consume palatable food [156]. Therefore, hyperactivation of this brain region may contribute to shift preference from standard food toward

more palatable food. In agreement with this concept, lesions of rat insula abolished this negative contrast effect and animals did not refuse to eat standard food [157].

Besides altered motivation, overeating may also originate from higher perception of food palatability. Consequently, it has been suggested that obese individuals may have higher preference for high-fat and high-sugar food taste. In the past, contrasting results were obtained from the comparison of food preference in obese and lean subjects. Whereas some studies showed enhanced preference for palatable food taste in obese patients, [158-160], other works did not show any difference [161]. However, more recent investigations have provided convincing evidence for altered taste sensitivity in obesity [162, 163], suggesting that overeating may be caused by changes in the hedonic perception of food properties.

Food consumption is also powerfully influenced by a variety of environmental factors that are unrelated to energy requirements [164]. In particular, we have already seen that cues, paired with eating, can be recognized, memorized and subsequently used to stimulate eating also in a satiated state. In agreement with several rodent studies, in humans the intense desire to eat a particular food (food craving) can be triggered by food-related cues [165-167]. In more recent imaging studies, the comparison of obese and overweight individuals with normal-weight controls has shown, in the former, enhanced food cue reactivity, especially in the insular and cingulate cortex [168-170]. These results are particularly interesting because they are reminiscent of those found in drug addicted subjects. In drug addiction it has been postulated that one of the reasons for the loss of control over drug use may be the dominance of cue-induced behaviors over the top-down cortical inhibition. Although, it is not yet proven, a similar mechanism may also be responsible for compulsive overeating observed in some cases of obesity and eating disorders.

Globally, there is considerable evidence for alterations of the reward system, at least in some classes of obese individuals. Nevertheless, it is not ascertained whether these abnormalities represent a cause or a consequence of body weight gain. Initially, it has been proposed that chronic exposition to palatable food may mediate these alterations of the reward system and drive overeating. However, not all people consuming dense calorie food present the same risk to develop obesity. Thus, preexisting individual differences in the motivational and hedonic response to palatable food may explain the higher risk of some individuals to eat beyond energetic requirement.

Two independent theories have been proposed as an attempt to explain these individual differences. The first theory, also defined as “reward-hyperfunction hypothesis”, considers that some people would have altered perception of the hedonic value of food, which would trigger

excessive motivation for food seeking and taking. Excessive activation of "liking" substrates might magnify the hedonic impact of food and therefore contribute to binge eating and obesity. The second alternative theory, the "dopaminergic hypofunction hypothesis", suggests that there would be individual differences in the expression of dopamine receptors in the reward system and, in particular, in the striatal region. As a result, those subjects expressing dopamine receptors at lower level would eat more palatable food to counterbalance the reduced dopamine signaling [171]. Accordingly, the A1 allele of the Taq1A gene that cause a 30% to 40% reduction in striatal D2 receptors shows greater preponderance in chronic drug users and in obese individuals compared with the general population [172].

Noteworthy, the concepts that repeated palatable food consumption could modify reward-related responses, and that some individuals may be genetically vulnerable to palatable food effects, are not mutually exclusive and it is likely that both conditions coexist for those individuals that develop compulsive overeating for palatable food [173, 174].

1.4 OBESITY-DEPRESSION INTERACTION

Since obesity has long been considered a metabolic illness, while depression was classified as psychiatric disorder, both basic science and medicine have long investigated these pathologies separately. Compelling clinical evidence suggests, however, that the relation between mood disorders and body weight alterations cannot be ignored. Indeed, while several depressive patients lose weight, others develop obesity. Although antidepressant therapy is likely responsible for positive energy balance in some of these patients, obesity occurs in other depressed subjects independently of medications. The overlap between obesity and depression was, for the first time, convincingly proposed by an extensive study reviewing clinical observations from 1966 to 2003 [175]. More recent publications, also reported relevant outcomes supporting a bidirectional link between obesity and depression, by which each disease may contribute to the other [176-178]. The current opinion is rather that obesity and depression are likely related diseases with distinct, but overlapping, pathophysiology. A more extensive discussion of biological underpinnings of the obesity-depression interaction can be found in the Article 3 at page 47. Hereafter, are debated some of those risk factors believed to participate in the etiology of both pathologies.

1.4.1 Common factors in the etiology of obesity and depression

1.4.1.1 Inflammation

A typical feature of obesity is the development of a low-grade chronic systemic inflammation that is mainly a consequence of excessive fat deposition in adipocytes. Indeed, upon fat accumulation, adipocytes release different factors, including adipokines, cytokines and chemokines [179, 180], which attract macrophages that are responsible for the inflammatory response. The systemic inflammation of obese individuals is attested by high serum levels of inflammatory factors, among which, interleukin-6, TNF-α and C-reactive protein [179, 180].

Cytokines are mostly produced in peripheral tissues and, due to their large molecular weight, can enter the brain only through cytokine-specific transporters or by crossing the BBB in permeable brain areas. The hypothalamus is one of these brain areas and, therefore, it is directly affected by the serum rise of inflammatory molecules. Accordingly, elevated levels of inflammatory cytokines have been found in *post-mortem* brain of obese patients [181]. Likewise, severe inflammation of the hypothalamus and compromised activity of NPY and POMC neurons, were found in rodent models of obesity [182-185]. Hence, it is likely that brain inflammation may impact on food intake regulation facilitating obesity progression.

Concomitantly, inflammation frequently accompanies depression. In this regard, chronic increase of pro-inflammatory and inflammatory markers has been repetitively observed in depressed patients [186-189] and it has led to the "cytokine hypothesis of depression" that considers depression as the result of a maladaptive response that occurs after a sustained and persistent cytokine release [190]. While there is convincing evidence of plasmatic increase of cytokines in depression, little is known about how these inflammatory modulators may affect mood. Research conducted on this topic proposes that chronic inflammation may affect central neurotransmission, in particular that of the serotonergic and dopaminergic system altering the synthesis, the release and the synaptic uptake of these monoamines [191-194].

It has been recently observed that the quality of diet can modulate the inflammatory response. In particular, the Mediterranean diet that privilege the consumption of fruits and vegetables lowers blood levels of interleukins and other inflammatory factors [195-197]. Although, the specific nutrients mediating this protective effect have not yet been clearly identified, the use of an appropriate diet able to minimize the development of inflammatory processes may be an

inexpensive and useful complementary approach to reduce the risk and the progression of both pathologies.

1.4.1.2 Stress

The hypothalamic-pituitary-adrenal (HPA) axis is a complex neuroendocrine system, composed of multiple brain structures and peripheral organs (Figure 7), allowing an animal to cope with stressful events [198]. Emotional and stressful stimuli, processed in the amygdala, activate a class of PVH neurons that, in turn, secrete the corticotropin releasing hormone (CRH) [199, 200]. The main effect of this hormone is to divert attention and energy toward those behaviors that allow dealing with the stressor. The principal CRH-target neurons are located in the anterior pituitary gland and release the adrenocorticotropic hormone (ACTH) in the bloodstream. This hormone stimulates the cortex of the adrenal gland to secrete glucocorticoids (GCs) hormones, notably cortisol in humans and corticosterone in rodents. GCs receptors are widely distributed in the body and their binding with GCs leads to the activation or repression of a plethora of genes that regulate the biological mechanisms able to restore the depleted energy, such as food intake, gluconeogenesis and fat deposition [201].

When the exposure to stressful stimuli is limited, the stress response has short duration because GCs exert a feedback inhibition on the HPA axis. Specifically, they act on the pituitary and hypothalamus limiting the release of CRH and ACTH, which reduces their own activity. GCs also stimulate GC receptors in the hippocampus, where inhibitory GABAergic projections to PVH neurons further block the CRH secretion [202, 203]. However, when stressful events are chronic, the HPA axis becomes hyperactive and the feedback control ineffective. Excessive or chronic stress, and the subsequent hyperactivation of the HPA axis, is considered a strong risk factor for developing a huge variety of diseases and disorders, including mood disorders, obesity and eating disorders [204].

During the past three decades, several groups reported compelling evidence showing that protracted stress response may represent a biological mechanism triggering depression [205-207]. Indeed, several abnormalities were found in stress hormone regulation in depressed patients. These abnormalities included: 1) hypercortisolemia [208], 2) increased CRH concentration in the cerebrospinal fluid [209] 3) increased number of CRH expressing neurons in the PVH and reduced number of pituitary and frontal cortex CRH receptors in *post-mortem* tissues of suicide victims [210, 211] and 4) blunted pituitary ACTH release after systemic administration of CRH [212]. Altogether, these findings have been interpreted as a consequence of the chronic sustained release of CRH. Indeed, it

has been suggested that in the long term, the excessive production of CRH would desensitize pituitary-CRH receptors and reduce ACTH release. In parallel, the adrenal gland, that has been chronically over-stimulated, would become hypertrophic and hyper-responsive to ACTH and would secrete abnormally higher quantity of cortisol [213].

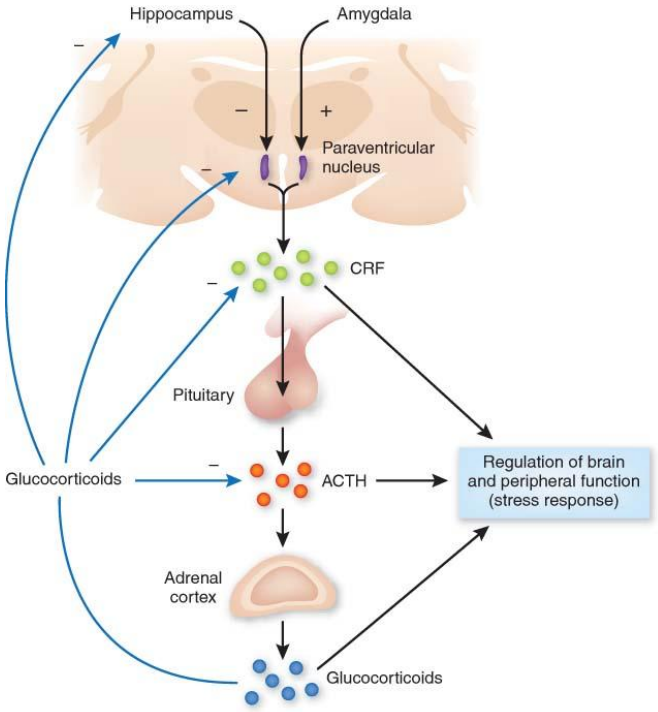

Figure 7. Principal brain structures and hormones of the hypothalamic-pituitary adrenal (HPA) axis. Emotional stressful stimuli activate amygdala that induces the release of CRF (or CRH) from neurons of the paraventricular nucleus of the hypothalamus. CRF stimulates the anterior pituitary to secrete ACTH that, in turn acts on the adrenal gland. The adrenal cortex release glucocorticoids that are responsible of stress response. When the stressful stimulus ceases, glucocorticoids inhibit the HPA axis through a feedback loop.

A plausible mechanism explaining how CRH may affect mood regulation is based on the interaction of this hormone with the noradrenergic and serotonergic neurotransmission. Indeed, injection of CRH in the rat locus coeruleus, the main source of NA in the brain, has been found to alter catecholamine turnover in the prefrontal cortex, and similar injection in the dorsal raphe caused substantial inhibition of the serotonergic neurotransmission to the forebrain [214, 215]. Findings resulting from pharmacological and genetic manipulation of the CRH system are also in agreement with a role of CRH in the mediation of anxiety and depressive symptoms. Indeed, mouse over-expression of CRH or its receptor CRH-R1 increased stress response whereas the knockdown of both, or the treatment with a CRH-R1 antagonist, reduced anxiety and depressive-like behaviors [216-218].

In the acute stress response, as said before, behaviors that can divert the attention from the stressor, such as food seeking, have to be temporally suppressed. This temporal inhibition of food intake is the result of the inactivation of NPY/AgRP neurons by CRH released in the ARC [219]. However, this effect of CRH is of short duration and it is subsequently reversed by the action of GCs, which allow replacing the energy used to face the fearful stimulus. To facilitate energy storage, GCs stimulate food intake by increasing the release of NPY/AgRP [220] and suppressing POMC gene expression [221] in the arcuate nucleus. GCs interact also with leptin and insulin but this interaction is not completely understood, because GCs increase the expression of leptin and insulin but, at the same time, seem to promote leptin and insulin resistance [222, 223]. In addition to food intake, GCs increase energy storage facilitating fat deposition in white adipose tissue, especially in the abdominal visceral adipose tissue [224].

Beside these actions, the persistent secretion of GCs during chronic stress increases preference for high-calorie food that, when consumed in high quantity, contributes to exacerbate body weight gain. Several animal models have highlighted these stress-induced changes in feeding preference [225, 226]. One explanation for this shift toward more palatable food may be the stress-dependent release of ghrelin. Elevation of plasmatic ghrelin levels has been observed in several studies of animals and humans submitted to stress [227-230]. Some of them have argued that altered ghrelin levels may explain the consumption of palatable food suggesting that the excessive consumption of palatable food, also defined as “comfort food”, would represent a mechanism allowing to cope with a stressful situation [231]. Interestingly, in a human study that enrolled individuals consuming more palatable food during stress (“emotional eaters”) and others whose food intake was suppressed or unchanged by stress (“non-emotional eaters”), it was observed that only the non-emotional eaters had a decline in ghrelin levels after food consumption. In line with this finding, authors proposed that higher plasmatic ghrelin in emotional eaters may be the cause for sustained hedonic eating in these subjects [232].

In conclusion, the repeated exposure to stressors affects the HPA axis and leads to anxiety and mood alterations, symptoms that characterize depressive disorders. Concomitantly, increased appetite, especially for calorie-dense food induced by excessive release of GCs would increase fat deposition and body weight gain [233]. Stress represents, therefore, a very potent risk factor for the parallel development of obesity and depression.

1.4.1.3 Reward system deficits

Deficits of the reward system as a cause for increased hedonic food intake in obesity have been already discussed in the paragraph 1.2.2.4. Hereafter, we will focus on anhedonia, a depressive symptom that has its origin in impairments of the reward system.

Anhedonia, defined as a reduced ability to experience pleasure, is a core symptom of depression [234] even though it is frequently associated to other neuropsychiatric disorders, including substance use disorders [235], Parkinson's disease [236] and schizophrenia [237]. Early studies on depression have interpreted anhedonia as a simple absence of pleasure [238]. However, depressive patients that underwent the "sweet taste test", which consist in rating the pleasantness of different sucrose concentrations, did not always show changes in the hedonic perception when compared to control subjects [239-241]. These and other contradictory findings have progressively led to the concept that anhedonia is more than an absence of pleasure. In the last two decades, neuroscience has greatly contributed to discern the different components of reward processing (pleasure, valuation, anticipation, motivation and decision-making) and to recognize brain structures that sustain these processes [77]. Based on these neurobiological findings, a current consensus now considers anhedonia as an inability to experience pleasure, reported in some depressed patient only, and also an inappropriate valuation of reward, associated with a lack of motivation to engage in reward seeking and compromised decision making capacities [242]. A great effort to bridge the gap between neurobiological and clinical studies has been recently done with the development of new laboratory-based procedures for anhedonia and reward-related deficits assessment in humans [243-245]. These tasks, which do not rely on subjective verbal responses from participants but are based on existing and well known rodent procedures, are expected to provide new insights into the involvement of reward-related processes in depressed patients. Improved knowledge of reward deficits in these psychiatric patients will allow to establish whether there is overlap between those that are emerging in obesity and eating disorders.

1.4.1.4 Circadian rhythm

Living organisms on this planet evolved in an environment characterized by 24-hour cycles of light and dark. In an attempt to adapt to this 24-hour cycling, they developed an endogenous circadian (from Latin: *circa*-approximately and *dies*-day) clock allowing to accomplish physiological functions at the most appropriate time during the day [246].

A circadian clock is essentially formed by: 1) a circadian oscillator, which generates the 24-hour oscillation, 2) an input system allowing its constant entrainment (synchronization) with the external environment and, 3) one or more output pathways leading to rhythmic fluctuations of different factors, notably neurotransmitter, hormones and enzymes, which ultimately affect metabolism and behavior.

Inside the cells that form a circadian clock, the oscillator is constituted by a group of genes having cyclic transcription. In mammalian cells, the circadian oscillation originates from the rhythmic transcription and translation of at least two clock genes called *Clock* (Circadian locomotor output cycles kaput) and *Bmal1* (Brain and muscle Arnt-like protein 1). CLOCK and BMAL1 are transcriptional activators that play a positive role in activating other genes, such as *Per* (Period) and *Cry* (Cryptochrome). PER and CRY proteins interact in the cytoplasm to form a heterodimer complex which is transported back to the nucleus. Here, this heterodimer complex binds a specific sequence of the promoter of *Clock* and *Bmal1* genes stopping their transcription. When CLOCK and BMAL1 decrease the inhibitory effect of PER and CRY lowers allowing the cycle to start again. This way, *Clock* and *Bmal1* are rhythmically expressed over a 24-h period and, in the same way, the genes under their control [246, 247].

In mammals, the first circadian clock was discovered in the suprachiasmatic nucleus (SCN) of hypothalamus (Figure 8). This nucleus is formed by ~20.000 neurons, each of them containing an independent circadian oscillator. The SCN is connected to the visual system through the retinohypothalamic tract (RHT) and when the light reaches the retina, the light-signal is transmitted through the RHT to the SCN neurons forcing their cellular oscillators to fluctuate in phase [248]. Besides the SCN also termed *master clock*, other similar oscillators are also found in many peripheral tissues including liver, muscle, kidney, gastrointestinal tract, heart and adipose tissue. All these clocks, called *slave clocks*, are synchronized to the day-night cycle by the SCN clock. Even though the mechanisms by which the SCN exerts this control on peripheral slave clocks are not completely understood, it is believed that this control occurs, at least, in two distinct ways [247, 248]. The first is through the activation of the parasympathetic and sympathetic system, whereas the second is through the release of humoral factors [249].

Under physiological circumstances the SNC clock regulates the temporal organization of several functions, keeping peripheral clocks aligned. Conversely, in absence of the SNC, the circadian system becomes disorganized and peripheral tissue activities drift out of phase. However, there is at least one important exception to this phenomenon. Indeed, it has been observed that, even in the

absence of SCN, food is able to synchronize some peripheral clocks, especially those of adipose tissue, gastrointestinal tract and liver [248, 250].

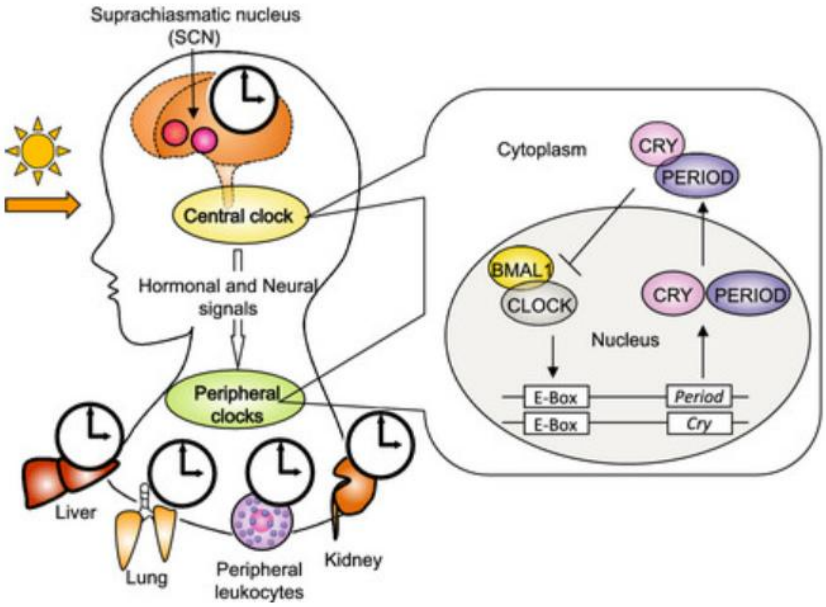

Figure 8. A canonical model of the mammalian circadian clock. The mammalian circadian clock consists of the central oscillator, located in the suprachiasmatic nucleus (SCN) of the hypothalamus, and peripheral oscillators present in virtually all cell types. Light activates a specific group of photoreceptors in the retina that are connected to the central SCN clock, which synchronizes and entrains peripheral circadian clock via neural and endocrine pathways. At the molecular level, CLOCK and BMAL1 heterodimers activate transcription of Period (Per) and Cryptochrome (Cry) genes. PER and CRY proteins in turn inhibit their own expression by repressing CLOCK/BMAL1 activity. This negative feedback loop, with additional post-translational modifications, generates ~24-h oscillations of clock protein levels and activity, which is translated into circadian behavior and physiology. The molecular mechanisms of rhythm generation are cell autonomous and highly conserved in the SCN (the central clock) and peripheral cells (the peripheral clocks). Please note that this model is classical and recent new data reveal that the molecular clock consists of more complicated network of clock genes (From Nakao A. et al, Allergy, 2015 [251]).

It is now accepted that between 10 and 30% of human genome is under the control of central and peripheral clocks [252] and that most of the processes regulating metabolism and behavior are rhythmic. This wide control exerted by the circadian system is indicated by the large number of hormones that have cyclic release, including melatonin, insulin, leptin, ghrelin, glucagon and glucocorticoids [253-257].

Circadian disruption or *chrono-disruption*, which is an imbalance between the 24-h environmental cycle and the temporal order in which the organism performs its physiological functions, is supposed to be at the origin of many pathologies (Figure 9). Chrono-disruption in humans occurs more and more frequently as a consequence of several conditions, such as jet lag, shift-work and light at night. Shift-work represents the most serious form of chrono-disruption in

which the normal synchrony between light-dark phase, sleeping and eating is strongly perturbed. Several studies have confirmed that people working at night are overweight or obese, present anxiety and depressive symptoms, and are a risk for metabolic syndrome [252, 258-261]. Chrono-disruption affects hormonal rhythmicity and impact on the central and peripheral functions of these hormones leading to a global metabolic desynchrony [262-265].

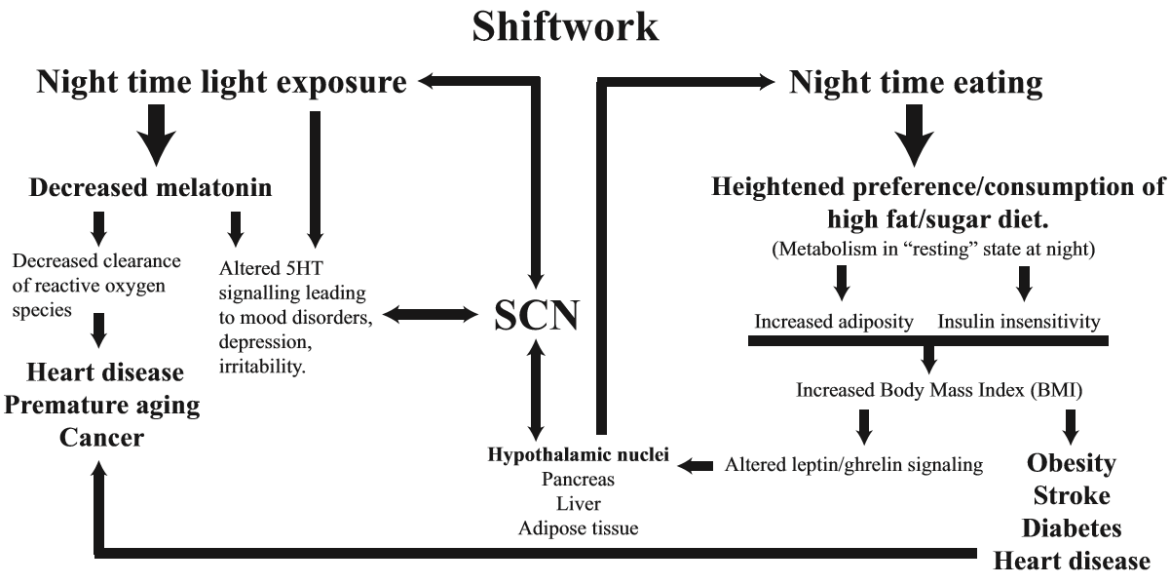

Figure 9 A detailed example of physiological functions altered by shift-work. Night-time shift work is associated with a reversal in the eating schedule and being exposed to light at night. Night-time light exposure is associated with altered 5HT processing in the SCN, which affects downstream structures related to cognition as well as hypothalamic nuclei that influence metabolism and peripheral circadian oscillators. In addition, light at night affects the secretion and receptor density of melatonin, a potent antioxidant. It is possible that the decreased efficacy of melatonin is involved in the development of premature aging, heart disease, or cancer. Working at night also results in night-time eating which is associated with a preference for high sugar and/or fat foods. In addition to the increased consumption of fats/sugars, lipid and glucose metabolism is altered resulting in increased adiposity and insulin insensitivity resulting in increased BMI. Increased BMI leads to altered leptin/ghrelin signaling that feeds back to the hypothalamus, further altering metabolism. When combined, various disease states begin to emerge such as diabetes, obesity, or cardiovascular disease. (From Zelinski et al., Neuroscience and Biobehavioral Reviews, 2014 [266])

The tight linkage of circadian system and energy metabolism has been confirmed by genetic manipulation of clock genes in the mouse. The deletion of *Clock* gene induces hyperphagia, obesity and a metabolic syndrome characterized by hyperleptinemia, hyperlipidemia and hyperglycemia [267]. Likewise, suppression of *Bmal1* in all tissues leads to behavioral arrhythmia, higher body weight and a greater adipose tissue mass before adult age [268, 269].

Like obesity, depression has a strong connection with the circadian system [270]. Depressed patients, despite heterogeneity of symptoms, share evident circadian alterations and seem to be

"phased advanced" compared to health controls. In line with this, they report that they feel better in the evening [271], rather than in the morning and they also have altered hormone circadian profiles, among which the morning rise of cortisol is the most frequent [272]. Additionally, the 90% of depressed patients have reduced nocturnal rise of melatonin [273, 274] and show disrupted sleeping time and structure [275]. All these disturbances are indicative of dysregulation of the central master clock. So far, it is not clear how the loss of clock rhythmicity can lead to depression. On the one hand, circadian dysfunction may affect directly neurobiological processes involved in mood regulation, whilst, on the other hand, circadian disturbances may lead to an abnormal sleep-awake cycle that itself could precipitate the depressive state [276]. Perturbations of the circadian system may represent a convincing biological mechanism responsible for the shared pathophysiology of obesity and depression

1.4.1.5 Gender

Sex differences are prominent in obesity, eating disorder and depression, as well. Depression is reported twice as much in women than in men [277, 278] and women seeking depression treatments report greater symptom severity and greater number of suicide attempts. Additionally, they experience sleep disturbances, increased appetite and weight gain [279-282]. Consistent with these symptoms, the atypical form of depression is more commonly found in women, whereas melancholic depression occurs equally in both sexes [283, 284]. Importantly, women are more likely than men to have comorbid eating disorders [285]. Depression in women is more frequent between puberty and menopause and the severity of depressive symptoms changes during the menstrual cycle suggesting that sex hormone fluctuation is a relevant factor in determining onset and progression of this psychiatric pathology.

Invaluable progress in the understanding of depression have been made using animal models, however, till now most of studies have been conducted only in male animals and very little is known about the causes of higher female sex vulnerability in depressive disorders [286]. Since clear sexual dimorphism has been observed in the rodent HPA axis regulation, similar differences in humans may contribute to explain the higher rates of depression in women [287].

Obesity and eating disorders are largely affected by sex differences. The clinical relevance of these differences is particularly evident when rates of obesity and eating disorders are compared between women and men. Women are approximately twofold more vulnerable to severe obesity [288] and threefold more vulnerable to eating disorders than men [19, 289].

A large body of literature concerning rodent studies attests for several differences between male and female in the physiology of eating. Many of these differences are attributable to the function of the hypothalamus-pituitary gonadal (HPG) axis and to the effect of estrogens and androgens. In rodents, estrogens, notably estradiol (ER), affect central and peripheral activity of several neuropeptides leading to a food intake reduction by acting on their principal receptor ERα. In rat females, food consumption changes according with estradiol release during the ovarian cycle.

Gender differences in human energy balance regulation are more difficult to establish because food selection, food intake and energy expenditure through physical activity are heavily influenced by social, cultural and economic factors. However, among normal weight subjects, women show significantly higher body adiposity than men and a different body fat distribution [290]. Moreover, like rodent females, women have the tendency to eat more or to prefer sweet food during the periovulatory phase, an adaptive mechanism allowing to store energy in case of pregnancy.

Whereas women accumulate fat mainly in subcutaneous depots, men have larger visceral fat accumulation. This sex specific fat distribution, which is regulated by estrogens, plays a critical role in the development of metabolic alterations that are frequently associated with obesity [291]. Indeed, regarding fat distribution, obese women with prevalent subcutaneous distribution are less vulnerable to metabolic comorbidity than men.

Differences in food preference and food cue reactivity were also reported in obese humans depending on sex and they may explain why women are more predisposed to obesity. Women preferred and craved more sweet foods, whereas men were more attracted by savory, protein-rich foods [292-294]. In agreement with the higher propensity of women to crave palatable food, two imaging studies comparing brain activation upon food images presentation have found stronger stimulation in the anterior insula and OFC, two craving and taste-related brain regions, in obese women compared to obese men [295, 296].

In conclusion, available clinical and preclinical data suggest that biological gender differences in mood and food intake regulation may explain the higher vulnerability of females for depression, obesity and eating disorders.

1.4.1.6 CREB-regulated transcription coactivator 1 (CRTC1)

Gene transcription requires the presence and activity of specific transcription factors that bind to DNA regulatory sequences and interact with the transcription machinery. CREB (cAMP response

element binding protein) is a ubiquitous and pleiotropic transcription factor, whose activity is regulated by co-activators [297]. More than fifteen years ago, a family of CREB coactivators, the CRTC (CREB-regulated transcription coactivator) family has been independently discovered by two research groups [298, 299]. Of the three members of this family, CRTC1 is the most abundant isoform expressed in the brain and early investigations revealed that the concomitant intracellular increase of calcium and cAMP, due to neuron depolarization, determines its dephosphorylation and nuclear translocation [300, 301]. Once in the nucleus, CRTC1 can bind CREB complex and promote the transcription of several CREB-related genes (Figure 10).

The first brain function attributed to CRTC1 has been the capacity to regulate long term synaptic plasticity [300, 301]. In the light of these findings, our group developed a *Crtc1* knockout mouse line to further investigate CRTC1 brain activity. CRTC1 deficiency affected mice behavior by inducing a depressive-like phenotype that was associated with a reduction of dopamine and serotonin turnover in the prefrontal cortex [302]. While we were characterizing the phenotype of *Crtc1* knockout mice, Montminy and colleagues [303] published a study showing the involvement of CRTC1 in energy balance regulation. This work revealed that *Crtc1* mutant mice developed obesity on standard food and exhibited signs of insulin and leptin resistance. Associated with the loss of brain sensitivity for leptin, these authors also found reduced fertility, a result that was in contradiction with our findings [304].

More recently, the involvement of CRTC1 in obesity has also been investigated in humans. A first study has highlighted the association of a specific *CRTC1* polymorphism with body mass index and fat mass, and has suggested that CRTC1 is involved in the high prevalence of overweight and obesity observed in psychiatric patients and in subjects from the general population [305]. In a second study, focused on patients with major depressive disorders, the same *CRTC1* polymorphism was analyzed for its association with three different obesity markers (fat mass, body mass index and waist circumference) in depressed and controls with no previous history of depression. Results indicated a role for CRTC1 in overweight and obesity in depressed but not in non-depressed subjects [306] underlying the possible existence of a neurobiological overlap between mood disorders and obesity.

Since the CRTC1-CREB pathway regulates several downstream genes and its impairment could affect a wide range of processes, many molecular targets could be implicated in energy balance and mood regulation. One of these molecular targets, which has attracted scientific attention in the last years, is the brain-derived neurotrophic factor (BDNF). BDNF, a member of the neurotrophin family

of growth factors, is well known for promoting neuronal differentiation and survival, neurogenesis, axonal and dendritic growth, and synaptic formation and plasticity [307]. Based on these varied functions, BDNF and its receptor TrkB (tropomyosin-related kinase B), have been primarily implicated in the etiology of depression. Indeed, BDNF is the central element of a recent theory of depression that considers chronic stress capable to reduce BDNF and its neurotrophic effect.

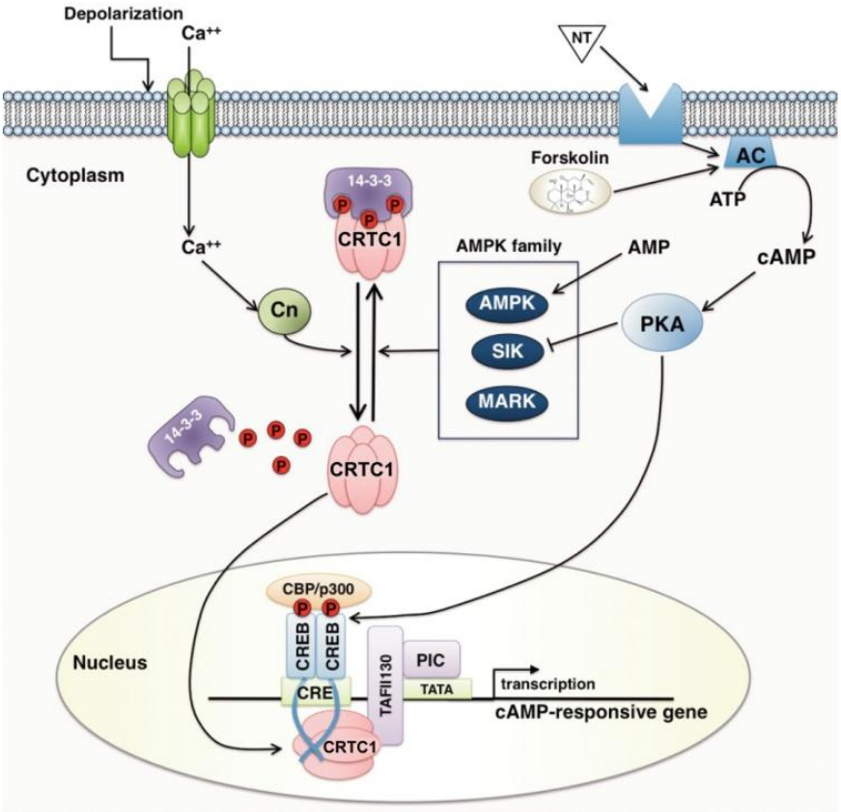

Figure 10. Schematic representation of CRTC signaling. The activity of CRTC1 is dependent on its phosphorylation state. Unlike most transcriptional coactivators, CRTC1 is sequestered in the cytoplasm by the scaffolding protein 14-3-3 when in the phosphorylated state and is thereby rendered inactive. Upon dephosphorylation, the tetrameric CRTC1 complex accumulates in the nucleus where it interacts with the dimerization and DNA binding domain of CREB and facilitates the recruitment of the preinitiation complex (including TAFII130), thereby potentiating transcriptional activation of CREB-dependent genes. The phosphorylation state of CRTC1 is controlled by the opposing effects of the calcium-sensitive phosphatase calcineurin (Cn), and the AMP kinase family of serine/threonine kinases (AMPK, SIK, MARK). In excitable cells, an increase in intracellular calcium via voltage-gated calcium channels activates calcineurin resulting in the dephosphorylation and nuclear translocation of CRTC1. Additionally, stimulation of adenylate cyclase (AC) via G protein-coupled receptors, or by forskolin, results in activation of PKA, which in turn inhibits members of the AMPK family such as SIK. This prevents CRTC1 sequestration in the cytoplasm, thereby resulting in greater nuclear accumulation of CRTC1. CBP, CREB binding protein; Ca^{++}, calcium; CRE, cAMP response element; MARK, microtubule-associated protein affinity-regulating kinase; NT, neurotransmitter; p300, E1A binding protein p300; PIC, preinitiation complex; TAFII130, subunit of transcription factor IID; TATA, conserved DNA promoter sequence 5-TATAAA-3; SIK, salt-inducible kinase (Adapted from Spencer and Weiser, Endocrinology, 2010 [308]).

The involvement of BDNF in depression is attested by the complex interplay between this neurotrophin and the serotonergic system. The neurotrophic hypothesis of depression suggests that disturbances in monoaminergic neurotransmission would interfere with BDNF function and cause reduced neurogenesis and synaptic connections [309]. This neural atrophy, especially in the hippocampus and prefrontal cortex would lead to emotional, mood and cognition disturbances that characterize depression. Accordingly, the expression of BDNF and its receptor were decreased in *post mortem* hippocampal and cortical tissues harvested from depressed human brains [310]. Also, depressed patients showed reduced plasma levels of BDNF [311-313] that were normalized in those subjects that successfully responded to antidepressant therapy [314].

Complementary evidence suggests also that BDNF plays a role in the development and survival of serotonergic neurons, and that impairment of its function alter monoamine neurotransmission [315]. The presence of BDNF receptors on serotonergic neurons in rat raphe nucleus suggests that this neurotrophin might regulate serotonergic neurotransmission [316, 317]. Further evidence of the influence of BDNF on serotonergic innervation is the presence of an ineffective serotonergic transmission in the hypothalamus and cortex of BDNF heterozygous mice [318]. Although these findings open new perspective in the understanding of depression etiology, a clear causal association between impairment of BDNF brain activity and depression has not yet been demonstrated.

Concomitant studies on obesity, have identified BDNF as a key component of mechanisms regulating energy intake and expenditure. Evidence for a role of BDNF in energy homeostasis comes from different lines of research. In humans, it has been reported that mutations in the gene encoding for BDNF, or for its receptor, lead to severe obesity [319, 320], while some BDNF polymorphisms were associated with higher risk for body weight gain [321]. Similar results were obtained in brain specific BDNF conditional knockout mice or in heterozygous BDNF constitutive knockout mice. In both, BDNF deficiency causes hyperphagia, obesity as well as insulin and leptin resistance [322, 323]. Moreover, BDNF i.c.v. administration or direct injection of this neurotrophin in VMH or PVN inhibited food intake, increased energy expenditure and reduced body weight in rats [324-329]. These results were consistent with the abundant expression of BDNF and its receptor found in the PVH, VMH, DMH and LH. According with a physiological role of BDNF in energy control, the expression of BDNF and TrkB was sensitive to the nutritional state. Indeed, food deprivation reduced BDNF mRNA expression in the rat VMH, whereas glucose injection rapidly induced BDNF and TrkB mRNA in the same region [330, 331].

Although collectively these findings point out the involvement of BDNF in energy homeostasis, it is not completely clear whether the alterations observed in case of BDNF deficiency are the result of a direct effect of this factor on homeostatic pathways or simply the result of a perturbed neurotrophic function. Only a few studies have addressed this question and two of them have found an interaction between BDNF and leptin showing that BDNF would act as a downstream factor of leptin signaling [59, 332]. Since the activation of MC_{4R} (melanocortin-4 receptor) by a melanocortin analogue was able to increase BDNF mRNA in mouse VMH [331], it is possible that the effect of leptin on BDNF is mediated by leptin-induced release of α-MSH by arcuate POMC/CART neurons.

Whereas the presence of BDNF in the VMH has been associated to food intake regulation, its production by PVH neurons seems to facilitate energy expenditure through activation of CRH-releasing neurons [325, 333]. Finally, a role for BDNF in hedonic regulation of food intake has been recently proposed. Cordeira and colleagues have demonstrated that the expression of BDNF and TrkB receptor in the VTA of wild-type mice was influenced by high-fat food consumption. Moreover, they also observed, in BDNF-depleted mice, blunted release of dopamine in the striatum and increased ingestion of high-fat diet. Although the participation of BDNF in dopamine regulation is supported by some drug addiction studies [334, 335], the role of this neurotrophin in hedonic feeding requires further investigations.

2 AIM OF THE PROJECT

In the first part of this project, we aimed at better understanding the role of CRTC1 in the obese profile reported in male and, in a lesser extent, female *Crtc1* knockout (ko) mice. In particular, we studied the effect of CRTC1 deficiency on the homeostatic and hedonic regulation of food intake, as well as on locomotor activity, the most relevant component of energy expenditure. In the second part of this work, we focused on compulsive overeating that characterizes binge-eating episodes, frequently present in obese individuals suffering of mental disorders. We first adapted a rat binge-eating model, developed around alternate accesses to standard and chocolate-flavored palatable food, and we assessed the behavioral, metabolic and molecular consequences of such a “yo-yo” dieting. In a third and last part, given the anxio-depressive-like behavioral profile exhibited by *Crtc1* ko mice, we tested their vulnerability to develop exacerbated binge eating like behaviors when submitted to alternate access to standard laboratory chow pellets and chocolate-flavored food.

3 ARTICLES

3.1 ARTICLE 1: Gender-specific alteration of energy balance and circadian locomotor activity in the *Crtc1* knockout mouse model of depression

Personal contribution: I participated in the scientific discussion that preceded the experimental work and defined the experimental design. I performed most of the behavioral and molecular tests and executed all the calculations and the statistical analysis. Finally, I entirely wrote the first draft of the manuscript.

Status: Accepted for revision, Translational Psychiatry

Gender-specific alteration of energy balance and circadian locomotor activity in the *Crtc1* knockout mouse model of depression

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ABSTRACT

Obesity and depression are major public health concerns, and there is increasing evidence that they share etiological mechanisms. CREB-regulated transcription coactivator 1 (CRTC1) participates in neurobiological pathways involved in both mood and energy balance regulation. *Crtc1*$^{-/-}$ mice rapidly develop a depressive-like and obese phenotype in early adulthood, and are therefore a relevant animal model to explore possible common mechanisms underlying mood disorders and obesity. Here, the obese phenotype of male and female *Crtc1*$^{-/-}$ mice was further characterized by investigating CRTC1's role in the homeostatic and hedonic regulation of food intake, as well as its influence on daily locomotor activity. *Crtc1*$^{-/-}$ mice showed a strong gender difference in the homeostatic regulation of energy balance. Mutant males were hyperphagic and rapidly developed obesity on normal chow diet, whereas *Crtc1*$^{-/-}$ females exhibited mild late-onset obesity without hyperphagia. Overeating of mutant males was accompanied by alterations in the expression of several orexigenic and anorexigenic hypothalamic genes, thus confirming a key role of CRTC1 in the central regulation of food intake. No alteration in preference and conditioned response for saccharine was observed in *Crtc1*$^{-/-}$ mice, suggesting that mutant males' hyperphagia was not due to an altered hedonic regulation of food intake. Intriguingly, mutant males exhibited a hyperphagic behavior only during the resting (diurnal) phase of the light cycle. This abnormal feeding behavior was associated with a higher diurnal locomotor activity indicating that the lack of CRTC1 may affect circadian rhythmicity. Collectively, these findings highlight the male-specific involvement of CRTC1 in the central control of energy balance and circadian locomotor activity.

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Running title: Diurnal hyperphagia and hyperactivity in *Crtc1*$^{-/-}$ males

INTRODUCTION

Obesity, resulting from an impairment of energy balance, represents a main public health concern because of its comorbidity with diabetes, cardiovascular diseases, cancer and psychiatric disorders [1]. Clinical studies report high prevalence of obesity in patients suffering of chronic mental illnesses, and among depressive subjects, those affected by atypical depression have the strongest odd to develop obesity [2]. If poor nutrition and psychiatric medication can explain, at least in part, why this psychiatric population develops obesity, growing evidence supports the hypothesis that the association of obesity and depression may originate from shared biological pathways [3-5]. Hence, in order to improve psychiatric outcomes, a better understanding of the neurobiological adaptations shared by these two pathologies is of the highest importance.

CREB-regulated transcription coactivator 1 (CRTC1) has recently been involved in mood regulation and energy balance [6-10]. This transcription coactivator, together with CRTC2 and CRTC3, constitutes a family of proteins that can detect cellular activation and stimulate CREB-dependent gene expression, independently of CREB phosphorylation [11-13]. CRTCs can sense many external inputs, such as hormones and neurotransmitters, by detecting cytoplasmic increase of calcium and cAMP levels. Upon cellular activation, they become dephosphorylated and translocate to the nucleus where they bind to CREB and activate the transcription of several tissue-specific CREB-regulated genes. Despite their different expression throughout the body, all the three CRTC members are involved in energy metabolism. CRTC3 is essentially expressed in the adipose tissue where it facilitates fat deposition [14], whereas CRTC2 has been found to regulate gluconeogenesis and insulin sensitivity in the liver and the pancreas, respectively [13, 15-17]. More recently, CRTC2 has also been detected in the brain, and in particular in the hypothalamus, where it is able to link glucose sensing with gene regulation [18]. Among the CRTC family members, CRTC1 is the most abundant in the brain, in particular in the prefrontal cortex, the hippocampus, the amygdala and the hypothalamus [19-21]. Inside the hypothalamus, a key region for regulating energy balance, CRTC1 is present in the arcuate nucleus (ARC), in the ventromedial and in the paraventricular hypothalamus [21].

Recent evidence collected in *Crtc1*$^{-/-}$ mice has established that not only the lack of CRTC1 induces hyperphagic obesity [6, 7], but it also triggers a depressive-like phenotype, which suggests that CRTC1 plays a role in mood disorder etiology and antidepressant response [8-10]. In agreement with these animal studies, two human investigations have highlighted an association of *CRTC1* polymorphisms with body mass index and fat mass and have suggested that CRTC1 is involved in the high prevalence of overweight and obesity observed in psychiatric patients and in subjects from the general population with major depressive disorder [22, 23]. Altogether, these findings suggest that CRTC1 is a transcriptional coactivator reciprocally involved in the bidirectional relation between obesity and depression.

In this study, we further investigated the consequences of the lack of CRTC1 on the energy balance of *Crtc1*$^{-/-}$ male and female mice, with the aim of better defining CRTC1-regulated molecular pathways involved in obesity, and possibly in mood disorders as well. Alterations in energy intake were assessed using three different approaches: (I) monitoring food consumption and body weight gain, (II) evaluating the expression of multiple genes in the ARC of the hypothalamus and (III) determining the integrity of the hedonic regulation of food intake by testing the preference and the conditioned response for saccharine. Moreover, the influence of CRTC1 on energy expenditure was assessed through the measure of the spontaneous and voluntary locomotor activity. Overall, our results confirm that CRTC1 is critical for the maintenance of energy balance and show, for the first time, the presence of a clear sexual dimorphism in the obesity of *Crtc1*$^{-/-}$ mice, because males develop a more severe obesity than females and are more active and hyperphagic during the resting (diurnal) phase of the cycle.

MATERIALS AND METHODS

Mice

Crtc1$^{-/-}$ mice and wild-type (WT) littermates were obtained and genotyped as previously described [7]. Mice were housed in a temperature and humidity-controlled environment and received water and standard rodent chow *ad libitum* under a 12-h dark-light cycle, unless otherwise specified. All behavioral experiments were carried out in the dark phase of the light cycle. The procedures were performed in conformity with the Swiss National Institutional Guidelines on Animal Experimentation and approved by the Cantonal Veterinary Office.

Body weight and food intake measurements

Six-week-old males and females were single-housed and their body weight and food intake measured weekly. Males were divided in two groups; the first was monitored until 8 weeks of age and the second until 36

weeks. Assessment of food intake and body weight of females was protracted until 52 weeks of age. Food consumption during the dark and light phase of the cycle was measured when males and females were 30 weeks old. The development of obesity in mutant females was also studied in an additional group of six-week-old mice fed ad libitum with a high fat diet (HFD 2127- KLIBA NAFAG: carbohydrates 41.1%, proteins 23.9%, fats 35%, 5.68 Kcal/g) during 50 days.

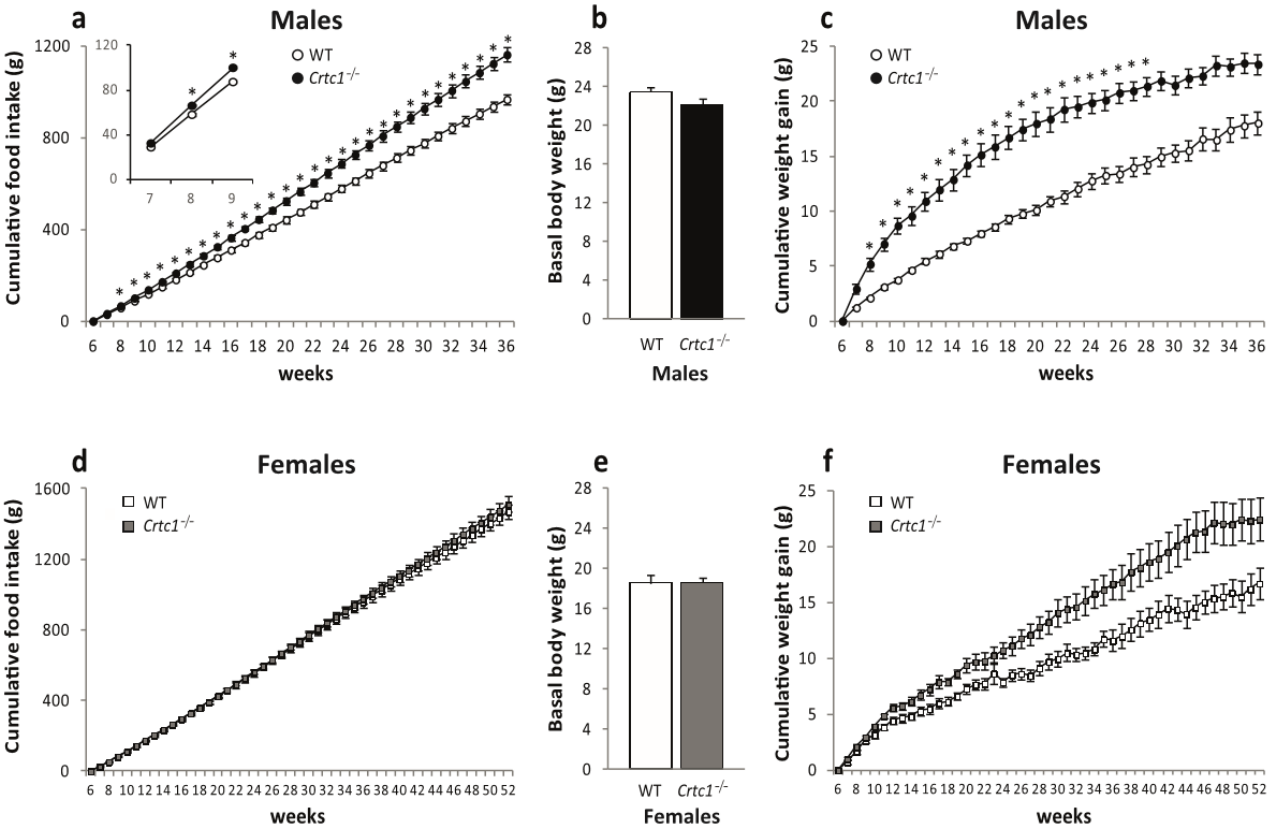


Figure 1. Effect of CRTC1 deficiency on food intake and body weight in male and female mice. (**a-c**) Cumulative food intake and body weight gain in *Crtc1*$^{-/-}$ (n=12) and wild-type (WT) males (n=14). (**a**) *Crtc1*$^{-/-}$ males eat significantly more than WT (repeated measures two-way ANOVA: *genotype*: $F_{(1,24)} = 20.737$, $P < 0.001$; *weeks*: $F_{(1.65,39.66)} = 34572.49$, $P < 0.001$; *genotype*weeks*: $F_{(1.65,39.66)} = 3.309$, $P = 0.055$) and their overeating is already present at 7 weeks of age, as shown in the inserted graph (**b**) Comparison of basal body weight in 6-week-old WT and *Crtc1*$^{-/-}$ males shows no significant difference ($P = 0.129$). (**c**) *Crtc1*$^{-/-}$ males gain more weight than WT (repeated measures two-way ANOVA: *genotype*: $F_{(1, 24)} = 53.537$; $P < 0.001$; *weeks*: $F_{(3.001,72.031)} = 513.07$; $P < 0.001$; *genotype*weeks*: $F_{(3.001,72.031)} = 513.073$; $P < 0.001$). (**d-f**) Cumulative food intake and body weight gain in *Crtc1*$^{-/-}$ (n=11) and WT females (n=15). (**d**) No hyperphagia was observed in *Crtc1*$^{-/-}$ females until 52 weeks of age (repeated measures two-way ANOVA: *genotype*: $F_{(1,24)} = 0.176$, $P = 0.678$; *weeks*: $F_{(1.041,24.982)} = 1944.94$, $P < 0.001$; *genotype*weeks*: $F_{(1.041,24.982)} = 0.43$, $P = 0.526$). (**e**) Comparison of basal body weight in 6 weeks old WT and *Crtc1*$^{-/-}$ females. Basal body weight was identical in both genotypes ($P = 0.969$). (**f**) Body weight gain of *Crtc1*$^{-/-}$ and WT females (repeated measures two-way ANOVA: *genotype*: $F_{(1,24)} = 10.527$, $P = 0.034$; *weeks*: $F_{(3.185,76.443)} = 263.167$, $P < 0.001$; *genotype*weeks*: $F_{(3.185,76.443)} = 0.508$; $P = 0.689$). *$P < 0.05$ Bonferroni post-hoc test.

Gene expression analysis

After body weight measurements, mice were killed and fresh brains were rapidly sliced into 1 mm-thick coronal sections in a mouse brain stainless steel matrix. The slice containing the hypothalamus was used to dissect the ARC by a micro-punch technique (0.98 mm diameter micro-punch, Stoelting). ARC-RNA was extracted with an RNeasy Plus Minikit (Qiagen, Valencia, CA, USA) and converted in cDNA by reverse transcription reaction using TaqMan® Reverse Transcriptase Reagents (Applied Biosystem). Real time PCR amplification was performed

with an ABIPRISM 7500 cycler and SYBER green PCR Master Mix (Applied Biosystem) using specific sets of primers (Mycrosynth AG). Forward and reverse primers for the tested genes are the following: *β-actin* = forward: 5'-GCT TCT TTG CAG CTC CTT CGT-3', reverse: 5'-ATA TCG TCA TCC ATG GCG AAC-3'; *AgRP* = forward: 5'-CGG AGG TGC TAG ATC CAC AGA-3', reverse: 5'-AGG ACT CGT GCA GCC TTA CAC-3'; *Cart* = forward: 5'-TTC CTG CAA TTC TTT CCT CTT GA-3', reverse: 5'-GGG AAT ATG GGA ACC GAA GGT-3'; *Fto* = forward: 5'-GGA CAT CGA GAC ACC AGG AT-3', reverse: 5'-AGG TGC CTG TTG AGC ACT CT-3'; *Glp1r* = forward: 5'-ACT TTC TTT CTC CGC CTT GGT-3', reverse: 5'-TTC CTG GTG CAG TGC AAG TG-3'; *LepRb* = forward: 5'-GCA TGC AGA ATC AGT GAT ATT TGG-3', reverse: 5'-CAA GCT GTA TCG ACA CTG ATT TCT TC-3'; *Npy* = forward: 5'-CAG AAA ACG CCC CCA GAA C-3', reverse: 5'-CGG GAG AAC AAG TTT CAT TTC C-3'; *Npy-rY1* = forward: 5'-CAA GAT ATA CAT TCG CTT GA-3', reverse: 5'-AGA TTG TGG TTG CAG G-3'; *Nor1* = forward: 5'-TGG CTC GAC TCC ATT AAA GAC-3', reverse: 5'-TGC ATA GCT CCT CCA CTC TCT-3'. All samples were analyzed in triplicates. Relative gene expression was measured with the comparative *ΔΔ Ct* method [24] and normalized with *β-actin* transcript levels.

Preference and conditioned response for saccharine

Two-bottle choice test

The preference for saccharine was assessed in *Crtc1*$^{-/-}$ and WT mice at 16 weeks of age. Individually housed, mice had simultaneously free access to two drinking bottles, one containing tap water and the other 0.2% saccharine solution. The position of the two bottles in the cage was interchanged every day. Water and saccharine consumption was measured daily, along 4 days. Saccharine preference was calculated as *preference ratio* = (saccharine consumption/total liquid consumption) x 100.

Saccharine operant conditioning

Saccharine self-administration was measured in 8-week-old mice using eight operant chambers (Med Associated Inc., St. Albans, VT, USA). Mice were trained to self-administer saccharine 0.2% liquid reward on a fixed ratio 1, time out 3 sec (FR1 TO3) schedule of reinforcement in the presence of an olfactory cue (apple aroma, Givaudan, Dübendorf, Switzerland) during 30-min daily sessions. A single nose entry in the active nosepoke activated a liquid dipper equipped with a 0.01 ml cup and a light cue located inside the nosepoke. The liquid reward remained available for 3 sec once access to the liquid dipper had been detected with head entry detectors. Supplementary entries in the active nosepoke in the absence of head entry detection above the liquid dipper and entries in the inactive nosepoke were recorded but had no further consequence. During the early phase of acquisition, mice were restricted to the 85% of their daily food consumption. Once fed ad libitum, mice were trained until stable levels of intake (≤ 25% variation of the mean responses for three consecutive sessions). Afterwards, mice underwent an extinction phase until completion of an extinction criterion (<30% of the mean responses obtained during the 3 days achieving the stabilization criteria across 3 consecutive extinction sessions). Animals were then tested for reinstatement of their nosepoking activities upon presentation of the olfactory and light cues, and the numbers of entries in the active and inactive nosepokes were recorded during 30-min sessions while no liquid reward was delivered. The number of nose-pokes was used to compare *Crtc1*$^{-/-}$ and WT mice along the entire procedure.

Measures of locomotor activity

Spontaneous activity

Spontaneous activity was recorded in an actimetry apparatus consisting of 12 transparent Plexiglas cages (30 x 50 x 40 cm). Each cage was equipped with an infrared movement detector on the top, a food dispenser and a bottle of water. Eight-week-old mice were individually placed in the activity cages and habituated one week to the novel environment before starting recording. Activity, measured as arbitrary units, was assessed every 15 min over 7 days.

Activity in running wheel

Voluntary wheel running was monitored in standard cages equipped with a stainless steel wheel. Each wheel measured 23 cm of diameter and had a running lane width of 7.5 cm (2B Biological Instruments, Varese, Italy). Animals of 8 weeks of age were individually housed in running wheel cages and acclimated for 7 days before starting measurements. Water and food were provided *ad libitum*. Running activity (number of wheel revolutions/hour) was recorded continuously for one week. For each mouse, the activity in the dark and light phase of the cycle was measured as the average of the last 3 days of the test, in which mice performance was stable.

Statistical analyses

All data reported in the text and figures are means ± s.e.m. The number of independent samples of each group is indicated in the figure legend. Before performing statistical comparisons, the normality of data distribution was verified with Shapiro-Wilk test. Since normality of

saccharine operant conditioning and spontaneous activity were not fulfilled, raw data underwent logarithmic transformation. Basal body weight, gene expression, saccharine preference, total spontaneous activity and total food intake over 24 hours were compared with Student's *t*-test for independent samples, after evaluation of homoscedasticity with Levene's test. Statistical analysis of body weight gain, cumulative food intake, saccharine operant conditioning, spontaneous activity and food consumption in the dark and light phases, was done using a repeated measure two-way ANOVA followed by Bonferroni post-hoc test for multiple comparisons, when appropriate. Sphericity assumption, for repeated measures, was verified with Mauchly's test and degrees of freedom eventually corrected by Greenhouse-Geisser epsilon value. Running activity in dark and light phase was analyzed with Mann-Whitney U test for non-parametric independent samples. Statistical power of all analysis was between 0.8 and 1.0. Level of significance was set at P≤ 0.05. All statistical analyses were performed with IBM SPSS Statistic 23.0 software (IBM, Armonk, NY, USA).

	8-week-old male mice			36-week-old male mice		
Gene	WT	*Crtc1*$^{-/-}$mice	*P* value	WT	*Crtc1*$^{-/-}$mice	*P* value
LepRb	1.000 ± 0.157	1.070 ± 0.187	0.778	1.000 ± 0.119	0.564 ± 0.092	**0.014***
Cart	1.000 ± 0.129	0.839 ± 0.127	0.408	1.000 ± 0.169	0.521 ± 0.032	**0.029***
Nor1	1.000 ± 0.107	0.554 ± 0.063	**0.008***	1.000 ± 0.085	0.382 ± 0.079	<**0.001***
AgRP	1.000 ± 0.299	2.851 ± 0.599	**0.020***	1.000 ± 0.543	0.732 ± 0.287	0.978
Npy	1.000 ± 0.217	0.918 ± 0.268	0.814	1.000 ± 0.109	1.774 ± 0.216	**0.005***
Npy-y1r	1.000 ± 0.210	1.371 ± 0.282	0.297	1.000 ± 0.256	1.031 ± 0.192	0.923
Fto	1.000 ± 0.061	0.767 ± 0.037	**0.004***	1.000 ± 0.038	0.844 ± 0.045	**0.019***
Glp-r1	1.000 ± 0.172	1.178 ± 0.104	0.388	1.000 ± 0.138	0.722 ± 0.106	0.397

Table 1. Relative mRNA levels of orexigenic and anorexigenic genes in *Crtc1*$^{-/-}$ and WT males at 8 and 36 weeks of age. *$P < 0.05$, Student's *t*-test.

RESULTS

Crtc1$^{-/-}$ *male mice are hyperphagic and develop obesity*

To characterize the obesity of *Crtc1*$^{-/-}$ mice, we monitored food intake and body weight until 36 weeks of age in males and 52 weeks in females. Measures of food consumption revealed that starting from 8 weeks of age *Crtc1*$^{-/-}$ males were hyperphagic and ate significantly more than controls (Figure 1a). Unlike males, females did not exhibit any overeating behavior (Figure 1d).

No significant difference was found in body weight between young *Crtc1*$^{-/-}$ and WT mice. Indeed, at 6 weeks of age males (*Crtc1*$^{-/-}$ = 22.10 ± 0.32, WT = 23.47 ± 0.41) and females (*Crtc1*$^{-/-}$ = 18.58 ± 0.46, WT = 18.55 ± 0.79) of both genotypes showed similar weight (Figures 1b and e). However, *Crtc1*$^{-/-}$ males compared to females, exhibited a stronger body weight gain with aging. According with their early overeating, *Crtc1*$^{-/-}$ males gained quickly more weight than controls (Figure 1c). Measures of body weight gain revealed that *Crtc1*$^{-/-}$ males were overweight from the 8th week of age (Bonferroni post-hoc, $P = 0.0019$).

In contrast to males, *Crtc1*$^{-/-}$ females, which did not show overeating, had a slower and more moderate body weight increase (Figure 1f), as attested by repeated measures two-way ANOVA (*genotype*: $F_{(1,24)} = 10.527$, $P = 0.034$; *weeks*: $F_{(3.185,76.443)} = 263.167$, $P < 0.001$; *genotype*weeks*: $F_{(3.185,76.443)} = 0.508$; $P = 0.689$), even though the Bonferroni post-hoc test did not show any significant comparison. The elevated number of multiple comparisons affected this post-hoc test, and therefore it did not reveal specifically at which weeks of age *Crtc1*$^{-/-}$ females had a significant higher body weight gain, as compared with WT females. Nevertheless, the significant effect of the genotype revealed by the ANOVA showed that mutant females globally gained more weight than WT females. An independent weight gain comparison of the 52-week-old *Crtc1*$^{-/-}$ and WT females actually confirmed this difference (*Crtc1*$^{-/-}$ = 22.4 ± 1.9 versus WT= 16.7 ± 1.4, Student's *t*-test, $P = 0.023$). Further

corroborating the absence of hyperphagia and early-onset obesity in mutant females, six-week-old mice were fed ad libitum with high fat diet (HFD) for 50 days (Supplementary Figure S1). *Crtc1*$^{-/-}$ and WT females consumed equivalent amount of HFD (repeated measures two-way ANOVA: *genotype*: $F_{(1,12)}$ =1.637, P = 0.225; *days*: $F_{(1.03,12.881)}$ = 833.32, P < 0.001; *genotype*days*: $F_{(1.03,12.881)}$ = 1.336; P = 0.278) and gained similar body weight (repeated measures two-way ANOVA: *genotype*: $F_{(1,12)}$ =0.62, P = 0.808; *days*: $F_{(1.233,14793)}$ = 37.464, P < 0.001; *genotype*days*: $F_{(1.233,14793)}$ = 0.421; P = 0.568). Taken together, these data show that the lack of CRTC1 seriously increases feeding behavior and body weight gain of male mice, whereas it has only a weak impact on energy balance of mutant females.

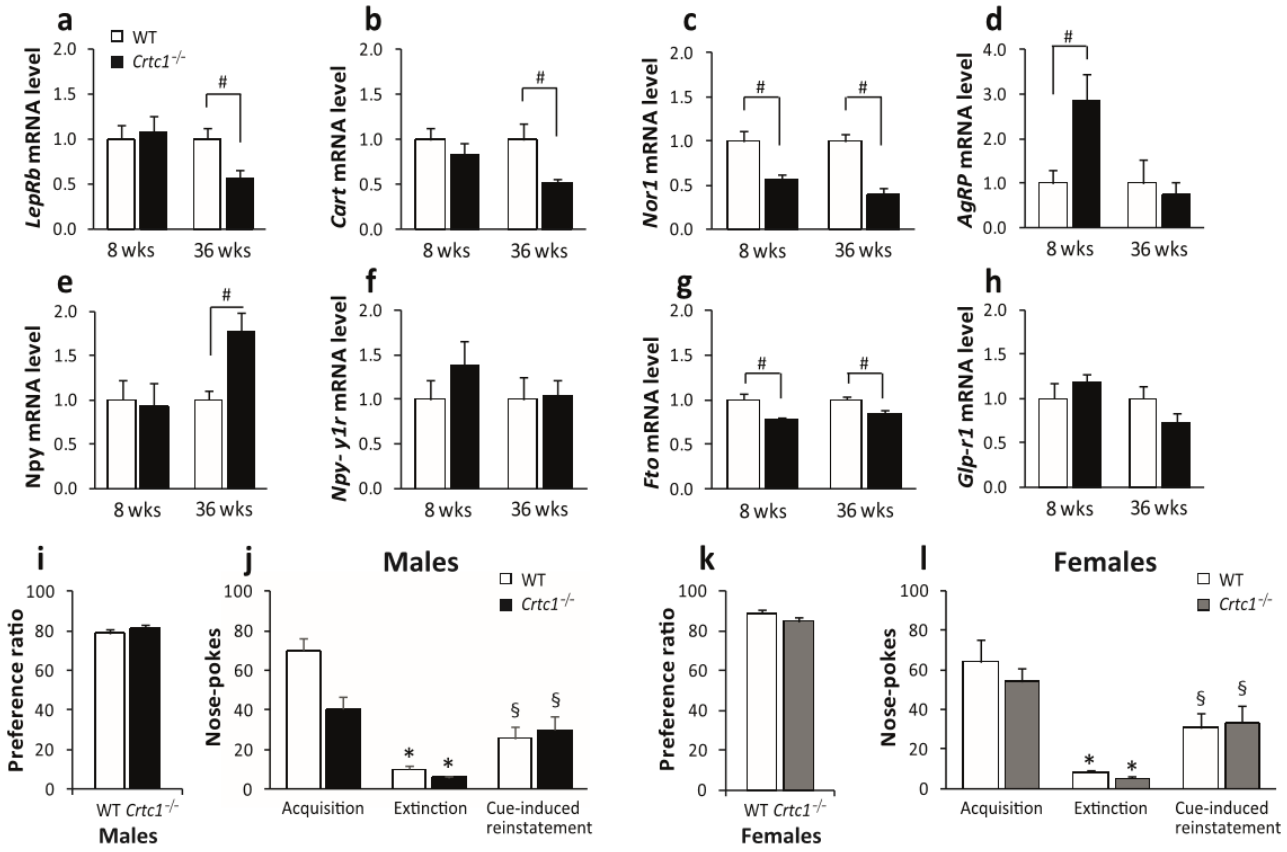


Figure 2. (a-h) ARC gene expression in 8- and 36-week-old *Crtc1*$^{-/-}$ (n=7-12) and WT males (7-13) (**a**) *LepBb* mRNA level was equal in young males but, according to leptin resistance was reduced in old mice (P = 0.0143). (**b**) *Cart* expression was similar in the two group at 8 weeks, whereas at 36 weeks *Crtc1*$^{-/-}$ males had reduced *Cart* mRNA compared to WT (P = 0.0294). (**c**) *Nor1* mRNA level was significantly reduced in *Crtc1* mutant males both at 8 weeks (P = 0.008) and 36 weeks (P < 0.001). (**d**) *AgRP* transcription was upregulated in young (P = 0.020) but not in old *Crtc1* mutant mice. (**e**) *Npy* mRNA level increased in 36-week-old mutant mice (P = 0.005) but not in 8-week-old mice. (**f**) *Npy-y1* expression was not impaired in *Crtc1* mutant males at both ages.(**g**) *Fto* mRNA level was lowered both in young (P = 0.004) and old *Crtc1* mutant males (P = 0.019). (**h**) *Glp-r1* transcript level was not affected by the lack of CRTC1. (**i-l**) Preference and conditioned response for 0.2% saccharine solution. (**i**) Both *Crtc1*$^{-/-}$ (n=5) and WT males (n=8) showed strong preference for saccharine in a two-bottle choice test. No difference was observed between the two genotypes (P = 0.521)**.** (**j**) Consumption of saccharine in the operant conditioning paradigm. *Crtc1*$^{-/-}$ males (n=8) collected less saccharine rewards during the acquisition phase, compared to WT (n=8), although the difference was not statistically different. Similar motivation for saccharine was observed during the cue-induced reinstatement phase (repeated measures two-way ANOVA: *genotype*: $F_{(1,14)}$ = 2.780, P = 0.118; *phase*: $F_{(2,28)}$ = 103.44, P < 0.001; *phase*genotype*: $F_{(2,28)}$ = 3.688, P = 0.038). (**k**) *Crtc1*$^{-/-}$ (n=9) and WT females (n=9) showed equal preference for saccharine in the two-bottle choice test (P = 0.246). (**l**) No statistically significant difference was found in motivated consumption of saccharine between *Crtc1*$^{-/-}$ (n=12) and WT females (n=13) neither in the acquisition phase nor in the cue-induced reinstatement phase (repeated measures two-way ANOVA: *genotype*: $F_{(1,23)}$ = 0.622, P = 0.438; *phase*: $F_{(2,46)}$ = 117.39; P < 0.001; *phase*genotype*: $F_{(2,46)}$ = 3.121; P = 0.054). $^{\#}P$ < 0.05, Student's *t*-test.*P < 0.05 Bonferroni post-hoc test vs acquisition. $^{\S}P$ < 0.05 Bonferroni post-hoc test vs extinction.

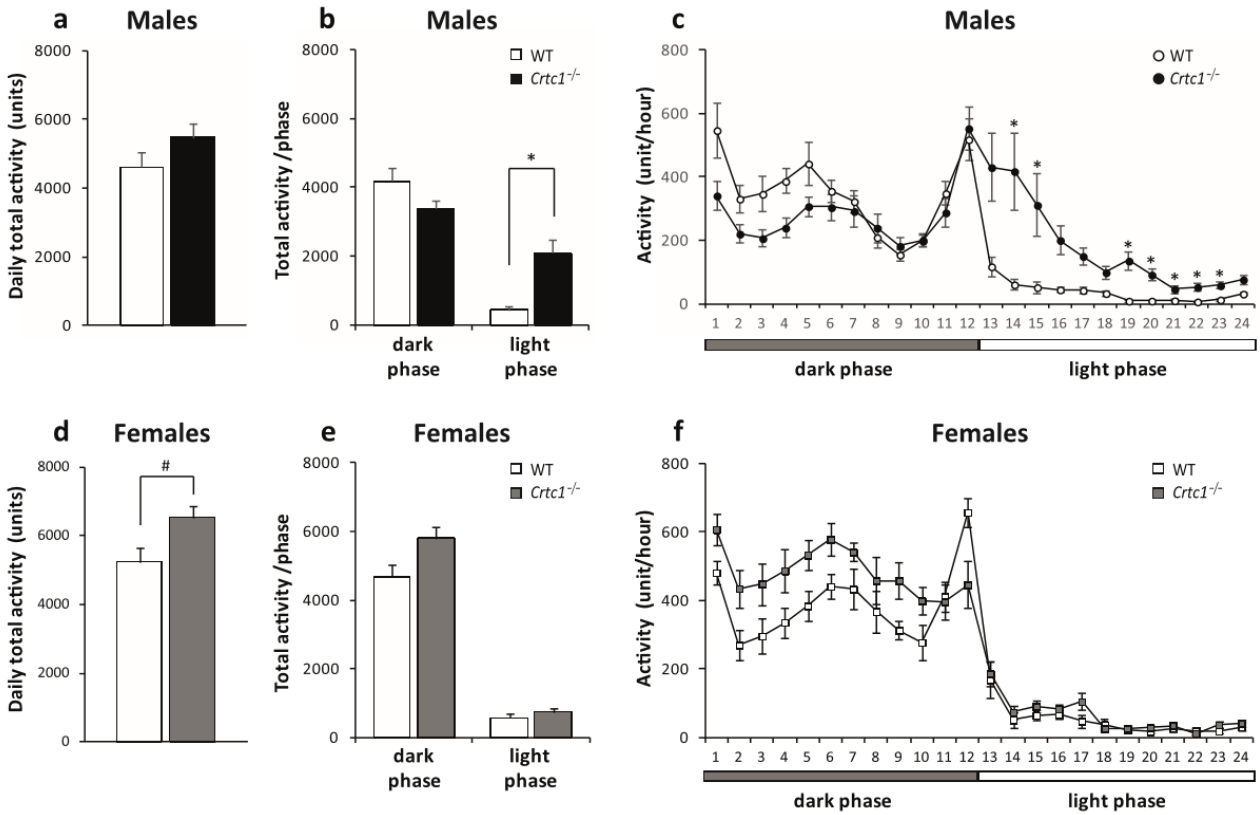


Figure 3. Spontaneous locomotor activity of *Crtc1* mutant mice.
(**a-c**) Spontaneous locomotor activity of *Crtc1*$^{-/-}$ (n=12) and WT males (n=12). (**a**) Total daily spontaneous locomotor activity of *Crtc1*$^{-/-}$ males was similar to that of WT (P = 0.162). (**b**) Spontaneous activity of *Crtc1*$^{-/-}$ and WT males during the dark and light phase. Mutant mice showed slightly reduced activity during the dark phase and increased activity during the light phase (repeated measures two-way ANOVA: *genotype*: $F_{(1,22)}$=71.175, *P* < 0.001; *phase*: $F_{(1,22)}$=64.713, *P* < 0.001; *phase*genotype*: $F_{(1,22)}$=108.43, *P* < 0.001). (**c**) *Crtc1*$^{-/-}$ and WT daily activity plotted hour-by-hour (repeated measures two-way ANOVA: *genotype*: $F_{(1,22\ )}$ = 21.029; *P* < 0.001; *hours*: $F_{(6.65,146.3)}$ = 44.42; *P* < 0.001; *hours*genotype*: $F_{(6.65,146.3)}$ = 7.85; *P* < 0.001). (**d-f**) Spontaneous locomotor activity of *Crtc1*$^{-/-}$ (n=11) and WT females (n=9). (**d**) *Crtc1*$^{-/-}$ females showed significantly higher daily activity than WT (*P* = 0.026). (**e**) *Crtc1*$^{-/-}$ females moved slightly more than control females in the dark phase (repeated measures two-way ANOVA: *genotype*: $F_{(1,18)}$ = 5.17; *P* = 0.035; *phase*: $F_{(1,18)}$ = 344.64; *P* < 0.001; *phase*genotype*: $F_{(1,18)}$ = 0.56; *P* = 0.464). (**f**) Daily activity of *Crtc1*$^{-/-}$ and WT females plotted hour-by-hour (repeated measures two-way ANOVA: *genotype*: $F_{(1,18)}$ = 6.017; *P* = 0.025; *hours*: $F_{(7.068,127.23)}$ = 58.55; *P* < 0.001; *hours*genotype*: $F_{(7.068,127.23)}$ = 1.411; *P* = 0.206) **P* < 0.05, Bonferroni post-hoc test. $^{\#}$*P* < 0.05, Student's *t*-test.

Orexigenic and anorexigenic gene expression is altered in the ARC of Crtc1$^{-/-}$ *males*

Given the presence of CRTC1 in the ARC and the pivotal role played by this structure in the homeostatic regulation of food intake, we investigated whether *Crtc1*$^{-/-}$ males' hyperphagia could be linked to an imbalance in the expression of anorexigenic and orexigenic genes. We compared ARC gene expression in 8-week-old and 36-week-old *Crtc1*$^{-/-}$ and WT males (Table 1), as well as the relative expression of four anorexigenic genes (*LepRb*, *Cart*, *Nor1* and *Glp-r1*), three orexigenic genes (*AgRP*, *Npy* and *Npy-y1*) and the fat mass- and obesity-related gene (*Fto*). In the ARC of 36-week-old *Crtc1*$^{-/-}$ males, we found significant downregulation of *LepRb*, *Cart*, *Nor1* and *Fto*, increased expression of *Npy* and no change in the expression of *AgRP*, *Npy-y1* and *Glp-r1*. At 8 weeks of age, the lack of *Crtc1* induced significant reduction of *Nor1* and *Fto* and upregulation of *AgRP*, as compared with WT males. On the other hand, we did not observe, at this age, any difference in the relative level of *LepRb*, *Cart*, *Npy*, *Npy-y1* and *Glp-r1*. Consistent with the absence of hyperphagia, 52-week-old mutant females did not exhibit any change of *Cart*, *AgRP*, *Npy* and *Glp-r1* expression, but only increased levels of *Npy-y1* (Supplementary Table S1 and Figure S2). Collectively, these results show that *Crtc1*$^{-/-}$ males present perturbed expression of both orexigenic and anorexigenic genes, whereas the lack of CRTC1 does not induce major alterations in the homeostatic regulation of food intake in mutant females.

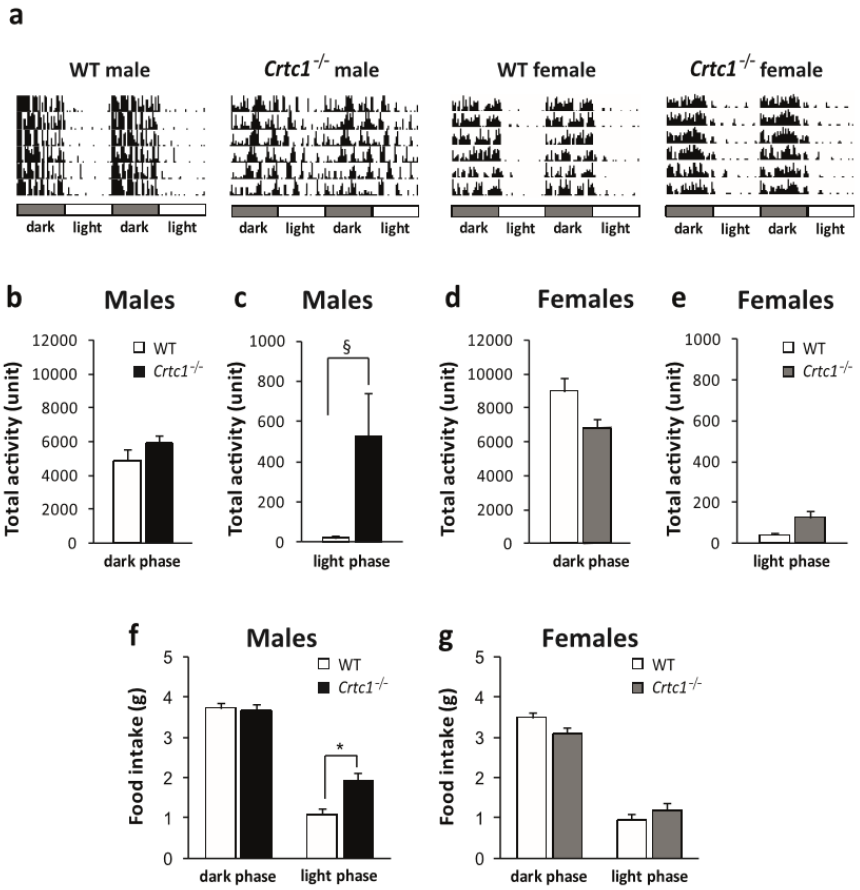


Figure 4. (a) Representative actograms of one *Crtc1* mutant and WT male and female mouse over 7 days. Dark phase and light phase are indicated by the black and the white bars, respectively on the top of each actogram. Each line of the actogram shows the activity of 48 hours. (**b-c**) Motivated wheel-running of *Crtc1*$^{-/-}$ (n=11) and WT males (n=9): (**b**) Total activity in the dark (P = 0.002) and (**c**) in the light phase (P = 0.449). (**d-e)** Motivated wheel-running of *Crtc1*$^{-/-}$ (n=12) and WT females (n=13): (**d**) Total activity in the dark (P = 0.077) and (**e**) in the light phase (P = 0.087). (**f-g**) Food consumption of *Crtc1* deficient mice in the dark and light phase of the cycle: (**f**) *Crtc1*$^{-/-}$ males ate significantly more during the light phase (repeated measures two-way ANOVA: *genotype*: $F_{(1,19)}$ = 10.90; P = 0.0037; *phase*: $F_{(1,19)}$ = 123.63; P < 0.001; *phase*genotype*: $F_{(1,19)}$ = 5.32; P = 0.032). (**g**) *Crtc1*$^{-/-}$ and control females consumed similar amount of food in both phases (repeated measures two-way ANOVA: *genotype*: $F_{(1,18)}$ = 0.278; P = 0.604; *phase*: $F_{(1,18)}$ = 145.23; P < 0.001; *phase*genotype*: $F_{(1,18)}$ = 2.95; P = 0.102). $^{§}P$ < 0.05, Mann-Whitney U test. *P < 0.05, Bonferroni post-hoc test.

The hyperphagia of Crtc1$^{-/-}$ *males does not depend on increased preference for food rewards*

Motivation to consume highly palatable food, which relies on the integrity of the reward system, is also essential for a proper feeding behavior. *Crtc1* is also expressed in brain structures belonging to the reward system, but so far, no study has established whether the functionality of this system is compromised in *Crtc1*$^{-/-}$ mice. Therefore, we compared *Crtc1*$^{-/-}$ and WT mice for saccharine preference in a two-bottle choice test and assessed their capacity to seek for saccharine reward in an operant conditioning test.

The two-bottle choice tests revealed that mutant mice and controls had similar preference for saccharine (Figures 2i and k), with a preference ratio for saccharine of 78.7% ± 2.5 in WT and of 81.2% ± 2.4 in *Crtc1*$^{-/-}$ males. As expected, females of both genotype exhibited a clear-cut preference for saccharine as well (WT = 88.9% ± 1.4, *Crtc1*$^{-/-}$ = 85.4% ± 2.5).

Both WT and *Crtc1*$^{-/-}$ males were then trained for self-administering saccharine in a fixed ratio-1 schedule until stable intake, and after a period of extinction, we assessed their ability to reinstate their saccharine seeking behavior upon presentation of the light and olfactory cues. A two-way repeated measure ANOVA revealed a significant main effect for phase ($F_{(2,28)}$=103.44, P < 0.001) and genotype x phase ($F_{(2,28)}$=3.688, P = 0.038), but no effect for genotype ($F_{(1,14)}$=2.780, P = 0.118). Although Bonferroni post-hoc tests did not reveal any significant difference between genotypes, the significant interaction revealed by the ANOVA most likely suggests that mutant

males may display a tendency to collect less rewards than controls (WT = 70.0 ± 6.45 versus *Crtc1*$^{-/-}$ = 40.5 ± 6.27 active nosepokes). As a consequence, despite both groups of mice exhibited a significant reinstatement of previously extinguished nosepoking behavior (WT = 25.9 ± 5.68 and *Crtc1*$^{-/-}$ = 30.0 ± 7.20 active nosepokes), *Crtc1*$^{-/-}$ mice manifested an enhanced reinstatement when expressed in function of their baseline intake of saccharine (WT= 36.8% ± 6.3 versus *Crtc1*$^{-/-}$ = 71.0% ± 12.3, Student's *t*-test, *P* = 0.026), most likely due to a ceiling effect observed during saccharine consumption. In contrast, mutant and control females both exhibited similar acquisition, extinction and reinstatement of saccharine seeking behaviors (Figure 2l), suggesting that, overall, the lack of CRTC1 most likely does not impair operant responding for saccharine reward in mice.

	52-week-old female mice		
Gene	WT	Crtc1$^{-/-}$ mice	P value
Cart	1.000 ± 0.125	0.987 ± 0.205	0.959
AgRP	1.000 ± 0.269	0.934 ± 0.247	0.862
Npy	1.000 ± 0.224	0.703 ± 0.155	0.284
Npy-y1r	1.000 ± 0.201	1.830 ± 0.226	**0.018***
Glp-r1	1.000 ± 0.055	1.030 ± 0.091	0.783

Supplementary Table S1. Relative mRNA levels of orexigenic and anorexigenic genes in *Crtc1*$^{-/-}$ and wild-type (WT) females at 52 weeks of age. *$P < 0.05$, Student's *t*-test.

Crtc1$^{-/-}$ *male mice move more during the light phase of the cycle*

Since reduced energy expenditure could induce body weight gain, we monitored spontaneous locomotor activity of *Crtc1*$^{-/-}$ mice in activity cages along 7 days. The total daily activity of mutant males (Figure 3a) was similar to that of controls (WT = 4613.80 ± 418.38, *Crtc1*$^{-/-}$ = 5465.70 ± 414.35), whereas *Crtc1*$^{-/-}$ females (Figure 3d) exhibited significant higher activity as compared to WT littermates (WT = 5245.10 ± 389.40, *Crtc1*$^{-/-}$ = 6535.40 ± 414.35, Student's *t*-test, *P* = 0.026). A deeper analysis of *Crtc1*$^{-/-}$ mice activity during the light and dark phase of the light cycle pointed out a strong difference in males' and females' behavior (Figures 3b and e). Mutant females behaved like control females in the light phase and moved a little bit more during the dark phase as shown by repeated measures two way ANOVA. (*genotype*: $F_{(1,18)} = 5.17$; *P* = 0.035; *phase*: $F_{(1,18)} = 344.64$; *P* < 0.001; *phase*genotype*: $F_{(1,18)} = 0.56$; *P* = 0.464). However, Bonferroni post hoc test did not show any statistical difference between WT and mutant females in this phase of the cycle (*P* = 0.237). Intriguingly, *Crtc1*$^{-/-}$ males presented opposite behavior moving as much as controls in the dark phase and significantly more in the light phase (Bonferroni post-hoc test, *P* < 0.001).

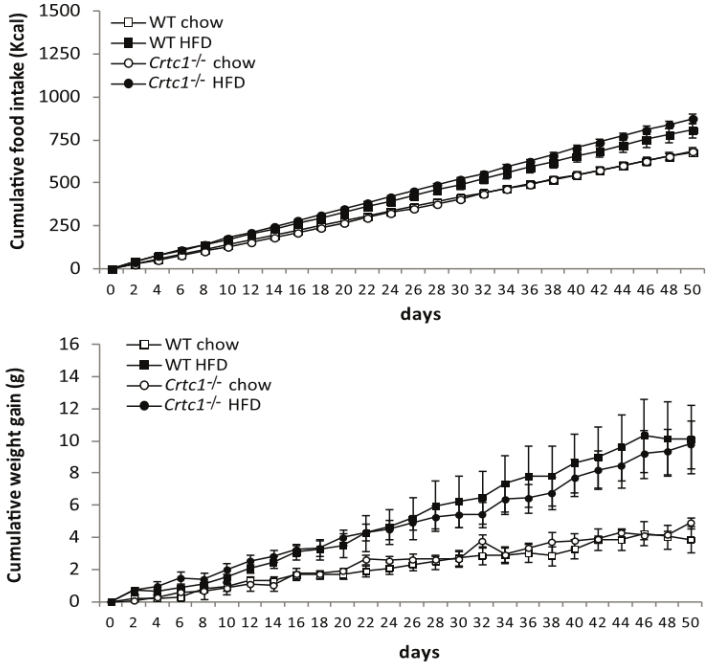
To better characterize this sustained activity of mutant males during the resting phase, we plotted the daily activity on a scale of one-hour intervals (Figure 3c). Mutant males, compared to controls, moved more when the light was switched on and maintained a significant higher activity along all the light phase (repeated measures two-way ANOVA: *genotype*: $F_{(1,22)} = 21.029$; *P* < 0.001; *hours*: $F_{(6.65,146.3)} = 44.42$; *P* < 0.001; *hours*genotype*: $F_{(6.65,146.3)} = 7.85$; *P* < 0.001). In contrast to what was observed with males, the same hour-by-hour analysis did not reveal any increase in activity of mutant females during the resting phase (Figure 3f). Seven-day-double-plotted actograms of a representative male and female mouse of both groups clearly showed that *Crtc1*$^{-/-}$ males, but not females, have a perturbed circadian locomotor activity (Figure 4a).

To confirm the increased mobility of *Crtc1*$^{-/-}$ males during the light period of the cycle we also measured their voluntary activity in cages equipped with a running wheel (Figures 4b-e). According to what was observed in the activity cages, mutant males ran more during the light phase, whereas their wheel turns in the dark phase were comparable to those of controls. Consistent with their spontaneous activity, mutant females (Figures 4d and e) ran as much as control females during the light phase. Taken together, these results show that mutant males, but not females, move more in the resting phase and have a perturbed light/dark activity.

Crtc1$^{-/-}$ *male mice eat more during the light phase*

To verify whether there was a relation between hyperphagia and light phase activity of *Crtc1*$^{-/-}$ males, we determined the quantity of food eaten at the end of the dark and the light phase of the daily cycle. 24-hour food intake of *Crtc1*$^{-/-}$ males was significantly higher than controls (WT = 4.35 ± 0.15, *Crtc1*$^{-/-}$ = 5.60 ± 0.17, *P* = 0.004, Student's *t*-test) confirming the overeating behavior observed during weekly measurements. When

we calculated the quantity of food ingested per phase, we found a consumption of 3.66 g ± 0.14 for *Crtc1*$^{-/-}$ males and of 3.36 g ± 0.13 for WT in the dark phase and of 1.93 g ± 0.19 and 1.00 g ± 0.14 (Bonferroni post-hoc test, $P < 0.001$) in the light phase, respectively (Figure 4f). These data support our hypothesis that the hyperphagia of *Crtc1*$^{-/-}$ males occurs only during the light phase of the cycle. As expected, *Crtc1*$^{-/-}$ females (Figure 4g), which did not show overeating along all measures of food intake, ate as much as their WT littermates in 24-hours (WT = 4.44 g ± 0.14, *Crtc1*$^{-/-}$ = 4.28 g ± 0.24). Food consumption in the active phase (WT = 3.49 g ± 0.13, *Crtc1*$^{-/-}$ = 3.09 g ± 0.12) and in the resting phase (WT = 0.95 g ± 0.11, *Crtc1*$^{-/-}$ = 1.19 g ± 0.17) was similar in both genotypes. In conclusion, these data suggest that the obesity of *Crtc1* mutant males depends, at least in part, on increased food consumption during the resting phase.



Supplementary Figure S1. Effect of HFD in mutant females. (a)Cumulative food intake of standard chow (n=10 WT, n=4 *Crtc1*$^{-/-}$) and HFD (n=7 WT, n=7 *Crtc1*$^{-/-}$). (**b**) Cumulative body weight gain in females fed with standard chow and HFD.

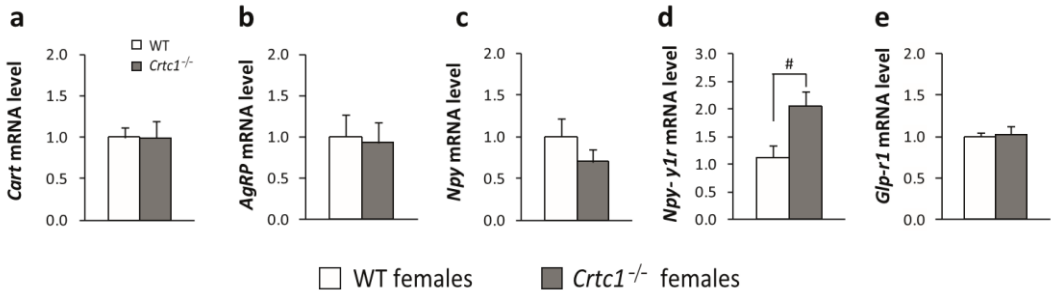
DISCUSSION

Overall, our work confirms the crucial role played by CRTC1 in energy balance, previously observed by Altarejos *et al.* [6], and shows that male mice lacking the CREB-coactivator 1 are hyperphagic and more vulnerable to develop obesity than mutant females, and this, from the beginning of adulthood. Indeed, mutant females exhibited a mild late-onset obesity without hyperphagia. We also found similar preference and self-administration responding for saccharine in both sexes. Finally, we observed an altered circadian activity in mutant males only.

So far, many neurobiological pathways responsible for food intake regulation have been identified in the brain [25]. The hypothalamus plays an essential role in controlling hunger and satiety, and the ARC, for its anatomical localization, is the first hypothalamic region that receives peripheral signals informing the brain about energy availability. Among these peripheral signals, leptin that is released by adipocytes, promotes fasting by stimulating CART/POMC neurons and inhibiting AgRP/NPY neurons through the binding to its receptor (LepRb) [26]. However, in obesity the large amount of leptin released by the adipose tissue leads to ARC-neurons leptin insensitivity (also called leptin resistance) and contributes to food overconsumption. Interestingly, Altarejos and colleagues have observed that 36-week-old *Crtc1*$^{-/-}$ mice are leptin resistant [6]. Many mechanisms have been proposed to explain leptin resistance, and one of them is a weak expression of the long form of the leptin receptors (LepRb) in the brain [27]. Accordingly, we found a significant reduction of the transcript levels for *LepRb* in overweight *Crtc1*$^{-/-}$ males, but not in young mutant males.

Recent studies have also shown that leptin can modulate the transcription of CREB-dependent genes directly facilitating CREB phosphorylation [28, 29] or by enhancing CRTC1 nuclear translocation [30]. Because *Cart* is a CREB-regulated gene, we expected to find reduced levels of *Cart* transcript in both young and old *Crtc1*$^{-/-}$ males. Surprisingly, only old and obese *Crtc1*$^{-/-}$ males have lowered levels of *Cart* transcript, whereas young mice

have not. Therefore, the deletion of *Crtc1 per se* does not seem to affect *Cart* transcription. Two mechanisms may explain this observation. First, our mice are constitutive knockout and the presence of CRTC2 in the ARC might compensate for CRTC1 deficiency [18]. Second, CREB phosphorylation is independent from CRTC1 activity and so, even in its absence, leptin-induced CREB activation may be sufficient to allow *Cart* transcription. Conversely, the impairment of leptin signal in old *Crtc1*$^{-/-}$ males, due to lowered *LepRb* expression, would have a more detrimental effect on CREB activation leading to reduced *Cart* transcription. Together, these results suggest that appropriate leptin signaling may be crucial for *Cart* expression.



Supplementary Figure S2. Effect of the lack of CRTC1 on gene expression of ARC neurons in female mice. (**a**) *Cart* (n=8 WT, n=8 *Crtc1*$^{-/-}$, *P* = 0.959), (**b**) *AgRp* (n=6 WT, n=8 *Crtc1*$^{-/-}$, *P* = 0.862), (**c**) *Npy* (n=7 WT, n=8 *Crtc1*$^{-/-}$, *P* = 0.284), (**d**) *Glp-r1* (n=8 WT, n=8 *Crtc1*$^{-/-}$, *P* = 0.783) mRNA levels were unchanged in old *Crtc1* mutant females. (**e**) Npy-y1 expression was upregulated in 52-week-old females (n=7 WT, n=8 *Crtc1*$^{-/-}$, *P* = 0.018). **P* < 0.05, Student's *t*-test.

Since young *Crtc1*$^{-/-}$ males, despite unchanged *Cart* expression, ate more than their age-matched controls, we explored the possibility that *Crtc1*$^{-/-}$ males overeating would depend on the impaired expression of other CREB-regulated genes. The neuron-derived orphan receptor 1 (NOR1), also known as NR4A3, is a protein belonging to the family of intracellular transcription factors [31]. The transcription of *Nor1* is under the control of CREB and we already reported lowered *Nor1* mRNA levels in the prefrontal cortex and in the hippocampus of *Crtc1*$^{-/-}$ mice [8]. Recently, a work of Kim *et al.* has shown that, in ARC neurons leptin seems to facilitate *Nor1* transcription through CREB recruitment and that in turn, *Nor1* would reduce appetite by antagonizing glucocorticoid-induced AgRP release and inhibiting *Npy* transcription [32]. According to this mechanism, we found that *Crtc1*$^{-/-}$ males, compared to their age-matched controls, have significantly lower levels of *Nor1* transcript. Moreover, this change in *Nor1* expression was associated with a rise of *AgRP and Npy* mRNA level in young and old *Crtc1*$^{-/-}$ males, respectively. Overall, our findings suggest that, the overeating of young *Crtc1*$^{-/-}$ males may be induced by the impaired expression of *Nor1* and that the appetite of older *Crtc1*$^{-/-}$ mice would be affected by a concurrent reduction of *Nor1* and *Cart* transcription.

The gene coding for the NPY receptor type 1 (NPY-1R) is another anorexic CREB-regulated gene [33]. The comparison between *Crtc1* mutant males and WT littermates at 8 and 36 weeks of age did not reveal any alteration in the expression of this gene indicating that it would not play a critical role in their hyperphagic behavior.

Fat mass- and obesity-related gene (*Fto*) is an mRNA demethylase whose enzymatic activity has been associated with increased body mass index and obesity vulnerability. Although its physiological functions remain largely unknown, recent investigations have begun to unravel the link between this enzyme and energy balance [34]. In rodents, it has been found that ARC-*Fto* expression is lowered during fasting [35]. Moreover, it seems that this enzyme would delay CREB-dephosphorylation and would inhibit food intake through the expression of *Npy-1r* and *Bdnf* [36]. In contrast to these previous works, we found a lower level of *Fto* transcript in both young and old *Crtc1*$^{-/-}$ males. Therefore, additional studies are required to understand more in detail the physiological role of FTO in energy balance regulation, and how CRTC1 interferes with the transcription of this mRNA demethylase.

The glucagon-like peptide 1 is a peptide that acts as a strong feeding suppressor in the hypothalamus upon the binding with its receptor (GLP-R1) [37]. The overlapping distribution of GLP-1R and CRTC1 in the hypothalamus, led us to investigate whether the lack of CRTC1 could affect the expression of this receptor, but our findings indeed show no alteration of its expression.

Unlike males, *Crtc1*$^{-/-}$ females do not exhibit any food overconsumption. Consistent with a normal feeding behavior, 52-week-old *Crtc1*$^{-/-}$ females do not show any modification in the gene expression pattern, except for a significant increase in *Npy-1r* transcript. This sexual

dimorphism was not described in the previous study of Altarejos and colleagues [6]. Further investigations are needed to understand why the lack of *Crtc1* impairs males preferentially, and in particular delineating a putative effect of sex hormones on the CREB-CRTC1 pathway would be of the highest relevance. Indeed, Choong and colleagues established that the vulnerability to develop obesity in patients bearing a CRTC1 polymorphism varied between men and women, suggesting that estrogen levels most likely modulate the effect of this CRTC1 polymorphism on fat accumulation [22].

Eating is a complex behavior driven by energy need and food rewarding properties. Whereas the homeostatic control of food intake depends on the functionality of the hypothalamic nuclei, the motivation to consume highly palatable food is governed by the reward system. Neurons of both systems are intimately interconnected [38]. In particular, lateral hypothalamic neurons, which integrate signals coming from other hypothalamic nuclei, project to VTA-dopaminergic neurons and regulate reward seeking behavior through the modulation of dopamine release. Leptin participates in this modulation by inhibiting dopamine release and consequently the rewarding properties of food [39]. Thus, impaired leptin signaling, as that observed in case of leptin insensitivity, may facilitate food overconsumption.

Considering the relevance of non-homeostatic feeding [26], the fact that CRTC1 is abundantly expressed in brain structures belonging to the reward system, and finally the leptin resistance showed by *Crtc1* mutant mice, we explored the possibility that their overeating could arise from impairments in reward appreciation. Noteworthy, regardless of their genotype and sex, all mice exhibited a drastic preference for sweetened taste over tap water, and the ability to acquire a stable saccharine intake in an operant conditioning paradigm. Furthermore, they all manifested a strong capacity to associate reward delivery and reward-paired cues, as attested by the reinstatement of their previously extinguished nosepoking behavior upon presentation of the olfactory and light cues.

Because *Crtc1*$^{-/-}$ males may have earned less rewards during acquisition of saccharine self-administration, an observation in line with a previous report of ours indicating that these mutant mice moved less in response to moderate stressful environments [8], we considered the possibility that *Crtc1*$^{-/-}$ males obesity could also depend on insufficient energy expenditure. To answer this question, and avoid confusion due to excessive overweight, we monitored spontaneous and motivated locomotor activity in young *Crtc1*$^{-/-}$ mice. Although *Crtc1*$^{-/-}$ and WT males had similar total activity, a more detailed analysis of their behavior showed a great difference between the dark and the light phase of the cycle. In fact, mutant males, as expected moved more during the light phase. Contrastingly, *Crtc1*$^{-/-}$ females were globally more active than control females but this extra activity was present only in the dark phase. Voluntary exercise in running wheels confirmed that *Crtc1*$^{-/-}$ males, but not females, run more than controls in the light phase. Collectively, these results highlight a different locomotor phenotype in *Crtc1*$^{-/-}$ females and males and show that these latter exhibit a perturbed night and day activity.

Because of this observation, we tested the hypothesis that diurnal activity of *Crtc1*$^{-/-}$ males could coincide with increased food seeking and food intake. Therefore, we measured food intake in the two phases of the light cycle. *Crtc1*$^{-/-}$ and WT females consumed similar amount of food both in the dark and light phase. In contrast, mutant males showed overeating only during the resting phase. Altogether, these finding show a shift in eating and locomotor activity of mutant males suggesting the presence of a misalignment of metabolic functions with the circadian clock. Interestingly, recent reports have pointed out the participation of CRTC1 in master clock entrainment [40, 41], but further investigations are required to unravel the role of this transcription coactivator as synchronizer of feeding time.

Accumulating evidence indicates that disrupted synchronization of feeding and sleeping time with the master clock results in dampening of metabolic and endocrine functions. Concerning obesity, alterations in adipogenesis, satiety signaling and energy metabolism have all been associated with circadian regulation [42]. Likewise, the lack of synchrony of the internal clock with the daily light cycle affect glucocorticoid levels and stress reactivity facilitating the development of emotional disturbances, namely depression [43]. In conclusion, improved knowledge of the mechanisms through which CRTC1 synchronizes metabolic functions with the light cycle may facilitate our understanding of the biological processes underlying the interaction between obesity and depression.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGMENTS

This work was partly funded by a grant from the Swiss National Science Foundation (31003A-135692), and supported by the National Centre of Competence in Research (NCCR) Synapsy.

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3.2 ARTICLE 2: Evidence for a compulsive-like behavior in rats exposed to alternate access to highly preferred palatable food

Personal contribution: I contributed to the scientific conceptualization of this work and to the choice of the binge-eating model. I conducted most of the behavioral manipulations, and all metabolic and molecular experiments. I also collected the results and made statistical calculations. Finally, I participated in the redaction of the article.

Status: Published in Addiction Biology, 2014 Nov; 19(6):975-985.

Addiction Biology

ORIGINAL ARTICLE

doi:10.1111/adb.12065

Evidence for a compulsive-like behavior in rats exposed to alternate access to highly preferred palatable food

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ABSTRACT

Converging evidence suggests that recurrent excessive calorie restriction causes binge eating by promoting behavioral disinhibition and overeating. This interpretation suggests that cognitive adaptations may surpass physiological regulations of metabolic needs after recurrent cycles of dieting and binging. Intermittent access to palatable food has long been studied in rats, but the consequences of such diet cycling procedures on the cognitive control of food seeking remain unclear. Female Wistar rats were divided in two groups matched for food intake and body weight. One group received standard chow pellets 7 days/week, whereas the second group was given chow pellets for 5 days and palatable food for 2 days over seven consecutive weeks. Rats were also trained for operant conditioning. Intermittent access to palatable food elicited binging behavior and reduced intake of normal food. Rats with intermittent access to palatable food failed to exhibit anxiety-like behaviors in the elevated plus maze, but displayed reduced locomotor activity in the open field and developed a blunted corticosterone response following an acute stress across the diet procedure. Trained under a progressive ratio schedule, both groups exhibited the same motivation for sweetened food pellets. However, in contrast to controls, rats with a history of dieting and binging exhibited a persistent compulsive-like behavior when access to preferred pellets was paired with mild electrical foot shock punishments. These results highlight the intricate development of anxiety-like disorders and cognitive deficits leading to a loss of control over preferred food intake after repetitive cycles of intermittent access to palatable food.

Keywords Anxiety, compulsive-like behavior, diet, palatable food, stress.

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INTRODUCTION

In developed countries, where nutrition and life-style have dramatically changed in the last decades, more than 1.5 billion adults are overweight. The World Health Organization reports that the increased body weight in the general population correlates with consumption of larger amounts of calorie-dense food and reduced physical activity (WHO 2009). A large body of evidence suggests that overweight and obese people have higher risks of developing diabetes, heart diseases and cancer (Allison *et al.* 1999). According to the current epidemiologic data, overweight/obesity, high blood glucose and high cholesterol have become the leading causes of preventable death behind high blood pressure and tobacco use worldwide (WHO 2009). In parallel, patients suffering from eating disorders, particularly anorexia nervosa, frequently experience mood disorders, including negative emotionality, neuroticism, generalized anxiety and depression (Kaye 2009; Luppino *et al.* 2010).

Current considerations on feeding regulation take into account two overlapping brain mechanisms, the *homeostatic* and the *hedonic* control of food intake (Berthoud 2007; Lutter & Nestler 2009). Clinical researchers have proposed that the hedonic control of food intake surpassed the homeostatic metabolic needs in overeaters, especially in compulsive overeaters (Zheng *et al.* 2009; Parylak, Koob & Zorrilla 2011). Concomitantly, recent evidence collected in pre-clinical models has suggested that compulsive overeating might share some neurobiological adaptations reminiscent of drug addiction (Avena, Rada & Hoebel 2008; Davis & Carter 2009; Kenny

2011b). Even if the debate remains open, and thoroughly discussed (Benton 2010; Wilson 2010; Ziauddeen, Farooqi & Fletcher 2012), most of recent clinical and pre-clinical data support the view that compulsive overeating and addiction share common neural substrates (Barry, Clarke & Petry 2009; Corsica & Pelchat 2010; Grosshans, Loeber & Kiefer 2011; Volkow *et al.* 2013). Of particular interest, sugar and sweet fat diets have been shown to stimulate the brain reward system (Hernandez & Hoebel 1988; Avena *et al.* 2008; Berridge *et al.* 2010) and it appears that hypersensitivity of reward circuitries may predispose an individual to overeating and weight gain (Kenny 2011b). In particular, the key role of dopamine receptor 2 has been demonstrated in both clinical (Stice *et al.* 2010, 2011) and pre-clinical studies (Johnson & Kenny 2010), emphasizing the plausibility of common cellular and molecular mechanisms in obesity and drug addiction (Volkow *et al.* 2013). Further, it has also been proposed that acute physical or emotional stress increased preference for fat and/or sugar contents even in absence of hungriness, thus defining the notion of comfort food (Dallman *et al.* 2003; Dallman 2010). Consumption of comfort food would alleviate signs of distress and anxiety by reducing the hypothalamic-pituitary-adrenal (HPA) axis activity (Dallman *et al.* 2003; Pecoraro *et al.* 2004; Gibson 2012). Such a relief, together with the direct activation of brain reward systems, would promote sweetened food seeking and exacerbate craving for calorie-dense food. This effect could be mediated by insulin and glucocorticoids (Dallman 2010). However, recent evidence demonstrated that the caloric load is not as critical as the hedonic aspect of palatable food consumption for reducing stress since saccharin-fed (or sexually active) rats had attenuated HPA axis responses to acute stress, like sucrose-fed animals (Ulrich-Lai *et al.* 2010). This observation is of significant importance since it suggests that the subjective feeling of pleasantness is fundamental for promoting palatable food consumption. This assumption may also explain why it is not the palatable food *per se* that promotes compulsive overeating, but most likely its pattern of consumption (Corwin & Grigson 2009; Corwin 2011). Polivy & Herman (1985) already reported that dieting often preceded binge eating chronologically. They proposed that recurrent excessive calorie restriction, in order to prevent weight gain, might cause binging by promoting behavioral disinhibition and overeating (Polivy & Herman 1985). Interestingly, these authors have also suggested that, with cognitive controls supplanting physiological regulatory processes, eating and weight begin to dominate the dieter's thought. Ultimately, dieting makes the dieter vulnerable to break the restrictive rules and lose control over incentive 'forbidden' calorie-dense food (Polivy & Herman 1985). In line with this interpretation, recent findings point out that alterations in cognitive processes, such as the appreciation of the rewarding value of palatable food and the capacity to inhibit hedonic feeding, may represent vulnerability factors promoting bingeing and overeating (Appelhans *et al.* 2011).

In parallel to these clinical observations, sugar binging models have been established in laboratory animals. However, escalation in sucrose consumption often required recurrent cycles of food restriction followed by periods of full access to sucrose or palatable food intake (Hagan *et al.* 2003; Corwin 2006, 2011; Avena *et al.* 2008), which raises the possibility of confounding stress-like effects due to chronic starvation. Noteworthy, recent pre-clinical evidence confirmed that alternate access to sweet fat food elicited binging behavior in non-food-deprived laboratory animals (Berner, Avena & Hoebel 2008; Cottone *et al.* 2008a,b, 2009a,b). Rats with alternate access to a palatable food exhibited binging behavior, metabolic alterations and anxiety-like behaviors (Cottone *et al.* 2009a,b). This model appears to be the most relevant for studying long-term consequences of 'yo-yo dieting', as defined as recurrent cycles of dieting followed by disinhibited hyperphagia. Indeed, intermittent access to rewards (palatable food or drugs of abuse) leads to negative emotional states when the rewarding substance is no longer available, and it is considered that such negative emotional states may be sufficient for motivating substance use and abuse via negative reinforcement mechanisms.

In summary, the present study investigated whether rats with a history of alternating access to highly preferred food (but only containing 5% more calories than regular chow pellets) would exhibit anxiety-like traits and cognitive alterations defined as a loss of control over palatable food intake.

MATERIALS AND METHODS

Animals

Thirty adolescent female Wistar rats (140–200 g, 45–60 days old at the beginning of the experiment) were individually housed and kept under reversed light-dark cycle conditions (12 hours light–dark cycle, lights off at 8 am) where humidity (50–60%) and temperature (22 ± 1°C) were controlled. Food and water were available *at libitum* during the procedure. All behavioral experiments were carried out during the dark phase of the cycle. The procedures were conducted in conformity with the Swiss National Institutional Guidelines on Animal Experimentation, and approved by the Swiss Cantonal Veterinary Office Committee for Animal Experimentation (authorization 1999 to B.B.)

Table 1 Diet composition and energy density.

Diet	*Energy density (kcal/g)*	*Macronutrient composition (kcal%)*		
		Carbohydrate	*Protein*	*Fat*
3436 Kliba (standard food)	3.129	77.0	18.5	4.5
5TCY Test Diets (preferred food)	3.290	63.6	22.5	13.9
Precision pellets BioServ (sweet pellets)	3.600	67.2	20.5	12.3

Alternate feeding protocol

After 1 week of habituation, animals matched for food intake and body weight were divided in two groups. One group (named C/C) received standard chow 7 days a week, whereas the second group (named C/P) was given 5 days of standard chow (defined as the C phase, for access to chow food) followed by 2 days of a more palatable food (defined as the P phase, for access to preferred food) for a total of seven consecutive weeks. Standard diet (3436 Kliba Nafag, Provimini Kliba AG, Kaiseraugst, Switzerland) and palatable diet (5TCY Test Diets, IPS Ltd, London, UK) had comparable energy density and macronutrient composition (see Table 1), and palatable diet was chocolate flavored. Daily caloric food intake and body weight were measured every day, around 1 hour after the onset of the dark phase.

Food operant conditioning

Apparatus

Food conditioning was conducted in six operant chambers (Med Associates Inc., St. Albans, VT, USA) placed in sound-attenuated cubicles equipped with ventilation fans. Inside cages, a food receptacle was located between two retractable levers and connected to a food dispenser. Presses on the 'active lever' resulted in the release of a 45 mg food pellet whereas presses on the 'inactive lever' had no consequence. A cue light illuminated for 1 second above the active lever signaled food delivery. The grid floor of the cage was connected to a shocker apparatus for administration of electrical foot shocks. Custom-made schedule programming and data acquisition were driven by a Med-PC software package (Med Associates Inc.).

Training for food reinforcement

During the 'C phase' of the third cycle of alternate feeding, rats were trained in the operant cages to acquire the lever pressing behavior and get food pellet rewards under a fixed ratio 1 time out 1 second (Dustless precision pellet 45 mg, rodent purified diet, BioServ, Frenchtown, NJ, USA). Training sessions lasted until the rat collected 60 rewards or terminated after a maximum of 30 minutes. Rats were trained twice a day and after five sessions, all rats succeeded to collect 60 pellets in less than 30 minutes.

Progressive ratio measurements

One week after, all animals were submitted to a progressive ratio (PR) schedule of reinforcement (meaning during the 'C phase' of the fourth cycle). Rats had to increase progressively the number of lever presses to receive one pellet reward. The progression of responses required was set to 1, 2, 4, 6, 9, 12, 15, 20, 25, 32, 40, 50, 62, etc. Both groups of rats underwent two PR sessions a day, one session with sweet pellets (45 mg Dustless precision pellets, BioServ) and the other session with chocolate pellets (45 mg 5TCY pellets, Test Diets). Rats were randomly assigned to sessions so that half of them received chocolate pellets in the morning and the others in the afternoon. Each session ended when no response was emitted for 20 minutes or after a total duration of 90 minutes. The number of lever presses was measured as an index of motivation. The test was performed twice, on the first and fifth day after home cage palatable food withdrawal.

Compulsive food seeking

Perseverance to get food pellets despite shock punishments was assessed in rats pressing a lever paired with the delivery of a 45-mg food pellet immediately followed by a moderate foot shock punishment (0.22 mA, 0.5 second) during a 30-minute test session, on the first and fifth day after preferred food withdrawal. Since repeated exposure to foot shocks may have induced confounding effects in rats, C/P and C/C groups were randomly divided in two subgroups. Rats were exposed to foot shocks only twice, on day 1 and day 5 as mentioned above, and thus were tested with one type of food reward only (half rats tested on chocolate pellets and half rats tested on standard pellets).

Anxiety-related behaviors

Rats were tested in the open field–novel object (during the fifth cycle) and in the elevated plus maze (during the sixth cycle) paradigms on the first day of palatable food withdrawal for evaluating anxiety-like behaviors. Both

experiments were conducted under a dim light (10–15 lx). Animal tracks were recorded by a digital video camera mounted above the maze and connected to a computer running a tracking software (Ethovision v.3.1—Noldus Technology, Wageningen, the Netherlands).

Open field–novel object

Rats were placed in a round arena (140 cm of diameter, 30 cm of depth) and their motor and exploratory activities were monitored for 10 minutes, after which a novel object (a white plastic bottle) was introduced in the center of the maze. The distance travelled in the arena, the time spent in the central part of the arena and the time spent touching the novel object were assessed for additional 5 minutes.

Elevated plus maze

The elevated plus maze consisted of two opposite open arms (50 cm L × 10 cm W × 42.5 cm H) and two opposite closed arms (50 cm L × 10 cm W) arranged in a cross and elevated 50 cm above the floor. In the center, a small platform (10 cm × 10 cm) gave access to all arms. Rats were gently placed in the center of the maze face to a close arm and their behavior was monitored for 5 minutes. The time spent on open arms was used as an index of anxiety.

Plasma corticosterone levels in response to acute stress

Plasma corticosterone levels were measured in response to an acute stress at the beginning (third cycle) and at the end (seventh cycle) of the entire protocol. The acute stress (rats were placed on an elevated platform 12 × 12 × 101 cm above the floor) was applied for 30 minutes on the first day of preferred food withdrawal. Corticosterone levels were assessed at three different timepoints, always in the middle of the dark (active) period: 24 hours before the acute stress (basal), 5 minutes and 60 minutes (t5 and t60) after the stress procedure. A blood sample (200–300 µl) was systematically collected using heparin Microvette CB 300 (Sarstedt AG, Sevelen, Switzerland) after tail vein incision. Blood samples were then centrifuged at 4°C for 20 minutes at 4500 rpm. Plasma samples were analyzed using a commercial enzymatic-immuno assay kit (Corticosterone EIA kit, Enzo Life Sciences, Lausen, Switzerland).

Statistical analysis

Data are expressed as mean ± standard error (SE). Food intake, body weight gain, operant conditionings and corticosterone measures were evaluated by using a two-way analysis of variance (ANOVA) with 'group' as between-subject factor and sampling 'time' as repeated-measures within-subject factor, followed by Fischer's PLSD *post-hoc* tests for multiple comparisons. Student's *t*-test was used to compare the distance moved in the open field and the time spent on the open arms of the elevated plus maze. For the novel object touching observation, a Shapiro–Wilks test revealed that the data were not normally distributed ($W = 0.8798$, $P = 0.0259$). Hence, a Mann–Whitney *U*-test was used to compare the duration of contact with the novel object. All statistical analyses were conducted using the statistical software STATISTICA 10 (Stat Soft, Tulsa, OK, USA). The level of significance was set at 0.05.

RESULTS

Alternate palatable food access changes food-taking behavior

During the last 3 days of habituation in individual cages, rats assigned to the standard laboratory chow pellets (C/C group) and rats exposed to alternating access to standard chow and preferred food (C/P group) exhibited similar body weight (180.7 ± 7.35 g and 182.8 ± 5.73 g, respectively). Their daily caloric consumption was also identical (49.9 ± 1.85 kcal/day and 47.2 ± 0.78 kcal/day, respectively).

Afterwards, as shown on Fig. 1, chow/preferred rats overate during the P phases and underate during the C phases, as compared to chow/chow control rats. Consumption of palatable food was particularly elevated on the first day of each 'P phase' [$F_{1.28}$ (group) = 43.55, $P < 0.001$]. Monitoring of food intake revealed that C/P rats displayed a binge eating behavior for the first 8 hours of access to preferred food compared to C/C rats [$F_{1,14}$ (group) = 12.20, $P < 0.01$; $F_{2,28}$ (time) = 217.57, $P < 0.001$; $F_{2,28}$ (group × time) = 3.84, $P < 0.05$; Fig. 1b]. When C/P rats returned to standard food, they displayed a significant reduction of food taking, again during the first day after preferred food withdrawal [$F_{1.28}$ (group) = 80.93, $P < 0.001$]. However, C/P rats ate progressively more food during the next days and, on the fifth day, their food intake was similar to controls. Overall, the mean caloric intake of C/P rats during 'P phases' (Fig. 1c) was significantly higher compared to C/C rats [$F_{1,28}$ (group) = 21.85, $P < 0.01$] and this effect was stable across the procedure [$F_{6,168}$ (group × phase) = 1.14, $P = 0.338$]. Meanwhile, when exposed to regular chow pellets ('C phases'), the C/P group displayed a drastic reduction of mean caloric intake [$F_{1,28}$ (group) = 20.43, $P < 0.01$; $F_{6,168}$ (group × phase) = 1.08, $P = 0.376$] compared to controls. As expected, body weight gain in C/P rats changed accordingly with palatable food availability. When rats had access to palatable food, their body weight gain was significantly increased compared to that of control rats [$F_{1,28}$ (group) = 7.72, $P < 0.01$]. Conversely, when exposed to laboratory chow

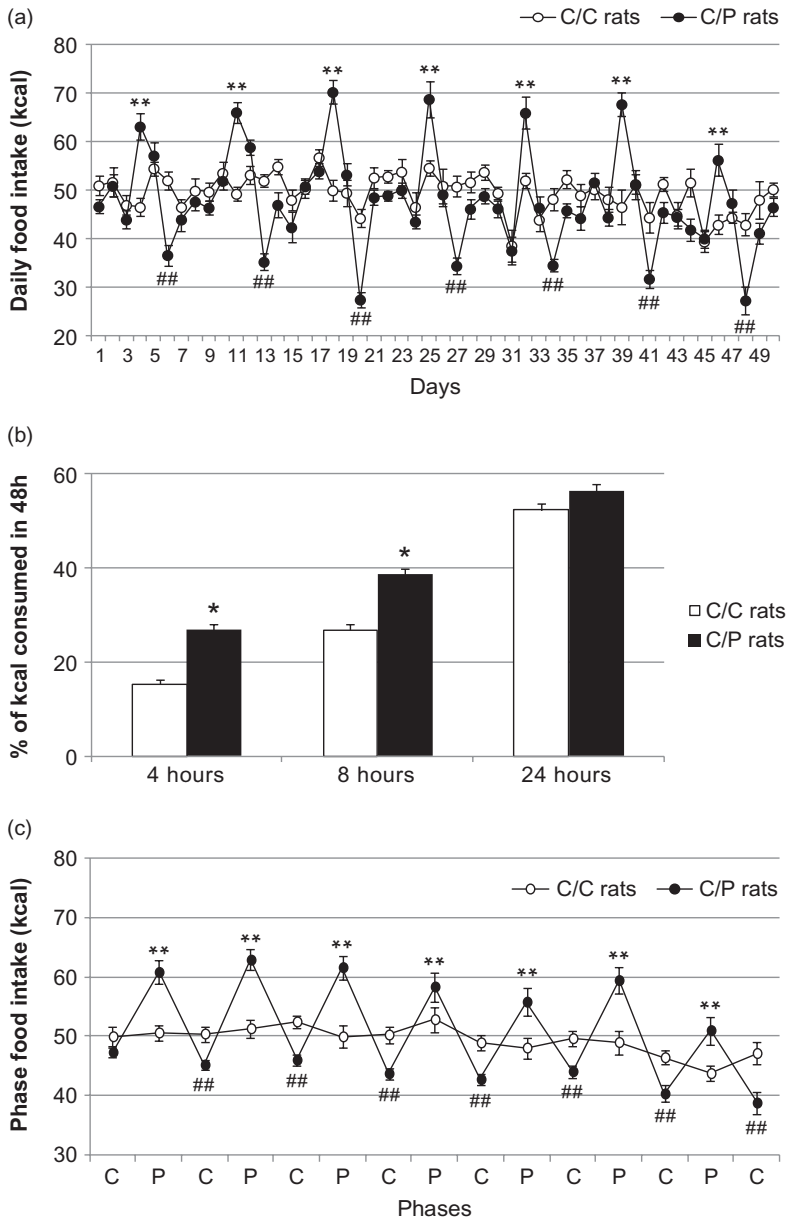

Figure 1 Intermittent access to preferred palatable food induced recurrent periods of food binging and restriction. (a) Data represent mean daily food intake (±SE) expressed in kcal. C/P rats ($n = 16$) had access to palatable food for 2 days followed by 5 days of standard chow pellet while C/C (control) rats had a continuous access to standard chow pellet (white circles, $n = 14$). (b) Data represent the mean cumulative food intake (±SE) expressed in percent of the total intake over the 48 hours of access to preferred food in C/P rats ($n = 8$) compared to standard food intake in C/C animals ($n = 8$) at the end of the procedure. (c) Data represent the average daily food intake (±SE) during the C (5 days of standard food) and P (2 days of preferred food) phases in C/P rats ($n = 16$) compared to controls ($n = 14$). Symbols (** and ##) indicate significant differences ($P < 0.01$) from controls (two-way repeated-measure ANOVA followed by Fisher's PLSD *post hoc* tests)

pellets, the body weight gain of C/P rats tended to be reduced [$F_{1,28}$ (group) = 3.92, $P = 0.057$].

As a result of these fluctuations in body weight gain, the cumulative body weight gain was similar in both groups of rats at the end of experimental procedure (Fig. 2).

Alternate palatable food access does not change motivation for preferred food in a PR schedule

All rats exhibited similar performances for collecting sweetened (BioServ) food pellets in a fixed ratio 1 schedule of reinforcement (C/C rats: 64.79 ± 1.20 and C/P rats: 64.31 ± 1.28 lever presses; *t*-test, $P = 0.791$). After acquisition of the operant conditioning procedure, C/C and C/P rats were tested on a PR schedule with both sweetened (BioServ) and chocolate (Test Diets) pellets. The experiment was repeated twice, on the first (test 1) and fifth day (test 2) of the 'C phase', meaning on the first and fifth day after preferred food withdrawal (Fig. 3a & b).

All rats exhibited similar performance during test 1 and test 2, suggesting similar motivation for the sweetened (BioServ) food pellets [$F_{1,56}$ (group) = 0.020, $P = 0.888$; $F_{1,56}$ (test) = 1.428, $P = 0.237$; Fig. 3a]. Interestingly, when exposed to the chocolate (Test Diets) food pellets, all rats again manifested similar performances, either during test 1 or test 2 [$F_{1,56}$ (group) = 0.433, $P = 0.512$; $F_{1,56}$ (test) = 0.374, $P = 0.543$; Fig. 3b], suggesting that C/P rats did not exhibit a higher motivation

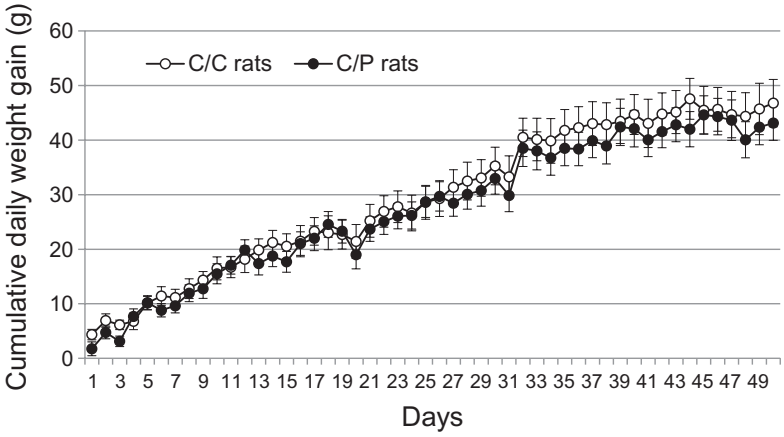

Figure 2 C/P ($n = 16$) and C/C (control) rats ($n = 14$) displayed similar cumulative weight gain curves during the seven weeks of the experimental procedure. Data represent mean weight (±SE) expressed in grams

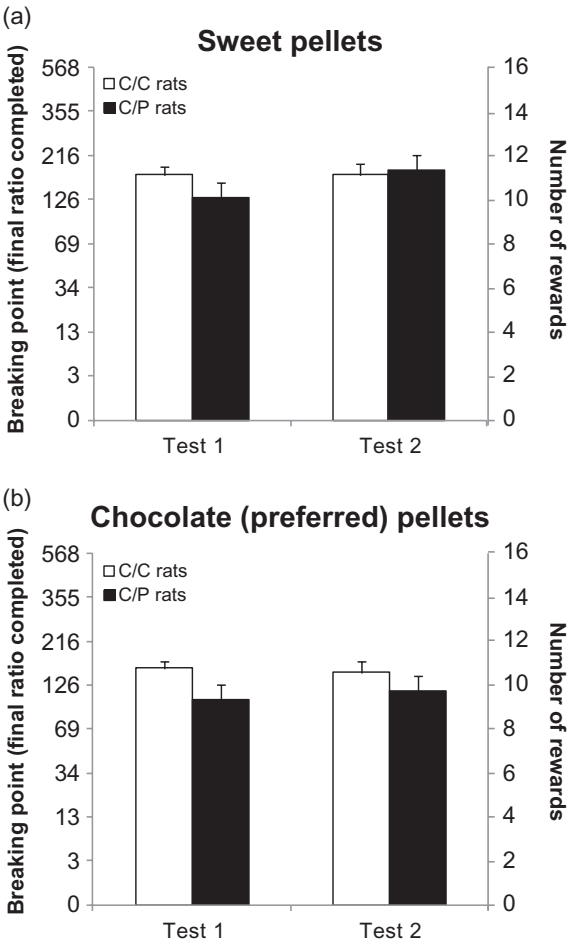

Figure 3 C/P ($n = 16$) and C/C (control) rats ($n = 14$) displayed similar motivation for sweetened and chocolate (preferred) pellets. Data represent mean breaking points (±SE) and concomitant mean rewards (±SE)

for preferred food compared to control animals. All rats, including controls for which access to palatable food was unusual, displayed a robust effort to collect sweetened food rewards, which reflects the high reinforcing properties of these pellets.

Noteworthy, standard laboratory chow pellets (3436 Kliba Nafag) were not available in 45 mg size. Consequently, we could not assess motivation for standard food using the PR schedule of reinforcement. Nonetheless, sated animals may not have worked thoroughly for non-rewarding/non-palatable food.

Alternate palatable food access promotes compulsive-like behavior

Perseverance to get food pellets despite shock punishment is considered to reflect compulsive-like behavior. Hence, rats were exposed to foot shocks twice, on day 1 and day 5 after preferred food withdrawal. They were tested with one type of food reward only, half rats were tested with Test Diet pellets, and the other half was tested with BioServ pellets.

All rats exposed for the first time to the foot shock procedure (test 1) accepted a few number of aversive stimuli for collecting sweet (BioServ) food pellets. However, when re-exposed to the experimental paradigm (test 2), all rats drastically reduced their number of food rewards and concomitant foot shocks. A two-way repeated-measure ANOVA revealed a significant main effect for test ($F_{(1,18)} = 16.87$, $P < 0.001$) but not for group ($F_{(1,18)} = 2.17$, $P = 0.157$) suggesting that the aversion for foot shocks surpassed motivation for BioServ food pellets in both C/C and C/P rats (Fig. 4a).

Strikingly, all animals exposed for the first time to the foot shock procedure (test 1) paired with preferred (Test Diets) food pellets exhibited an enhanced perseverance for reward-associated punishments compared to rats exposed to sweet (BioServ) pellets ($F_{(1,24)} = 6.95$, $P = 0.014$; Fig. 4a & b), whatever their group ($F_{(1,24)} = 0.854$, $P = 0.364$). However, only rats with a history of intermittent access to preferred food persisted to lever press during test 2. A two-way repeated-measure ANOVA revealed a significant group × test interaction ($F_{(1,18)} = 5.14$, $P < 0.05$), and Fisher's PLSD *post-hoc* tests among means demonstrated a significant decrease in reward

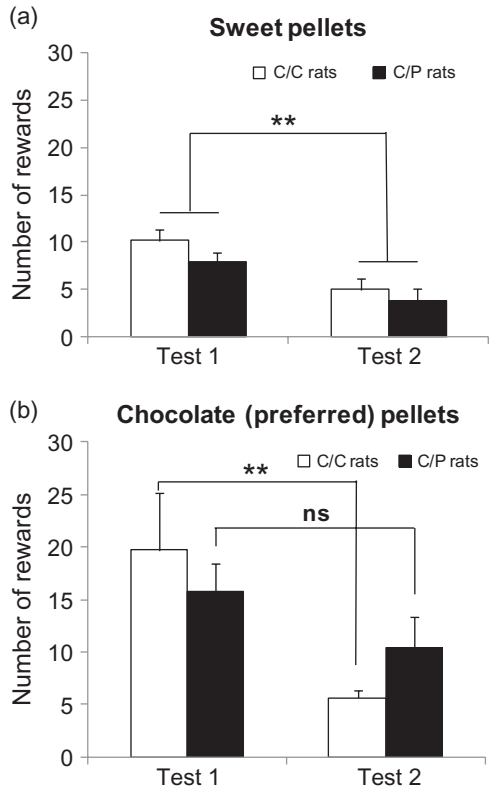

Figure 4 C/P rats persisted in taking preferred food pellets despite shock punishment compared to C/C (control) rats. Data represent the mean number of reward/punishments (±SE). (a) Both groups of rat (C/C, $n = 6$; C/P, $n = 5$) significantly reduced their lever pressing behavior on the second test for sweet pellets. (b) C/C rats ($n = 7$) significantly reduced their lever pressing behavior on the second test for preferred food pellets whereas C/P ($n = 10$) rats persisted in seeking palatable food despite aversive consequences. Symbols ** indicate a significant difference ($P < 0.01$) from test 1 (two-way repeated-measure ANOVA followed by Fisher's PLSD *post hoc* tests)

taking between test 1 and test 2 in C/C rats, whereas C/P rats displayed unchanged reward/punishment taking. The incentive for preferred (Test Diets) food pellets surpassed the aversion for foot shocks in C/P rats only, thus demonstrating that intermittent access to highly rewarding food promotes persistent palatable food-seeking behavior despite aversive consequences.

Alternate palatable food accesses moderately affect anxiety-like behaviors

Previous reports established that intermittent access to palatable food elicits signs of anxiety in rats. Hence, we evaluated anxiety-like behaviors in C/C and C/P rats at the end of the experimental procedure (fifth and sixth food cycles).

Open field–novel object

Although both groups spent similar amount of time in the central zone of the arena, C/P rats exhibited a reduced exploratory behavior assessed by the decreased distance travelled in the entire arena compared to C/C rats (5921.47 ± 162.91 cm versus 6473.81 ± 189.76 cm, respectively; *t*-test, $P = 0.0413$; Fig. 5a). Further, both groups displayed similar approaching behavior after introduction of the novel object in the central zone of the open field (10.00 ± 3.25 and 18.80 ± 4.53 seconds contacting the object, respectively; Mann–Whitney test, $P = 0.1097$; Fig. 5b).

Elevated plus maze

Both groups of rats spent an average of 20 to 25% of the total time on the open arms of the elevated plus maze suggesting similar emotional states while performing this test (C/C rats: 61.73 ± 19.47 and C/P rats: 68 ± 21.16 seconds; *t*-test, $P = 0.8117$; Fig. 5c).

Alternate palatable food access alters corticosterone response after an acute stress

We measured corticosterone levels in all rats at the beginning (third cycle) and at the end (seventh cycle) of the experimental procedure. At the beginning of the feeding protocol (Fig. 6a), both C/C and C/P rats displayed similar basal corticosterone levels (85.6 ± 13.7 versus 62.0 ± 10.3 ng/ml, respectively). Plasma levels strongly increased after the acute stress in both groups (321.3 ± 49.0 ng/ml and 276.3 ± 58.7 ng/ml, respectively) and returned to basal values later on (105.0 ± 26.5 ng/ml and 120.5 ± 50.3 ng/ml, respectively). A two-way repeated-measure ANOVA showed a main significant effect for time ($F_{2,40} = 28.75$, $P < 0.001$) but no difference between groups ($F_{1,20} = 0.175$, $P = 0.679$) suggesting that all rats responded similarly to the acute stress.

When rats were again tested at the end of the feeding protocol (Fig. 6b), C/C and C/P rats still exhibited similar basal levels of corticosterone (41.94 ± 10.6 versus 58.41 ± 8.11 ng/ml, respectively). However, whereas C/C rats still exhibited a drastic response the acute stress procedure, C/P rats displayed blunted corticosterone levels (298.4 ± 48.7 versus 186.8 ± 21.0 ng/ml, respectively; Fig. 6b). A two-way repeated-measure ANOVA revealed a main significant effect for time ($F_{2,40} = 47.52$, $P < 0.001$) and a significant interaction time × group ($F_{2,40} = 4.35$, $P = 0.019$), and Fisher's PLSD *post-hoc* tests among means demonstrated a significant decrease in corticosterone levels after acute stress in C/P rats compared to C/C controls. Taken together these results revealed an adaptation of the HPA axis in animals with a history of intermittent access to preferred palatable food.

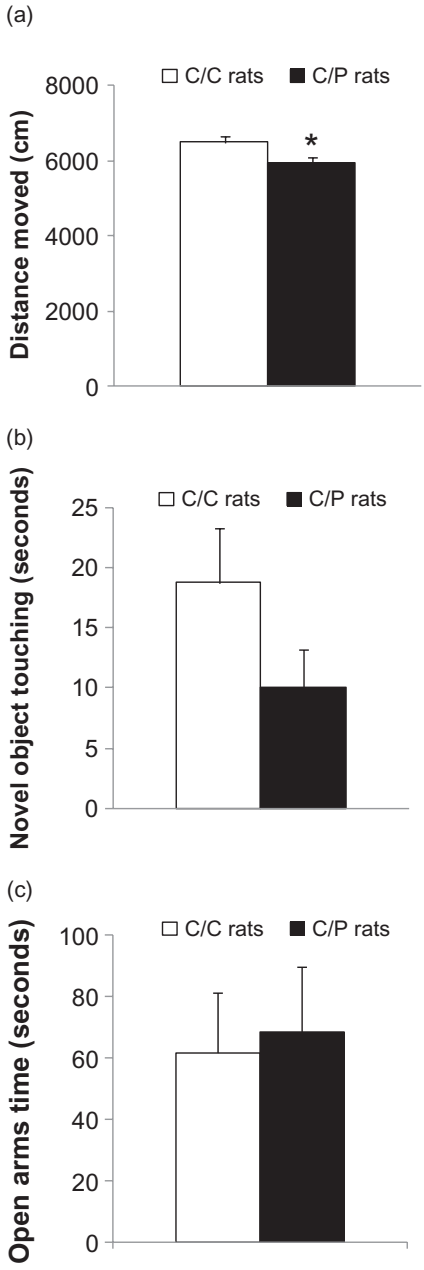

Figure 5 Measures of anxiety like-behaviors in C/C ($n = 14$) and C/P ($n = 16$) rats. (a) Data represent the mean distance (±SE) travelled in cm. C/P rats exhibited a reduced exploratory behavior compared to C/C rats in the open field (*t*-test, $^{*}P < 0.05$). (b) Data represent the mean duration (±SE) of contact with the novel object expressed in second. C/P rats showed a reduced, but not significant, interaction with the novel object compared to C/C rats (Mann–Whitney test, $P = 0.1097$). (c) Data represent the mean duration (±SE) expressed in second of the time spent on the open arms of the elevated plus maze. Both groups of rats spent similar amount of time on the open arms (*t*-test, $P = 0.8117$)

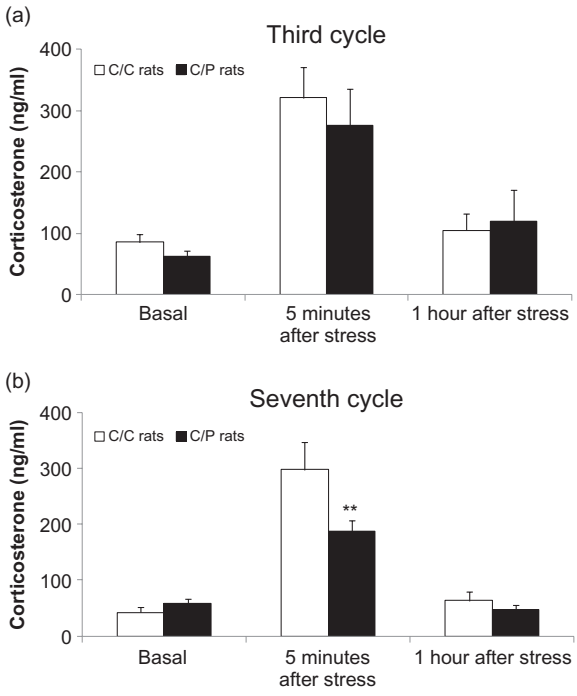

Figure 6 C/P ($n = 16$) rats exhibited blunted corticosterone levels after an acute stress compared to C/C (control) rats ($n = 14$). Data represent mean levels of corticosterone (±SE) expressed in ng/ml. (a) At the beginning of the procedure, C/P and C/C rats responded to the acute stress with increased levels of plasma corticosterone compared to basal measures. (b) At the end of the procedure, C/P rats displayed blunted corticosterone levels following the acute stress compared to C/C (control) rats. Fisher's PLSD *post hoc* tests, $^{**}P < 0.01$ versus C/C rats

DISCUSSION

Rats with a history of intermittent access to highly preferred food developed a 'yo-yo dieting', defined as recurrent cycles of reduced feeding behavior followed by disinhibited hyperphagia. We confirmed that alternate access to sweet fat food elicited binging behavior in non-food-deprived laboratory animals (Cottone *et al.* 2008a,b, 2009a,b). Furthermore, we also confirmed that not only did this diet cycling procedure elicit binge-like intake of preferred food, but it also caused a reduced intake of less preferred food between palatable food accesses (Cottone *et al.* 2008a,b, 2009a,b). It could be argued that this 'yo-yo dieting' is no more than a homeostatic control of feeding since both groups exhibited similar weight gain across the procedure. This assumption is most likely correct, at least at the beginning of the experiment, but our demonstration emphasizes the long-term consequences of this diet cycling on the stress response. Indeed, the blood corticosterone release following an acute stress was normal in rats exposed to a 3-week period of alternate diet, but was significantly reduced after 7 weeks. This apparent stress hyporesponsiveness, considered as a hallmark of persistent disruption of HPA function, is in line with a recent report demonstrating that intermittent access to palatable diets induces an allostatic shift in brain stress systems (Cottone *et al.* 2009a). In our hands

though, rats with intermittent access to palatable food did not exhibit any increased anxiety-like behaviors assessed with the elevated plus maze (both groups of rats spent around 20% of the total time on the open arms). This observation differs from that of Cottone *et al.* (2009b) in which control rats spent twice as much time on the open arms. Therefore, we may have missed the diet cycling-induced anxiety effect due to a possible floor effect. Nonetheless, in line with the idea that intermittent access to preferred food leads to negative emotional states, we report a compulsive-like behavior in C/P rats since they persisted to accept punishments when access to preferred food pellets was paired with mild electrical foot shocks. Overall, our results highlight the intricate development of disrupted HPA function and cognitive deficits leading to a loss of control over preferred food intake after repetitive cycles of intermittent access to palatable food.

Consumption of sugar and high-fat contents is considered to lower the stress response (Dallman 2010). According to this assumption, occasional binge eating may reflect a coping strategy aimed at alleviating signs of anxiety. This behavior could trigger the development of 'bad' habits leading to obesity due to recurrent and exaggerated accesses to high caloric foods (Dallman 2010). Meanwhile, recurrent withdrawal from calorie-dense contents could precipitate an increased stress state further promoting calorie-dense food craving (Teegarden & Bale 2007). However, the present findings support that food inclination, and not caloric load, is sufficient for promoting emotional changes upon cessation of preferred food access (Cottone *et al.* 2009b; Ulrich-Lai *et al.* 2010). Indeed, as reported in Table 1, preferred food content is quite similar to that of standard laboratory chow pellet, and its caloric load is unlikely to explain our behavioral observations. A more plausible explanation is that carbohydrate-rich foods may directly activate brain reward systems that produce a concomitant sensation of wellbeing, inherently reducing subjective feelings of anger and tension. Repeated overconsumption of palatable foods may also produce long-term neuroadaptations in brain reward and stress pathways that ultimately may promote depressive or anxious responses when those foods are no longer available or consumed (Parylak *et al.* 2011). Repeated overconsumption of highly palatable foods may initiate a downregulation of dopaminergic reward circuitry via mechanisms that mirror those commonly observed in drug addiction: reduced striatal dopamine D2 receptor availability and blunted dopamine release (Kenny 2011b; Volkow *et al.* 2013). Such a reduced dopaminergic tone in the striatum may lead to impaired inhibitory control over food intake and thereby increase risk of overeating (Johnson & Kenny 2010).

In parallel, a concurrent amplification of brain stress or 'antireward' systems may contribute to the shift from a positive to a negative reinforcement of palatable food, thus exaggerating the vulnerability to lose control over food intake (Parylak *et al.* 2011). Indeed, converging evidence revealed a key role for corticotropin releasing factor (CRF) in promoting food seeking. Infusion of CRF into the nucleus accumbens shell enhanced the incentive salience of a contextual cue previously paired with palatable food availability (Pecina, Schulkin & Berridge 2006; Berridge *et al.* 2010). Further, mice with extended access to palatable high-fat diet exhibited decreased expression of CRF in the central amygdala (CeA), whereas mice undergoing a palatable food withdrawal had increased CRF expression in this brain area (Teegarden & Bale 2007). Mice with increased CRF expression in the CeA also spent significantly more time in an aversive environment to obtain palatable food compared to control animals fed with regular chow pellets (Teegarden & Bale 2007).

In our hands, C/C and C/P rats exhibited similar breaking points in the PR schedule. This is not surprising considering that C/C rats had been briefly exposed to sweetened food during acquisition of the operant conditioning. They logically manifested an elevated motivation for both types of sweetened food pellets when given access to, since enhanced motivation does not correspond to loss of control. This high motivation was again observed on the first exposure to foot shocks; however, the key observation is that all rats drastically decreased their food-seeking behavior on the second exposure to punishments at the notable exception of C/P rats working for chocolate (Test Diets) pellets. This persistent food-seeking behavior despite adverse consequences is strikingly reminiscent of that observed in cocaine addict rats (Koob & Le Moal 2001, 2008; Deroche-Gamonet, Belin & Piazza 2004; Parylak *et al.* 2011).

Interestingly, a recent study established that food seeking in spite of harmful consequences depended on noradrenergic neurotransmission in the prefrontal cortex (Latagliata *et al.* 2010). Noteworthy, the nucleus of the tractus solitarius (NTS) contains neurons producing catecholamine neurotransmitters (including noradrenaline) and is involved in the regulation of feeding behavior. The NTS relays visceral signals to homeostatic feeding centers in the hypothalamus, and also projects to brain areas involved in the regulation of stress and reward processing, including the nucleus accumbens, the amygdala and the prefrontal cortex. Hence, neuroadaptations within the NTS could contribute to an altered perception of food reward (Kenny 2011a) and could play a key role in the intricate networks of brain nuclei involved in the stress-induced loss of control over palatable food intake after chronic episodes of binging and dieting.

With regards to the blunted corticosterone levels displayed by rats with a history of dieting and binging, it is

important to mention that apart from two brief exposures on elevated platforms, all rats remained in a quiet environment for the duration of the entire procedure. Thus, these blunted corticosterone levels cannot be attributed to external factors or stressful situations. Strikingly, blunted cortisol, and concomitant blunted cardiac reactions to acute psychological stress, have been frequently reported in patients with eating disorders (Pirke *et al*. 1992; Koo-Loeb *et al*. 2000; Ginty *et al*. 2012), but also in abstinent alcoholic and polysubstance-abusing patients (Lovallo *et al*. 2000; Lovallo 2006). It seems therefore that a common spiraling distress (defined as a progressive dysregulation of the brain reward function and concomitant development of counter adaptive processes within the brain stress system) may grow with repeated cycles of binging (or drug abuse) and dieting (or protracted abstinence), producing an allostatic state that drives further consumption, and ultimately compulsive intake (Koob & Le Moal 2001, 2008). Blunted cortisol in humans (and corticosterone in rats) may represent a biological marker of this allostatic state that may increase the vulnerability to develop negative affect and depressive-like behaviors. Indeed, blunted cardiac reactions to acute psychological stress have been correlated with an increased vulnerability to depression in humans (Phillips *et al*. 2011). And it has been recently demonstrated that rats with a history of intermittent access to palatable food exhibited signs of depression-like behaviors (Iemolo *et al*. 2012).

Overall, the present study is a novel contribution to the concept of dark side of food addiction (Parylak *et al*. 2011), in which a downregulation of brain reward systems that subserve appetitive responses to rewards and a concomitant amplification of brain stress or 'antireward' systems concur to elicit anxiety-like disorders and cognitive deficits potentially responsible for the loss of control over palatable food intake after repetitive cycles of dieting and binging.

Acknowledgements

Supported by the Swiss National Science Foundation (31003A-133056 to B.B). The authors thank Dr. Christopher V. Dayas for English proofreading and his helpful comments and corrections on the manuscript.

Financial Disclosures

All authors report no biomedical financial interests or potential conflicts of interest.

Authors Contribution

CR, OH and BB were responsible for the study concept and design. CR, GS and BB acquired the data and performed the analysis. CR and BB drafted the manuscript. All authors critically reviewed the content and approved the final version for publication.

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3.3 ARTICLE 3: Controversies about a common etiology for eating and mood disorders

Personal contribution: I performed the analysis of the literature, and wrote the first draft of the manuscript, and further participated to the revised versions.

Status: Published in Frontiers in Psychology, 2014 Oct 27; 5:1205.

frontiers in
PSYCHOLOGY

REVIEW ARTICLE
published: 27 October 2014
doi: 10.3389/fpsyg.2014.01205

Controversies about a common etiology for eating and mood disorders

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Obesity and depression represent a growing health concern worldwide. For many years, basic science and medicine have considered obesity as a metabolic illness, while depression was classified a psychiatric disorder. Despite accumulating evidence suggesting that obesity and depression may share commonalities, the causal link between eating and mood disorders remains to be fully understood. This etiology is highly complex, consisting of multiple environmental and genetic risk factors that interact with each other. In this review, we sought to summarize the preclinical and clinical evidence supporting a common etiology for eating and mood disorders, with a particular emphasis on signaling pathways involved in the maintenance of energy balance and mood stability, among which orexigenic and anorexigenic neuropeptides, metabolic factors, stress responsive hormones, cytokines, and neurotrophic factors.

Keywords: depression, obesity, reward, overweight, palatable food

INTRODUCTION

Most of antidepressant medications have long been known to induce weight gain, although the obesogenic mechanisms of these treatments remain largely unknown. Most interestingly, a large body of evidence supports the idea that non medicated depressed patients with mood disorders have increased risks of developing obesity compared to the general population, while specific interventions aiming at reducing body weight (including bariatric surgery and increased physical activity) have been found to improve mood. Hence, empirical observations have long suggested a common etiology between obesity and depression: (1) unhealthy diets favoring energy dense foods promote the development of both pathologies; (2) reduced physical activity and sedentary lifestyle are commonly observed in obese and depressed patients; (3) impaired sleep and/or circadian cycles deteriorate mood and increase body weight; (4) recurrent psychological stress and early life trauma have been shown to contribute to a late-onset obesity and depression. Considering the increased prevalence of obesity and depression worldwide, a better comprehension of their common biological bases has become crucial for identifying novel therapeutic targets.

Indeed, obesity-related diseases, including type II diabetes, high blood pressure, and cancer, have become one of the leading causes of preventable death worldwide, reducing life expectancy by 10 years or so. With depression, obesity represents a serious public health concern that contributes to significantly deteriorate the quality of life in people of most countries. Although researches concerning obesity and depression have been conducted for decades separately, recent evidence has shown that these two pathologies might share some neurobiological underpinnings, and possibly a common etiology. Here, we propose to review biological adaptations within several signaling pathways potentially underlying the increased risks of developing both diseases.

Obesity is a pathological condition characterized by excessive body fat accumulation resulting from an imbalance between caloric intake and expenditure. The World Health Organization (WHO) has regularly monitored the worldwide prevalence of overweight and obesity, and a recent estimation suggests that 11% of the world population (more than half a billion people) is obese, and 35% overweighed. Some monogenic forms of obesity have been described (e.g., mutations of leptin and leptin receptor, proopiomelanocortin and melanocortin-4-receptor genes) but they are very rare and not sufficient to explain the worldwide distribution of this disorder (Andreasen and Andersen, 2009). As a matter of fact, recent clinical findings rather suggest that obesity is most likely related to common genetic variants and/or single-nucleotide polymorphisms, representing a genetic risk factor of vulnerability (Frayling et al., 2007; Andreasen et al., 2008; Haupt et al., 2008; Hunt et al., 2008).

Depression is the commonest psychiatric disorder, characterized by severe negative mood and inability to experience pleasure from usually pleasurable activities (anhedonia). Depressed mood is associated with fatigue, incapacity to concentrate, altered appetite, sleep disorders, and metabolic complications (Ustun et al., 2004; Kessler and Bromet, 2013). Depressive symptoms often occur at younger age and, in the most serious cases, lead to suicide attempts. The most recent survey conducted by WHO in 2012 estimates that depression affects more than 350 million people worldwide, hence constituting a major threat to public health (Kessler and Bromet, 2013). Major depression is a heterogeneous disease comprising several subtypes, but the atypical depression is one of the most diagnosed forms. Symptoms like psychomotor retardation, insomnia, increased appetite, and body weight gain differentiate atypical depression from the other forms

(Gold and Chrousos, 2002). This subtype of depression is often accompanied by visceral adiposity and shares with obesity certain metabolic, endocrine and behavioral alterations (Vogelzangs et al., 2007; Lasserre et al., 2014). Interestingly, fat accumulation, commonly measured by the body mass index (BMI; weight in kilograms divided by height in squared meters, BMI), has long been used to monitor weight gain. Increasing evidence rather suggests that abdominal obesity [as referred as the waist-to-hip ratio (WHR)], presents a stronger predictive value than total body fat for predicting obesity-depression co-morbidity (Zhao et al., 2011; Wiltink et al., 2013).

Like obesity, the underlying biological causes of depression remain largely unknown, but genetic predisposition is considered to account for ∼40% of cases (Uhl and Grow, 2004). The genetic approach suggests that depression is a polygenic disease, determined by environmental influences in genetically predisposed individuals (Charney and Manji, 2004). Early life stress has been considered as one of the most critical factor triggering depression (Nemeroff and Vale, 2005). Many longitudinal studies have shown that depression is a consistent predictor of metabolic syndrome since its progression is often associated with cardiovascular diseases, diabetes, obesity, and chronic inflammation (Katon and Ciechanowski, 2002). One possible explanation for this observation would take into account the fact that depressed patients often adopt poor health behaviors, including smoking and drinking habits (Davis et al., 2008), reduced physical activity and unhealthy diets (Cizza, 2011).

The aim of this review is to summarize the current knowledge about the obesity-depression relationship and discuss plausible biological mechanisms underlying this association; first by reviewing the clinical findings supporting the intermingled interaction between obesity and depression, second by discussing the possibility that obesity may be a cause of depression and *vice versa*. In particular, we will be focusing our attention on signaling pathways involved in the maintenance of energy balance and mood stability, among which orexigenic and anorexigenic neuropeptides, metabolic factors, stress responsive hormones, cytokines, and neurotrophic factors.

THE OBESITY-DEPRESSION ASSOCIATION AND MOST RELEVANT MEDIATORS

Since obesity has long been considered a metabolic illness, while depression was classified a psychiatric disorder, both basic science and medicine have long investigated these two pathologies separately. Most of the epidemiological and clinical investigations conducted before the end of the 20th century provided only confounding outcomes, unable to prove a direct association between obesity and depression. Nevertheless, a seminal observation proposed that "socio-demographic, psychosocial and genetic factors may render certain obese individuals more prone to depression and *vice versa*" (Faith et al., 2002). The overlap between mood disorders and obesity was later supported by an extensive study reviewing clinical studies from 1966 to 2003 (McElroy et al., 2004). The authors claimed that obesity and mood disorders were likely related disorders with a distinct, but overlapping, pathophysiology. As a consequence, only some forms of obesity and mood disorders that share pathogenic factors would be related. Further supporting this assumption, obesity was found to be strongly associated with a range of common mood and anxiety disorders, suggesting that social and cultural factors may represent possible mediators and/or modulators of the obesity-depression relationship (Simon et al., 2006). More recent publications, including prospective cohort studies and cross-sectional researches, also reported relevant observations supporting a bidirectional causal link between obesity and depression, by which each disease may contribute to the other (Markowitz et al., 2008; Luppino et al., 2010; Faith et al., 2011). In a recent systematic review of the literature, undertaken to identify biopsychological variables associated with the relationship between obesity and depression (Preiss et al., 2013), physical health, decreased social activity, family depression history, childhood abuse, body image distorsion, severity of obesity and binge eating were listed consistent risk factors for developing comorbid obesity and depression. Collectively, these reports suggest that specific risk factors may confer to subgroups of obese individuals higher probability to develop depression, and to subgroups of depressed patients greater vulnerability to become obese. However, it is important to note that the conclusions drawn from of Lupino's, Faith's, and Preiss's works may be limited by quite a strong heterogeneity, in the methodology, the patient identification and the measures of depression and body weight. In particular, the evidence for a mutual influence of depression and obesity relied on reported weight and not on objective body weight measures (Faith et al., 2011), and the negative impact of obesity on depression depended on the evaluation of clinical depression instead of depressive symptoms (Luppino et al., 2010).

Hence, despite limitations, current data suggest a mutual influence between obesity and depression (Atlantis and Baker, 2008), even if a few studies on adolescents would argue that depression represent a clearer risk factor for obesity (Korczak et al., 2013), whereas obesity may not confer any higher risk for later developing depressive symptoms (Roberts and Duong, 2013). A reasonable conclusion would imply that obesity might represent a risk factor for depression only under particular conditions, which are binge eating behavior or abdominal fat deposition (Weber-Hamann et al., 2002; Araujo et al., 2010; Zhao et al., 2011; van Reedt Dortland et al., 2013a,b).

To summarize, the obesity-depression association seems to be strongly dependent on several risk factors that have been clearly established. Reduced physical activity and sedentary lifestyle have been frequently observed in depressed (Azevedo Da Silva et al., 2012; Song et al., 2012) and obese individuals (Bailey et al., 2007; Tucker and Tucker, 2011). Conversely, when depressed patients were encouraged to do physical exercise, improvement of their mood has been repeatedly reported (Conn, 2010; Carek et al., 2011; Rimer et al., 2012). Moreover, the efficacy of exercise in reducing risk of depression has also been observed in overweight and obese adults (Vallance et al., 2011). Sleep disorders and circadian cycle alterations have been shown to be associated with mood changes (Costa e Silva, 2006; Turek, 2007; Kronfeld-Schor and Einat, 2012; Avila Moraes et al., 2013) and weight gain (Bray and Young, 2007; Shi et al., 2013). Gender has also been considered an important factor since a stronger vulnerability in women has been well documented for both diseases (Peveler et al.,

2002). Unhealthy diets characterized by excessive consumption of energy dense foods have been associated with an increased risk of depression (Jeffery et al., 2009; Sanchez-Villegas et al., 2012; Sanchez-Villegas and Martinez-Gonzalez, 2013) as well as obesity (Fulton, 2010; Mozaffarian et al., 2011). In contrast, adherence to Mediterranean diet or to others diets comprising high amounts of vegetables and fruits have been shown to reduce risks of depression (Sanchez-Villegas et al., 2009; Jacka et al., 2010). Finally, stress and early life trauma have been shown to significantly contribute to both late-onset obesity (Gustafson and Sarwer, 2004; Gunstad et al., 2006; D'Argenio et al., 2009) and depression (Cirulli et al., 2009).

In the next part of this article, we will be essentially focusing on two increasing risk factors: stress and diet.

OBESITY AS A CAUSE OF DEPRESSION

The decision to eat is not only ultimately influenced by the internal state of the caloric equation but also by non-homeostatic factors, including food palatability and environmental cues known to trigger conditioned responses (Lutter and Nestler, 2009; Williams and Elmquist, 2012). Hence, a current consensus acknowledges that feeding behaviors are not only influenced by caloric needs, but also by other layers of regulation that involves the processing of reward, notably through dopamine signaling and its ability to pair food consumption to the context predicting its availability (see for review, Volkow et al., 2013a). In an evolutionary perspective, this property of palatable foods used to be critical for surviving in environments where food sources were scarce. Large amounts of food were eaten when available, enabling energy to be stored in the body (as fat) for future use (Spiegelman and Flier, 2001). However, food habits have profoundly changed over the past decades. Energy-dense foods, especially high fat diets, have a reduced satiety capacity and a higher hedonic value compared to that of meals richer in proteins and/or complex carbohydrates, which may explain their excessive consumption and their role in promoting overweight and obesity (Lawton et al., 1993; Blundell and Macdiarmid, 1997). Moreover, in modern societies, food has become plentiful and ubiquitous. As a consequence, the evolutionary adaptation inducing energy storing has become a dangerous liability (Erlanson-Albertsson, 2005a,b) promoting disinhibited and uncontrolled food seeking habits. Energy dense foods are potentially harmful for human health not only for their unbalance contents but also for their capacity to promote overeating behaviors (Berthoud et al., 2011; Egecioglu et al., 2011).

Recent evidence has established that disruption of energy homeostasis can affect the reward circuitry and that overconsumption of palatable food can lead to changes in the reward circuitry that result in compulsive food intake (Bassareo and Di Chiara, 1999; Kenny, 2011). The peripheral signals include peptides and hormones like leptin, insulin, cholecystokinin (CCK), tumor necrosis factor- α (TNF-α) but also nutrients (sugars and lipids), that are transported via the vagus nerve to the nucleus solitary tract and directly through receptors located not only in the hypothalamus, but also in autonomic and limbic brain regions. These multiple signaling pathways ensure that food is consumed when needed. However, with repeated access to highly palatable food, some individuals may eventually override the inhibitory processes that signal satiety and begin to compulsively consume large amounts of food despite nutrition overload (Zheng and Berthoud, 2007; Johnson, 2013). This loss of control and compulsive pattern of food intake is reminiscent of the drug intake patterns seen in addiction and has led to the description of some types of eating disorders inducing obesity as a form of "food addiction" (Volkow et al., 2013b).

Smith and Robbins proposed that overconsumption of palatable foods would lead to habit-driven responses by initiating a devolution from goal-directed to habitual behavior. According to this hypothesis, a process of signaling transfer would devolve impulsively driven and hedonically motivated actions from ventral to dorsal striatal control (Smith and Robbins, 2013). As a consequence, the consumption of high-fat/high-sugar foods would become less pleasurable and instead would turn into a compulsive response triggered by cues such as advertisements, mood, and context. The prefrontal cortex is considered to be critical for self-control, inhibition, and goal representation, and reduced activity in this region is associated with higher levels of impulsivity and compulsivity. Since executive function difficulties have been reported in overweight and obese individuals, and a decrease in orbito-frontal cortex volume correlated with disinhibited eating in obese adolescents, the shift from ventral to dorsal striatal control described above may also be associated with impairments in executive functions such as cognitive control, flexibility, decision-making, and working memory. Ultimately, individuals who experience a lack of control in the face of food persistently overuse their preferred food despite severe health, social, legal, and financial problems; and are unsuccessful at attempting to cut back or reduce their consumption. These behaviors are typically accompanied by feelings of guilt, remorse, sometimes triggering excessive food restriction and distress that in turn, promote palatable food intake to alleviate these dysphoric signs, further accentuating the spiraling distress (Fulton, 2010).

In other words, negative mood, provoked by palatable food withdrawal or by the incapacity to lose weight, often results in the adoption of an abnormal eating behavior characterized by recurrent cycles of dieting and overeating (Polivy and Herman, 1985; Petroni et al., 2007; Stice et al., 2008). Consequently, overeating episodes may turn into an overt binge-eating disorder (BED) that is frequently associated with a feeling of distress. Consistent with this, obese patients suffering from BED often present severe depressive symptoms (Heatherton and Baumeister, 1991; Stice et al., 2000; Spoor et al., 2006; Araujo et al., 2010; Blumenthal and Gold, 2010; Faulconbridge and Bechtel, 2014). These clinical findings are largely supported by experiments in which compulsive overeating has been induced in laboratory animals. For example, the capacity of palatable foods, when taken in a discontinuous pattern, to promote binge eating behavior has been repeatedly demonstrated in rodents (Colantuoni et al., 2002; Bello et al., 2003; Cottone et al., 2008; Corwin et al., 2011). Interestingly, several of these works have also proved that long-term intermittent access to palatable food is accompanied by depressive-like phenotypes and metabolic alterations (Cottone et al., 2009b; Rossetti et al., 2013).

Traumatic stress experiences, especially in early life, are known to affect mood and feeding behavior and therefore, they constitute

a strong element conferring higher vulnerability for developing obesity and depression. Compelling evidence suggests that stress increases palatable food preference and consumption both in humans (Gluck et al., 2004; Gluck, 2006; Adam and Epel, 2007; O'Connor et al., 2008; Tryon et al., 2013a,b) and in rodents (Foster et al., 2006; Moles et al., 2006; Machado et al., 2013; Patterson and Abizaid, 2013). A likely explanation for such stress-induced preference is that palatable food acting as a "comfort food" would be able to alleviate discomfort (Jeffery et al., 2009). In agreement with this hypothesis, a large body of preclinical studies has demonstrated that, not only chronic stress promotes palatable food intake (Dallman et al., 2003; Pecoraro et al., 2004) but its withdrawal increases stress sensitivity and depressive-like behaviors (Avena et al., 2008; Teegarden and Bale, 2008; Iemolo et al., 2012).

Industrial foods that progressively replace healthier fresh cooked meals worldwide are harmful for human health not only for their strong rewarding properties but also for their low content of poly-unsatured fatty acids (PUFAs). Accordingly, higher intake of saturated fatty acids has been found to promote visceral fat deposition and mood alterations (Schulze et al., 2006; Akbaraly et al., 2009; Molenaar et al., 2009; Jacka et al., 2010). Diets containing low levels of PUFA also increase the risk of depression (Appleton et al., 2010), while lower levels of omega-3 PUFA have been recently reported in the blood of depressed patients (Ross et al., 2007; Lin et al., 2010). Similar conclusions about the role of PUFA in depression have been indirectly drawn from people adhering to a Mediterranean diet. This diet, privileging vegetables, fruits and fish and containing high levels of PUFA, has been shown to limit excessive weight gain and obesity (Schroder et al., 2004, 2007; Romaguera et al., 2009) as well as depression (Sanchez-Villegas et al., 2009). Beside these human studies, the relevance of PUFA in the prevention of depressions has also been reported in rodents (Fedorova and Salem, 2006; Huang et al., 2008) and non human primates (Chilton et al., 2011).

In sum, protracted consumption of unhealthy diets and recurrent stress could interfere with the homeostatic control of the energetic balance and modify the reactivity of the rewarding system leading ultimately to a loss of control over food intake. The resulting compulsive overeating might trigger a BED and eventually lead to abdominal fat deposition, two factors tightly associated with depression (Weber-Hamann et al., 2002; Rivenes et al., 2009; Zhao et al., 2011; van Reedt Dortland et al., 2013a,b).

LEPTIN SIGNALING

Leptin is a peptide hormone belonging to the adipokines family that is secreted primarily from adipocytes of the white adipose tissue. Leptin serum levels change depending on the feeding/fasting state and correlate with body mass (Seeley and Woods, 2003). This peptide is coded by the obese gene *ob* and exerts its physiological function mainly in the brain. After release in the bloodstream, leptin pass across the blood–brain barrier (BBB) using a receptor-mediated transport (Banks et al., 1996). LepRb is the long form of the leptin receptor and is critical for leptin signaling cascade (Banks et al., 2000). LepRb was first identified in the hypothalamus and its activation was related to the anorectic effect of leptin, that is its ability to reduce food intake and increase energy expenditure (Chehab, 2000). Later, the presence of this receptor in other brain structures, namely the hippocampus, prefrontal cortex, ventral tegmental area (VTA) and amygdala has suggested additional roles of leptin in other physiological functions, among which learning and memory (Farr et al., 2006; Harvey et al., 2006), motivation for reward (Farooqi et al., 2007; Grosshans et al., 2012), and mood regulation (Guo et al., 2013; Milaneschi et al., 2014).

The role of leptin in the orchestration of food intake and energy expenditure in the hypothalamus has been largely described in the literature (Cone, 2005; Coll et al., 2007). Briefly, leptin is released by the adipose tissue, crosses the BBB and reaches the arcuate nucleus (ARC) of the hypothalamus where interacts with its receptors localized on two neuron populations: the orexigenic neuropeptide Y (NPY)/agouti related peptide (AgRP) neurons and the anorexigenic proopiomelanocortin (POMC)/cocaine and amphetamine regulated transcript (CART) neurons. Leptin inhibits the former and activates the latter, leading to a satiety signal (Spiegelman and Flier, 2001). It has long been known that impairment in leptin signaling causes obesity, increased food intake and fat deposition (Pelleymounter et al., 1995a; Speakman et al., 2007). These observations gave rise to the hypothesis that leptin could be used as anti-obesity treatment, but clinical investigations have shown that obese patients have chronically high levels of circulating leptin (Lu et al., 2006). This apparent contradiction suggests the emergence of a leptin resistance syndrome, a phenomenon also observed with insulin in diabetic patients. The precise mechanism responsible for leptin resistance is not yet known but may be linked to down-regulation of leptin receptors, reduced transport of leptin across the BBB or altered intracellular transduction of leptin signaling (Jung and Kim, 2013).

Abnormalities in leptin functioning are also believed to enhance the motivation for palatable food and promote its overconsumption. This assumption comes from the discovery that leptin receptors are also localized in brain reward structures (Figlewicz and Benoit, 2009). In particularly, leptin receptors have been detected in dopaminergic neurons of the VTA and electrophysiological studies in rodents have established that leptin decreases the firing rate of these neurons reducing dopamine release and food intake (Hommel et al., 2006). There is also evidence that leptin is able to regulate the incentive salience of reward because food restriction in rodents (that corresponds to a reduction of leptin signal) increases the preference for sucrose and other drugs in a conditioned place-preference paradigm (Figlewicz and Benoit, 2009). In other words, leptin impairment may result in a higher stimulation of dopaminergic neurons that, in turn, may increase the incentive for palatable food and ultimately its consumption. In support of this hypothesis, some animal studies demonstrated that injection of leptin in the VTA reduced food intake whereas a viral-mediated knockdown of leptin receptor in the same brain structure had opposite effect (Hommel et al., 2006).

In the last years, the scientific interest for leptin has also been extended to psychiatric disorders, such as anxiety and depression. Mutant mice that lack leptin signaling have been found to develop depressive symptoms (Collin et al., 2000; Sharma et al., 2010; Yamada et al., 2011) whereas systemic and central administration

of leptin in wild-type mice reduced anxiety and depressive-like behaviors (Asakawa et al., 2003; Lu et al., 2006; Finger et al., 2010; Liu et al., 2010; Yamada et al., 2011; Guo et al., 2013). In addition, other preclinical studies have shown that circulating leptin levels are modulated by stress since chronic unpredictable stress or chronic social defeat, but not acute stress, decreased basal levels of leptin in rats (Lu et al., 2006; Ge et al., 2013).

Although these findings seem to support a reduction of leptin levels in animal models of depression, the current knowledge on the role of leptin signaling in human depression remains unclear. Leptin levels in depressed patients have been reported to be lower, higher or equal to those observed in control patients (Deuschle et al., 1996; Antonijevic et al., 1998; Rubin et al., 2002; Esel et al., 2005; Zeman et al., 2009). However, studies with larger size samples reported a negative correlation between plasma leptin levels and major depression, hence supporting the idea that decreased leptin signaling may be a shared biological alteration in both obesity and depression (Kraus et al., 2001; Atmaca et al., 2002; Westling et al., 2004; Jow et al., 2006; Lawson et al., 2012). In conclusion, there is a current consensus suggesting a reduced leptin signaling in human depression, even though, this association might be stronger in certain subtype of depression or might be influenced by different factors including age, sex, body mass, and co-morbidity with other disorders (Gecici et al., 2005).

Nevertheless, the involvement of the leptin hormone in the emergence of depressive symptoms remains unclear. In this regards, some findings suggest that leptin may exert an antidepressant effect by modulating the hypothalamic-pituitary-adrenal (HPA) axis function. An early *in vitro* study on primary cultures of bovine adrenocortical cells demonstrated the capacity of leptin to reduce the transcription of cortisol (Bornstein and Chrousos, 1999). Mutant mice with altered leptin signaling exhibit hypercorticosteronemia, whereas leptin replacement reduces corticosterone levels in these mice (Chen et al., 1996; Chua et al., 1996; Arvaniti et al., 2001). Similarly, an inverse correlation between basal levels of leptin and release of glucocorticoids (GCs) has been found in humans (Licinio, 1998; Komorowski et al., 2000). Beside a direct effect on GCs, it has also been speculated that leptin may limit the activity of the HPA axis by suppressing the hypothalamic corticotropin-release factor (CRF) release (Ahima et al., 1996; Huang et al., 1998; Arvaniti et al., 2001).

A second hypothesis in favor of the antidepressant role of leptin is linked to the neurotrophic hypothesis of depression, since this adipokine may facilitate neurogenesis. In particular, mutant mice exhibiting an altered leptin signaling present reduced brain volume and abnormal expression of neuronal and glial proteins. These morphological changes have been attributed to the lack of leptin, since leptin administration was shown to normalize the brain morphology (Vannucci et al., 1997; Ahima et al., 1999; Steppan and Swick, 1999). Consistent with a neurotrophic role of leptin, it has been shown that this hormone facilitated the formation of specific neuronal projection pathways inside the hippocampus (Bouret et al., 2004). It also has been shown that leptin increased the motility and density of dendritic filopodia, and enhanced the number of hippocampal synapses (O'Malley et al., 2007). Further, convergent evidence demonstrated the importance in the hippocampus: first, mice with a selectively ablation of Lep-Rb in glutamatergic hippocampal neurons showed long-term potentiation (LTP) impairment, anhedonia-like phenotype, behavioral despair and enhanced social avoidance, and second, specific reduction of leptin receptors in the dentate gyrus, where neurogenesis is considered critical, was shown to induce marked depressive-like behavior (Guo et al., 2013).

In summary, accumulating findings established multiple and complex implications of leptin signaling in physiological and cognitive functions that extend energy balance regulation, and a compromised leptin signaling represents a serious candidate contributing to the development of pathological adaptations underlying the overlap between obesity and depression.

INFLAMMATION AND CYTOKINES RELEASE

Obesity is associated with a low-grade chronic systemic inflammation and in particularly interleukin-6 (IL-6), TNF-α and C-reactive protein (CRP) are present at high levels in the serum of obese people (Shelton and Miller, 2010; Gregor and Hotamisligil, 2011). Very recent epidemiological studies reported that increased levels of CRP correlated with depressive symptoms and abdominal obesity (Alvarez et al., 2013; Daly, 2013; Wium-Andersen et al., 2013) and other clinical works have found CRP to be the most consistent marker of the obesity-depression association (van Reedt Dortland et al., 2013a,b).

Two factors are responsible for chronic increase of circulating cytokines in obese patients. The first is fat deposition. Fat is stored in the adipocytes of the white adipose tissue and an increased adiposity, especially around the abdomen, stimulate these cells to release inflammatory factors, including adipokines, cytokines, and chemokines (Shelton and Miller, 2010; Gregor and Hotamisligil, 2011). Macrophages attracted into the adipose tissue by chemokines massively produce inflammatory factors, leading to the systemic inflammation observed in human obesity (Clement et al., 1997; Wellen and Hotamisligil, 2003). The second factor is diet quality. In a study conducted on a sample of healthy Greek population, the consumption of several food items was scored during a year. Higher scores, reflecting a stronger adherence to the Mediterranean diet, were inversely associated with biomarkers of systemic inflammation (Chrysohoou et al., 2004). The protective effect of the Mediterranean diet was more recently confirmed by other studies revealing that lower plasma concentration of CRP, IL-6, TNF-α were closely related to this type of diet (Camargo et al., 2012; Rimer et al., 2012; Urpi-Sarda et al., 2012). To date, the identification of the Mediterranean diet nutrients supposed to alleviate signs of chronic inflammation remain unclear. A recent study reviewed 26 randomized clinical trials and established that ω-3 PUFA was of particular interest to lower inflammatory markers (Bloch and Hannestad, 2012; Kiecolt-Glaser et al., 2012; Calder, 2013).

Cytokines are mostly produced in peripheral tissues and due to their large molecular weight cannot freely pass cellular membranes, but they can enter the brain through the leaky regions of the BBB or via cytokine-specific transporters. Cytokine signal also arrives inside the brain by afferent nerve fibers (the vagus nerve for example) or infiltration in the brain parenchyma of peripherally activated monocytes (Plotkin et al., 1996; Rivest et al., 2000;

Quan and Banks, 2007; D'Mello et al., 2009). Evidence for elevated levels of neuroinflammatory cytokines has been found in the hypothalamus of rodents after 4 weeks of high-fat feeding, and in *post mortem* brain samples of obese patients as well (Thaler et al., 2012). Noteworthy, whether it is diet-induced or genetically programmed, animal models of obesity present severe inflammation of hypothalamus (Velloso et al., 2008; Wisse and Schwartz, 2009). As a consequence, hypothalamic neurons are injured and the activity of some neurotransmitters, NPY and POMC, compromised (Thaler and Schwartz, 2010; Thaler et al., 2010). Hence, it is likely that excessive amounts of cytokines may exaggerate hypothalamic neuroinflammation and consequently, alter food intake regulation and energy expenditure, worsening signs of obesity in a spiraling down mechanism.

Concomitantly, the neuroinflammation response may exacerbate signs of depression as well, since chronic increase in pro-inflammatory and inflammatory markers has been repetitively observed in depressed patients. It is important to note that inflammation is a protective mechanism for the body to fight microbial and viral attacks. The short-term release of pro-inflammatory cytokines in the blood, including interleukin-1α (IL-1α), interleukin-1β (IL-1β), TNF-α, and IL-6, triggers fatigue, psychomotor retardation, sleep alteration and anhedonia. The "sickness behavior" is supposed to save energy, minimize risks and promote recovery. However, similitudes with the depressive symptoms are quite striking and suggest that depression could be associated with a long-term activity of cytokines (Maes et al., 1995; Charlton, 2000).

In other words, the "cytokine hypothesis of depression" considers depression as the result of a maladaptive response that occurs after a sustained and persistent cytokine release (Dantzer et al., 2008). In favor of this theory, many recent clinical data have shown elevated concentrations of inflammatory markers (including TNF-α, IL-6, IL1-β, and CRP) in the blood and in the cerebral spinal fluid of patient with major depression (Kim et al., 2007; Simon et al., 2008; Howren et al., 2009; Dowlati et al., 2010).

It is also important to note that psychiatric disorders, among which depression, have frequently been described in patient afflicted by pathologies associated with chronic inflammation; it is particularly true for rheumatoid arthritis and autoimmune disorders. Systemic lupus erythmatosus for instance is frequently associated with episodes of major depression. And further, elevated IL-6 spinal fluid levels found in these patients often correlate with the severity of their neuropsychiatric symptoms (Fragoso-Loyo et al., 2007).

The assumption that inflammatory processes may take part in the etiology of depression is also supported by the observation of patients receiving cytokine treatments (e.g., interferon-α) as an anti-cancer or anti-viral therapy. Despite the clinical efficacy of such therapy, more than 45% of treated subjects developed a major depression (Capuron and Miller, 2004; Raison et al., 2006). Moreover, with regards to antidepressant therapies, it has also been noted that their efficacy was related to a reduction of plasmatic levels of inflammatory markers (Lanquillon et al., 2000; O'Brien et al., 2007; Hannestad et al., 2011).

Meanwhile, preclinical findings have demonstrated that mice with targeted deletions of the gene coding for IL-6 or for TNF-α receptor show depressive-resistant phenotypes (Chourbaji et al., 2006; Simen et al., 2006). In other genetically and pharmacologically induced inflammation models, the presence of depressive-like behaviors has been demonstrated in several paradigms including the tail suspension and forced swim tests, as well as in protocols based on social interaction monitoring (Anisman et al., 2005; Moreau et al., 2008; Sukoff Rizzo et al., 2012). Collectively, these findings suggest that depression may represent a maladaptive response to a sustained cytokines release, which may occur in predisposed subjects when the activation of the immune system is heightened in intensity or duration.

While the plasmatic increase of cytokines in depression is confirmed by several studies, little is known about how these modulators of the immune response may affect mood regulation. Recently, it has been proposed that chronic inflammation can affect central neurotransmission, in particular, those of the serotonergic and dopaminergic systems. Particular interest has been accorded to the interaction between cytokines and serotonin because of the serotonergic hypothesis of depression (Coppen and Wood, 1978; Blokland et al., 2002; Wichers and Maes, 2004).

Briefly, cytokines seem to interfere with the serotonin system at different levels: synthesis, release and synaptic reuptake (Dunn and Wang, 1995; Dunn et al., 1999; Anisman et al., 2005). The amount of serotonin in the brain is highly dependent on the availability of the amino acid tryptophan and its transformation in 5-hydroxytryptophan by the enzyme tryptophan hydroxylase (TH; Delgado et al., 1990). Tryptophan levels also depend on an alternative metabolic pathway via its conversion in kynurenine upon the activation of the enzyme indoleamine 2,3-dioxygenase (IDO) localized in multiple cell types including macrophages, astrocytes, and microglia. Hyperactivation of this pathway leads to a depletion of tryptophan and ultimately, decreases the brain amounts of serotonin (Schwarcz and Pellicciari, 2002; Schrocksnadel et al., 2006). Intriguingly, several cytokines (e.g., TNF-α, interferon-α and interferon-γ) and their signaling pathways have been found to activate IDO, hence limiting tryptophan availability (Takikawa et al., 1999; Popov et al., 2006). Consistent with these *in vitro* observations, high plasmatic levels of pro-inflammatory cytokines have been associated with IDO induction in mice displaying depressive-like phenotypes (O'Connor et al., 2009a,b). In line with this interpretation, it also has been shown that: (1) IL-1, IL-6, and TNF-α are able to reduce brain levels of serotonin by accelerating its catabolism (Clement et al., 1997; Dunn, 2006) and (2) cytokines may facilitate serotonin synaptic reuptake (Blakely and Berson, 1992; Bull et al., 2009; Lotrich et al., 2009) hence reducing serotonin neurotransmission.

In parallel, chronic inflammation was shown to alter dopamine neurotransmission as well. Symptoms of depression have been shown to correlate with a reduced prefrontal and striatal dopamine activity (Dunlop and Nemeroff, 2007) and repeated interferon-α administration has been shown to decrease dopaminergic neural activity in the mouse brain (Shuto et al., 1997). Cytokines may also interfere with the dopamine transporter, or affect dopamine synthesis by inhibiting tetrahydrobiopterin (BH4), an important co-factor for tyrosine hydroxylase enzyme that converts tyrosine into L-DOPA (Kitagami et al., 2003). Finally, cytokines also affect

the HPA axis functioning. Hyperactivity of the HPA axis is a hallmark of depression (see next section) and several observations have demonstrated that cytokines and cytokine-inducers can potently activate the stress cascade. Administered acutely, cytokines have been shown to increase the expression and the release of CRF, adrenocorticotropic hormone (ACTH), and cortisol (Besedovsky and del Rey, 1996; Pariante and Miller, 2001; Pace and Miller, 2009).

Even though these findings linking cytokines to depression have to be confirmed, the idea that neuroinflammation might be a leading cause of depression, represents a fascinating prospective connecting mood to eating disorders.

DEPRESSION AS A CAUSE OF OBESITY

The hyperactivation of the HPA axis and reduced neurogenesis and/or brain plasticity are two current hypotheses trying to reconcile the past and recent assumptions about the pathogenesis of depression. Interestingly, these biological processes have been demonstrated to participate to the etiology of obesity as well, and thus may represent a thread union between these two pathologies (Sinha and Jastreboff, 2013; Sominsky and Spencer, 2014).

HYPERACTIVATION OF THE HPA AXIS

A large body of clinical and preclinical evidence has led to the general consensus that hyperactivation of the HPA axis following a chronic stress experience is a leading cause of depression. Animal and human survival depends on the capacity to recognize and face harmful stimuli (Chrousos, 2009). In this context, stress response allows to react to potentially harmful threat and to maintain body homeostasis through transient physiological and behavioral adaptations (Chrousos and Gold, 1992; McEwen, 1998). This temporary adaptive stress response is directed to increase energy availability in those organs of the body involved to counteract the stressor. As a consequence, cardiac output and respiration are accelerated, catabolism is increased and blood flow is potentiated in the brain and muscles (Gilbey and Spyer, 1993).

These adaptive responses rely on the activation of the autonomic nervous system and the HPA axis, a complex neuroendocrine system composed of multiple brain structures and peripheral organs (Tsigos and Chrousos, 2002). Emotional and stressful stimuli, processed in the amygdala, activate the paraventricular nucleus of the hypothalamus (PVN) and trigger a cascade of physiological adaptations through the release of corticotropin-releasing hormone/factor (CRH/CRF; de Kloet, 2000; Charmandari et al., 2005). CRF target neurons are located in the anterior pituitary gland and release the ACTH in the bloodstream. This hormone stimulates the cortex of the adrenal gland to secrete GCs, cortisol in humans, and corticosterone in rodents. GCs receptors are widely distributed in the body and their binding with GCs leads to the activation or repression of a plethora of genes (Bamberger et al., 1996; Dostert and Heinzel, 2004), among which those coding for enzymes involved in promoting the hepatic synthesis of glucose from non-glucidic substrates (i.e., lactate, pyruvate, and amino acids). GCs also contribute to increase blood glucose levels, and antagonize the anabolic activities of insulin, growth, and thyroid hormones (Rizza et al., 1982).

When the exposure to stressful stimuli is limited, the stress response has short duration because GCs exert a feedback inhibition on the HPA axis. Specifically, they act on the pituitary and hypothalamus limiting the release of CRF and ACTH, which reduces their own activity. GCs also stimulate GC receptors in the hippocampus, where inhibitory GABAergic projections to PVN neurons block the CRF release (Boudaba et al., 1996; Herman et al., 2002). Higher cortical structures, including the dorsomedial prefrontal cortex and the prelimbic cortex, also control the stress response (Diorio et al., 1993; Radley et al., 2008).

During the past three decades, several groups reported compelling evidence showing that prolonged stress response (in particular sustained CRF and GCs release) may represent a biological mechanism triggering depression (Carroll, 1982; Holsboer, 2000; Pariante, 2003; Strohle and Holsboer, 2003) and obesity (Bjorntorp and Rosmond, 2000; Pasquali and Vicennati, 2000), even though psychosocial stress would exert only a modest effect on weight gain (Wardle et al., 2011). CRF has been particularly studied and its role in the emergence of depressive symptoms has been well documented (Grigoriadis, 2005). The activity of CRF depends on its binding on two types of receptors, both widely express in the brain and body, called CRF-R1 and CRF-R2. Three other endogenous ligands with different affinity for CRF receptors have been revealed and named urocortin1 (UCN1), UCN2, and UCN3 (Nakayama et al., 2011). Consistent with the CRF hypothesis of depression, some studies have shown that depressive patients exhibit high levels of CRF in the cerebrospinal fluid (Nemeroff et al., 1984), increased number of CRF expressing neurons in the PVN, elevated expression of CRF mRNA in the same neurons and reduced CRF receptor density (Banki et al., 1987; Raadsheer et al., 1994; Merali et al., 2004). Early observations in rodents also found that intracerebroventricular (ICV) administration of CRF induced anxiety and depression-like behaviors, whereas the injection of CRF antagonists produced the opposite effect, confirming the potential antidepressive properties of CRF ligands (Deak et al., 1999; Zobel et al., 2000; Seymour et al., 2003). Beside pharmacological data, genetic manipulations in rodents confirmed these observations: overexpression of the CRF gene in mice led to increased anxiety-like behaviors and impaired stress (Stenzel-Poore et al., 1994). Interestingly, these behavioral changes were accompanied by enhanced food intake, weight gain and insulin (Coste et al., 2001). However, whereas transgenic mice lacking CRF-R1 showed a reduced stress response and blunted anxiety-like behaviors (Smith et al., 1998; Timpl et al., 1998), those lacking CRF-R2 exhibited pronounced anxiety-like behaviors and stress hypersensitivity (Bale et al., 2000; Coste et al., 2000).

Compared to depression, less is known about a direct role of CRF signaling on obesity. The involvement of the CRF family peptides (CRF, UCN1, UCN2, and UCN3) in energy balance and food intake regulation has been documented (Chalew et al., 1995; Richard et al., 2002), but most of the data are rather contradictory (Levine et al., 1983; Negri et al., 1985; Asakawa et al., 1999; Ushikai et al., 2011). Similarly to what is observed with anxio-depressive like behaviors, the impact of the CRF system on energy balance largely depends on CRF ligands, the type of receptor and the brain structure targeted. Indeed, recent observations reported

that blocking CRF-R1 signaling in the central nucleus of the amygdala prevented palatable food intake and anxiety-like behaviors in the rat (Cottone et al., 2009a; Iemolo et al., 2013). Meanwhile, CRF-R2 activation with UCN2 was shown to reduce high-fat food consumption when injected ICV in the rat, confirming a former observation in mice lacking CRF-R2, which consume larger meals compared to wild type mice (Bale and Vale, 2003; Tabarin et al., 2007).

Further confirming a role of the HPA axis dysfunction in the emergence of excessive food intake and obesity, it has been recently demonstrated in humans that the systemic administration of a low dose of CRF stimulated food intake, likely through GCs release (George et al., 2010). The Cushing's syndrome, a disease resulting from pituitary/adrenal tumors or chronic treatment with corticosteroids, also represents a coincident model of depression and obesity (Sonino et al., 1998). Clinical observations of these patients revealed that cortisol hypersecretion is accompanied by visceral adiposity, metabolic syndrome and mood related disorders. In order to explain this, early and more recent researches have pointed out that the excessive release of GCs impairs the ability of insulin to promote glucose uptake, induces metabolic syndrome and promotes body fat deposition (Brindley and Rolland, 1989; Black, 2006). Likewise, chronically elevated GCs contribute to visceral fat accumulation in primates (Shively, 1998; Shively et al., 2009) and humans (Marin et al., 1992; Rosmond et al., 1998; Epel et al., 2000; Spencer and Tilbrook, 2011). Finally, the diurnal variations of circulating cortisol levels positively correlated with the hip-to-waist ratio in obese patients (Weaver et al., 1993; Lasikiewicz et al., 2008). Beside insulin and leptin resistance (Zakrzewska et al., 1997, 1999; Jéquier, 2002a,b), GCs may alter the energetic balance and stimulate food intake acting on different brain structures. GCs receptors are widely distributed in hypothalamic nuclei implicated in energy homeostasis such as ARC, LH, and PVN (Morimoto et al., 1996) and long-lasting stimulation of GC receptors in these brain regions is known to potentiate orexigenic signals modulating the expression of genes involved in energy balance. In particular, the expression of one gene contributing to satiety signaling, the POMC gene, has been found to be reduced following GC receptor stimulation and increased after adrenalectomy (Cavagnini et al., 2000; Savontaus et al., 2002).

While clinical data strongly support that chronically elevated GCs receptors affect the hedonic regulation of food intake and increase the preference for palatable food, which most likely contributes to excessive fat deposition (Dallman et al., 2003, 2006; Pecoraro et al., 2004; Germano et al., 2007), enhanced levels of corticosterone have been found in different animal models of obesity including fa/fa rats, db/db, and ob/ob mice (Guillaume-Gentil et al., 1990). Conversely, the absence of GCs, due to adrenalectomy, provoked body weight loss and food intake reduction in rodents, effects that could be reversed by corticosterone administration (Saito and Bray, 1984; Pralong et al., 1993; Makimura et al., 2000). Accumulating evidence suggests that diet composition may also play a role in modulating the HPA axis functions. Although, only a few studies have covered this topic, some of them indicate that unbalanced fat diets may perturb lipid metabolism and, as a consequence, increase circulating levels of GCs. Consistent with this hypothesis, a recent study on woman health has reported a positive correlation between consumption of saturated fatty acids and diurnal variation of cortisol (Garcia-Prieto et al., 2007). In addition, converging evidence has found that patients who received diets supplemented with PUFA or fish oil had reduced ACTH and cortisol rise after acute stress (Delarue et al., 2003; Michaeli et al., 2007).

Collectively, these observations emphasizes that stress alters the HPA axis homeostasis and most likely triggers the onset and worsening of depression. Since the hypothalamus is the main brain orchestrator regulating energy balance and food behavior, it is not surprising that alterations in the HPA axis may lead to overeating and obesity.

NEUROGENESIS AND THE BRAIN-DERIVED NEUROTROPHIC FACTOR (BDNF)

The monoaminergic hypothesis of depression has long proposed that depression originates from decreased monoamine neurotransmission. Despite the impressive number of pre-clinical and clinical studies that have been conducted over the last 40 years, the validity of this theory remains debatable (Mulinari, 2012; Blier and El Mansari, 2013). In particularly, the discrepancy between the rapid increase of monoamine signaling occurring upon antidepressant treatment and their delayed therapeutic effect remains unclear. Moreover, inconsistent clinical findings have failed to demonstrate a decrease in monoamine basal levels in depressed patients compared with health people. Thus, the altered monoamine neurotransmission classically reported in depression is now considered to reflect the consequences of neuronal damage. Historically neurons have been presumed to not regenerate. However, recent evidence demonstrated that the mammalian central nervous system retains the capacity to produce new neurons that can integrate neural network (Ming and Song, 2011). Two brain regions have been extensively studied: the subventricular zone of the lateral ventricles and the subgranular region of the dentate gyrus in the hippocampus (Duman et al., 2001; Duman, 2002; Kempermann and Kronenberg, 2003). Hippocampal neurogenesis, which is pivotal for cognitive functions and mood control, is strongly affected by chronic stress which causes shortening and pruning of neuron dendrites and reduction of the final number of newborn neurons (McEwen, 1999; Duman, 2004a,b). Accordingly, morphologic and morphometric studies of brains of depressed patients have found a reduction of hippocampal volume due, at least in part, to an impaired neurogenesis (Sheline, 1996; Bremner et al., 2000; Sheline et al., 2003; Stockmeier et al., 2004; Lucassen et al., 2010). Interestingly, antidepressant treatments have shown to reverse neural atrophy, cell loss and stimulate neurogenesis (Duman and Monteggia, 2006). Similar observations have been reported with preclinical investigations, confirming that unpredictable chronic stress protocols suppressed hippocampal neurogenesis in rodents (Watanabe et al., 1992; Malberg and Duman, 2003; Pham et al., 2003; Mineur et al., 2007).

Neurotrophins constitute an important class of signaling molecules in the brain, playing a pivotal role in brain development, neuron survival and synaptic plasticity. The brain-derived neurotrophic factor (BDNF) has received particular attention,

since it is considered a relevant biomarker of depression and suicidal behavior, and a possible downstream target of a variety of antidepressant drugs (Dwivedi, 2009; Lee and Kim, 2010). Hippocampal neurogenesis is an important process that appears to be involved in maintaining balanced mood. A large body of evidence identifies BDNF, and its TrkB receptor, as two key elements in the orchestration of different phases of neurogenesis such as cell proliferation, migration, differentiation and death. The rationale for the involvement of BDNF in depression comes from the observation that stress can down-regulate the expression of this neurotrophic factor in brain structures known to control emotions (Dwivedi, 2009; Lee and Kim, 2010). Stress-related disorders, among which major depression, is considered to induce morphologic damages in the hippocampus, which is intimately connected to the HPA axis (Bremner et al., 2000). Accordingly, the expression of BDNF, BDNF regulated genes and TrkB receptors are decreased in *post mortem* hippocampal tissues harvested from depressed human brains (Tripp et al., 2012). Similar findings have also been reported for the prefrontal cortex, another brain region essential for mood regulation. Moreover, BDNF protein levels are reduced in the serum of depressed patients (Castren et al., 2007; Castren and Rantamaki, 2010; Thompson Ray et al., 2011). It is important to note that, *post mortem* hippocampal BDNF levels and serum concentrations were normalized in depressed patients successfully treated with antidepressants, suggesting that the therapeutic effect of these compounds is related to BDNF activity (Duman and Monteggia, 2006).

However, human studies are only correlative and a clear causal association between impairment of BDNF brain activity and depression has not been yet demonstrated in human patients. Thus, preclinical studies have attempted to validate this theory. Converging studies have shown that stress (both physical and psychological) is able to lower BDNF expression levels in rat hippocampus (Duman and Monteggia, 2006) and that direct BDNF infusion in this area reduces stress-induced depressive-like behaviors (Hoshaw et al., 2005; Hu and Russek, 2008). Viral-mediated deletion of BDNF or TrkB genes in the VTA resulted in a significant antidepressant-like response as well. However, results discrepancy made complicated the interpretation. For instance, mice exposed to a chronic social defeat exhibited increased depressive-like behaviors and increased BDNF protein levels in the nucleus accumbens and amygdala (Berton and Nestler, 2006; Yu and Chen, 2011). Therefore, the current hypothesis, suggesting a strong link between low BDNF brain levels and depression, appears to be too simplistic and has to be reconsidered. One possible explication is that BDNF gene expression may be down regulated in some brain structures (like hippocampus and prefrontal cortex) and up regulated in others.

Interestingly, compelling evidence demonstrated that BDNF has a direct role in the regulation of homeostatic and hedonic eating as well (Lyons et al., 1999; Kernie et al., 2000; Rios et al., 2001; Xu et al., 2003). With regards to the homeostatic regulation, early studies showed that ICV injection of BDNF in rats led to a reduction of body weight, suggesting that BDNF could take part in the central control of feeding behavior (Lapchak and Hefti, 1992; Pelleymounter et al., 1995b). More recently, BDNF deficiency has been associated with increased weight in mice (Noble et al., 2011; Schwartz and Mobbs, 2012), while leptin injected in the ventromedian hypothalamus (VMH) would exert an anorexigenic effect activating the expression of BDNF (Komori et al., 2006). The relevance of BDNF in energy balance was also confirmed in mutant mice. Heterozygous BDNF +/- mice show hyperphagia, body weight gain, insulin resistance, dyslipidemia, and hyperglycemia. In addition, these mice were more sensitive to negative effects of a high fat diet (Lyons et al., 1999; Kernie et al., 2000). Similarly, mice expressing only about 25% of TrkB receptors display excessive feeding (Xu et al., 2003). Inversely, BDNF infusion in the VMH of adult wild-type mice resulted in decreased food intake and body weight (Wang et al., 2007). Consistent with a role in homeostatic mechanisms, levels of expression of BDNF and trkB in hypothalamus and hindbrain regions are influenced by the energy status (Xu et al., 2003; Bariohay et al., 2005; Tran et al., 2006; Unger et al., 2007). Preclinical studies also support a role for BDNF in regulating hedonic feeding by modulating the mesolimbic dopamine (Seroogy et al., 1994; Numan and Seroogy, 1999; Cordeira et al., 2010). Meanwhile, only a limited number of human studies managed to correlate BDNF expression to obesity. Human BDNF haploinsufficiency was linked to elevated food intake and obesity (Gray et al., 2006; Han et al., 2008). Recent evidence also associated a functional polymorphism of the Bdnf gene, Bdnf Val66met which impedes a correct secretion and signaling of BDNF, with obesity predisposition (Beckers et al., 2008; Skledar et al., 2012), and the missense mutation in the TrkB gene, which prevents TrkB function, has been identified in patients exhibiting overweight and severe obesity (Yeo et al., 2004).

Interestingly, adhering to a balanced diet was shown to influence neurogenic factors, and potentially alleviates signs of depression and obesity. Depressed patients following a Mediterranean diet exhibited signs of remission concomitant to increased plasma BDNF concentrations (Sanchez-Villegas et al., 2011; Sanchez-Villegas and Martinez-Gonzalez, 2013). Meanwhile, overweight and obese patients exposed to a 3-months calorie restricted diet displayed increased levels of serum BDNF (Araya et al., 2008). On the other hand, high fat meals were shown to decrease plasma BDNF of almost 30% in healthy patients (Karczewska-Kupczewska et al., 2012). These observations have been confirmed in preclinical studies since BDNF level decreased in rats maintained on a high carbohydrate diet (Maioli et al., 2012), and high fat diet (Yamada-Goto et al., 2012), whereas caloric restriction increases BDNF expression (Lee et al., 2002; Duan et al., 2003).

Further investigation has revealed that ω-3 PUFA may play a role in neurogenesis. In animals, ω-3 PUFA supplementation provided protection against reduced plasticity and normalized BDNF after traumatic brain injury (Wu et al., 2004), whereas diets deficient in ω-3 PUFA lowered BDNF brain levels (Rao et al., 2007; Bhatia et al., 2011).

Taken together these data suggest that BDNF pathway may be a relevant biological substrate underlying the pathogenesis of both mood and eating disorders. However, considering the emerging data on complexity of BDNF pathway, further findings are needed to better understand whether BDNF is a real causal factor for the depression-obesity association.

CONCLUDING REMARKS

Obesity and depression represent a global health burden, and the diagnostic is even more severe for those individuals suffering from both diseases. The increasing prevalence of depression-obesity co-morbidity strongly suggests that these disorders may share a common pathogenesis.

Earlier and current findings clearly demonstrated a link between the two pathologies but the nature of their association is still to be fully understood. The complexity comes from the fact that both obesity and depression are heterogenic diseases, influenced by multiple environmental and genetic factors. Clinical works have identified environmental factors that seem to facilitate the development of obesity as well as that of depression. In particular, factors like stress and diet quality have a strong impact on both pathologies and may constitute key mediators for the obesity-depression association. Other risk factors, reduced physical activity, sleep impairments and altered circadian rhythms, are also considered relevant but most likely as factors worsening the acquired pathologies rather that factors influencing the emergence of the diseases.

Collectively, clinical studies reviewed in this article suggest that obesity and depression are closely related but may not be globally interconnected. Indeed, only subgroups of obese patients are at higher risk for developing depression, and *vice versa*. Accumulating evidence emphasizes that mainly patients with BEDs and those with abdominal fat deposition and metabolic syndrome are at higher risk for depression. The identification of these two subgroups of obese individuals is promising for discovering the biological mechanisms underlying the obesity-depression association.

In the last years, clinical and preclinical studies have also found that leptin may represent a biological substrate underlying the pathogenesis of both obesity and depression. Circulating leptin increases proportionally with body mass and is significantly elevated in obese patient in comparison with non-obese individuals. However, persistent high levels of the hormone alter leptin signaling in the brain, most likely due to leptin resistance compensatory mechanisms. This deficit is believed to trigger a maladaptive functioning of the brain homeostatic system, ultimately leading to obesity. Since converging evidence suggests that impaired leptin signaling may affect mood regulation and food reward perception in humans, impaired leptin signaling cascades may represent a biological mechanism binding obesity and depression, in particular when obesity is paired with compulsive overeating. This assumption opens novel perspectives for the development of therapeutic medications able to correct clinical signs of obesity and depression.

Substantial fat deposition is known to stimulate inflammatory processes, which in turn, promote peripheral inflammatory cytokines release. These molecules enter the brain, not only affecting the hypothalamic control of food intake but also impacting other brain functions that are pivotal for mood regulation. Consistent with this hypothesis, some findings demonstrated that high levels of inflammatory cytokines might interfere with serotonin and dopamine neurotransmission, and with HPA axis as well. Although they need to be confirmed, these findings suggest that the inflammatory hypothesis of eating and mood disorders should get a larger attention in the near future.

The stress response is known to alter the HPA axis functioning and to trigger depressive symptoms and eating disorders. Deciphering the contribution of CRF signaling in these two pathologies remains quite difficult though. Indeed, CRF has opposite effects on depressive symptoms depending on which brain receptors are activated, but CRF binding to CRF receptor 1 seems to exacerbate signs of depression while increasing food intake. The development of CRF receptor 1 antagonists may represent another relevant strategy for improving mood and eating disorders. Inhibitors of GCs are currently used for treating the Cushing's syndrome and some cancers of the adrenal gland, but no medications are available to date for treating depression or obesity.

The neurothrophic hypothesis of depression has received particular attention, but the current knowledge remains elusive and recent evidence, rather than establishing a clear-cut role for BDNF to causally link depression to obesity, mainly claim for further studies before delineating any mechanism underlying the association obesity-depression.

In conclusion, compelling evidence shows that obesity and depression are two overlapping pathologies. However, the causal link between eating and mood disorders needs to be clarified. Most likely, different mechanisms may contribute to the worsening of each disease, and a global cure does not sound realistic. Instead, a better understanding of the molecular and cellular adaptations occurring in subgroups of obese (or depressed) patients identified as highly vulnerable to develop comorbidities should be a clinical priority. Meanwhile, improving animal models of eating and mood disorders is critical for unraveling the underpinnings of the obesity-depression association.

However, given the deleterious impact of stress and junk food consumption on the onset and progression of these two pathologies, the most effective prevention program remains public campaign defending the adherence to healthy balanced diets and promoting effective programs of stress management.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 31 May 2014; accepted: 06 October 2014; published online: 27 October 2014.
*Citation: Rossetti C, Halfon O and Boutrel B (2014) Controversies about a common etiology for eating and mood disorders. Front. Psychol. **5**:1205. doi: 10.3389/fpsyg.2014.01205*
This article was submitted to Eating Behavior, a section of the journal Frontiers in Psychology.

4 SUPPLEMENTARY MATERIAL

4.1 SUPPLEMENTARY METHODS

4.1.1 Additional behavioral and molecular measures in rats submitted to alternate access to palatable food

An additional experiment was performed in rats to expand our previous observations obtained with the binge-eating model. The aim of this experiment was to verify whether basal impulsivity of rats could explain compulsive overeating of palatable food and whether brain gene expression alterations could be associated with the behavioral and metabolic adaptations formerly found. A new group of female rats (150-200 g) was trained in operant chambers to collect sweet sugar pellets as already described (Rossetti et al., 2014). Once the training for food reinforcement was completed, rat impulsivity was assessed in the 5 Choice Serial Reaction Time Test (5-CSRTT).

4.1.1.1 5 Choice Serial Reaction Time Test (5-CSRTT)

Six operant chambers (305 x 241 x 292 mm) were used for the 5-CSRTT experiment (Med Associates Inc., St-Albans, Vermont, USA). Each cage contained five nosepoke cavities (2.5x2.5 cm) on one side of the cage, each spaced by 2.5 cm, and a food receptacle on the other side. Nosepoke cavities and food receptacle were equipped with lights and infrared beams to monitor activity. Cages were controlled by a Med Associates software (Med-PC IV) and electronic interface. Sucrose pellets (Dustless precision pellet 45mg, rodent purified diet, Bioserv, Frenchtown, NJ) were used as reinforces. After habituation to the chambers, rats were trained to make a nosepoke in one randomly illuminated hole (1 out of 5) to earn a food pellet. A correct response was recorded when rats made a nosepoke in the illuminated hole, whereas a nosepoke in the other cavities was recorded as incorrect, but had no scheduled consequence. Ultimately, rats were trained to postpone their seeking behavior for 5 sec (during presentation of a tone) before exploring a randomly illuminated cavity to earn a food pellet. Premature responses consisted in nosepoking during this 5-sec delay before illumination of one hole, and were considered to reflect motor impulsivity. Perseverative responses were scored when rats made supplementary nosepokes before collecting the food pellet reward. Omissions were scored in absence of behavioral response within the 5 sec after hole illumination. The impulsivity score was defined as the ratio between the premature responses and the sum of the premature responses plus correct, incorrect responses and omissions.

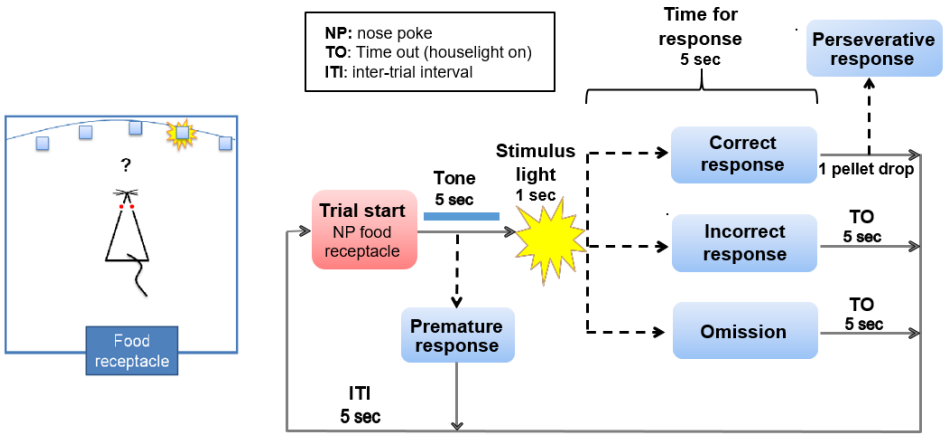

Figure 11. Schematic representation of the 5-Choice Serial Reaction Time Task procedure (Adapted from Robbins, Psychopharmacology, 2002 [336]**)**

4.1.1.2 Body weight and food intake measures

Animals matched for food intake, body weight and impulsivity score, were individually housed and assigned to two groups. C/C rats (meaning Chow/Chow) were provided with standard food along the entire procedure, whereas C/P (meaning Chow/Palatable) rats received standard food for five days and chocolate-flavored food pellet (aka palatable food) for two consecutive days. This alternation of diets was repeated for 8 weeks (i.e. 8 cycles), as shown in Figure 12.

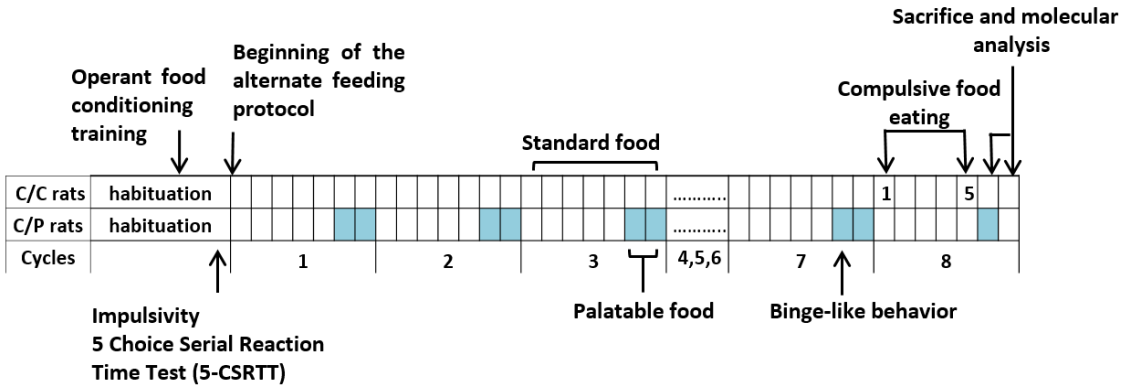

Figure 12. Schematic representation of the alternate feeding protocol used for rats. Chow/Chow rats means rats fed with standard chow only. Chow/Palatable rats means rats fed with standard and palatable food. For compulsive food eating assessment (8th cycle), 1 means first day of palatable food withdrawal, and 5 means last day of palatable food withdrawal.

Food intake and body weight measures were taken daily. The first day of palatable food access of the 7th cycle, the food consumed by C/P rats was measured every two hours for eight hours and compared with the amount of standard food eaten by C/C rats. The food consumed in the first two hours was taken as a measure of binge-like behavior.

4.1.1.3 Compulsive food eating

At the end of the last cycle of the feeding protocol, rat willingness to accept shock punition for sweet or chocolate flavored pellets was assessed. In this experiment the procedure previously used to determine compulsivity (Rossetti et al, 2014) was modified because we tested all rats, the first and the fifth day of palatable food withdrawal, with both kinds of pellets. We introduced this change because we observed that, due to the very low intensity of the shock punishment, rats did not decrease their seeking behavior when tested twice the same day. Therefore, to avoid biased results due to which pellets were used first rats were randomized in the test following a *latin square* design. The comparison of the number of sucrose and chocolate-flavored pellets collected by C/P rats on the first and the fifth day of palatable food withdrawal was considered to reflect their compulsive-like behavior. C/C rats were trained in parallel, and were exposed to the same operant conditioning sessions, within 5 days.

4.1.1.4 Gene expression measures

At the end of the procedure, all rats were sacrificed by rapid decapitation at two different time points defined in the C/P group of rats to assess gene expression in two conditions, either in the brain of rats exposed to food withdrawal, or in the brain of rats exhibiting food binging. Hence, rats were sacrificed either 4 hours after withdrawal of palatable food, or 4 hours after continuous access to chocolate-flavored food pellets. After extraction, brains were rapidly sliced in a rat brain matrix (rat coronal, 1mm, stainless matrix) in 2mm thick slices under RNAse-free conditions. Three different brain structures, basolateral amygdala (BLA), lateral hypothalamus (LH) and hippocampus (HIP), were collected by micropunching (micropunch Ø = 1mm; Stoelting Co., Illinois, USA) using the Paxinos and Watson Atlas as reference. Collected tissues were processed for RNA extraction with RNeasy Plus Minikit (Qiagen). RNA samples were quantified with a Nanodrop (Thermoscientific) and converted in cDNA by reverse transcription reaction using TaqMan®Reverse Transcriptase Reagents (Applied Biosystem). cDNA samples were amplified with an ABIPRISM 7500 cycler and SYBR green PCR Master Mix (Applied Biosystem) using specific primers (Microsynth AG). For each tested gene the forward and reverse primer are listed in table 1. All samples were analyzed in triplicates and the relative gene expression was measured with the comparative ΔΔ *Ct* method and normalized with β-*actin* transcript level.

Gene	Forward	Reverse
β-actin	5'-CACACCCGCCACCAGTTCG-3'	5'-CTAGGGCGGCCCACGATGGA-3'
Crtc1	5'-AGACAGACAAGACCCTTTCTAAGCA3'	5'-CAGGACTTGGGCCTGGAA-3'
Bdnf	5'-AAAACCATAAGGACGCGGACTT-3'	5'-GAGGCTCCAAAGGCACTTGA-3'
LepRb	5'-GCATGCAGAATCAGTGATATTTGG-3'	5'-CAAGCTGTATCGACACTGATTTCTTC-3'
Crh-R1	5'-TGCCTGAGAAACATCATCCACTGG-3'	5'-TAATTGTAGGCGGCTTGTCACCAAC-3'
Crh-R2	5'-AACGGCATCAAGTACAACACGAC-3'	5'-CGATTCGGTAATGCAGGTCATAC-3'
Gr	5'-GCCCTGGGTTGGAGATCATAC-3'	5'-CATGCAGGGTAGAGACATTCT-3'
Orexin	5'-AGATACCATCTCTCCGGA-3'	5'-CCAGGGAACCTTTGTAGA-3'

Table 1. Forward and reverse primer sequences for genes tested in rat brain structures

4.1.2 Effect of alternate access to palatable food in *Crtc1* ko female mice

Stress and negative emotionality have been shown to impact palatable food consumption. We decided to test *Crtc1* ko female mice in a feeding protocol with intermittent access to palatable food, given the anxio-depressive-like behavior they display. The protocol we applied to mice was adapted from that used for rats, with shorter alternate accesses to laboratory chow and palatable food pellets. Here, mice were exposed to standard food for three days, and palatable food for one day only, and the procedure was repeated for 12 cycles (Figure 13). We formed four experimental groups: WT mice fed with standard food (WT C/C), WT mice fed with standard and palatable food (WT C/P), *Crtc1* ko mice fed with standard food (*Crtc1* ko C/C) and *Crtc1* ko mice fed with standard and palatable food (*Crtc1* ko C/P). All mice were 6-week-old at the beginning of the procedure.

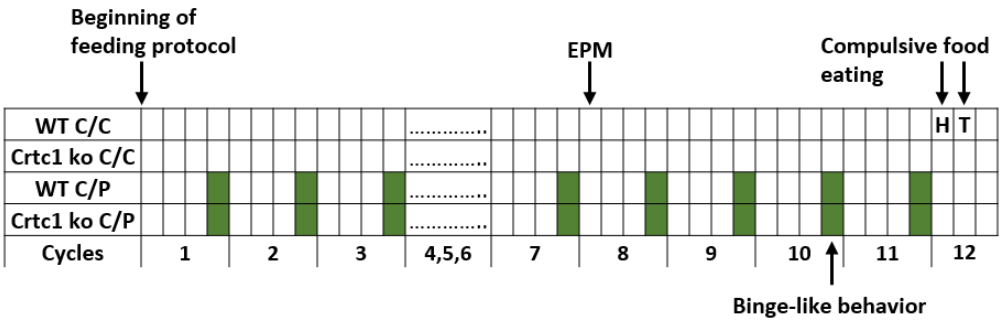

Figure 13. Schematic representation of the alternate feeding protocol used for mice. WT C/C = wild-type mice fed with standard chow. *Crtc1* ko C/C = *Crtc1* ko mice fed with standard food. WT C/P = wild-type mice fed with standard and palatable food. *Crtc1* ko C/P = *Crtc1* ko mice fed with standard and palatable food. EMP = Elevated plus maze test. H = habituation day of the compulsive food eating procedure. T = test day of the compulsive food eating procedure.

4.1.2.1 Body weight and food intake measures

Food consumption and body weight was measured daily in all mice. On the 10th cycle, calorie intake in C/P mice exposed to palatable food was measured every two hours for 8 hours and compared to the standard food consumed in appropriate controls, as a measure of binge-like behavior.

4.1.2.2 Measure of anxiety-like behavior

During the 8th cycle, on the first day of standard food reintroduction, anxiety-like behavior was assessed in the elevated plus maze (EPM). The maze consisted of two opposite open arms (30.5 x 5.5 cm) and two opposite closed arm (30.0 x 5.0 x 16 cm) arranged in a cross. The maze was elevated 60 cm above the floor and placed in a room under dim light (12-15 Lx). A digital camera was mounted above the maze and animal movements were recorded trough the Ethovision tracking system (v.3.1 Noldus technology, Wageningen, The Netherlands). Animals were gently placed in the central platform of the maze facing one of the closed arms. Each animal was observed for 5 min. The total distance travelled in the maze and that travelled in the open arms were calculated as an index of anxiety.

4.1.2.3 Compulsive food eating

As for rats, we tested whether C/P mice were willing to face aversive conditions in order to eat palatable food. Compulsive-like eating was evaluated in a light/dark box (45cm L x 20cm W x 30cm H) in which the bright aversive compartment (20cm W x 30cm L) was illuminated by 60 lux light. The dark side (20cm W x 15cm L) containing bedding was covered with a black opaque lid and had ~0 lux light. The two compartments were connected by an open doorway which allowed mice to freely move from one to the other. The experimental procedure was performed in two consecutive days. The first day, mice were habituated to the light/dark box for 15 min, whereas the second day (test day) a plastic cup containing pre-weighed food was placed in the middle of the bright side and mice behavior was recorded for 15 min through a digital camera. Whereas C/P mice received in the cup palatable food C/C mice received standard food to avoid feeding inhibition due to the presentation of an unknown diet. Animal tracks were analyzed with the Ethovision tracking system. The distance moved in the bright compartment during habituation and test day, as well as the amount of palatable food eaten the day of the test were compared among mice groups.

4.1.2.4 Statistical analysis

All data are expressed as mean ± standard error. Rat experiments were analyzed using two way repeated measures ANOVA, followed, when appropriated, by Fisher's PLSD *post hoc* tests for multiple comparisons. Student's *t-test* was used to compare gene expression results. In mice experiment, the comparison between groups was done with one factor ANOVA except for the binge-like behavior which was analyzed with a two way repeated measures ANOVA. When required multiple comparison between groups was done with Bonferroni *post hoc* test. Level of significance was set at $P \leq 0.05$.

4.2 SUPPLEMENTARY RESULTS

4.2.1 C/P Rats exhibit compulsive- and binge-like behaviors for palatable food

As previously reported, while C/C rats maintained a stable food intake along the feeding protocol, C/P animals showed drastic transitions in food intake, with increased consumptions of chocolate-flavored food pellets followed by decreased intakes of standard food (Figure 14a).

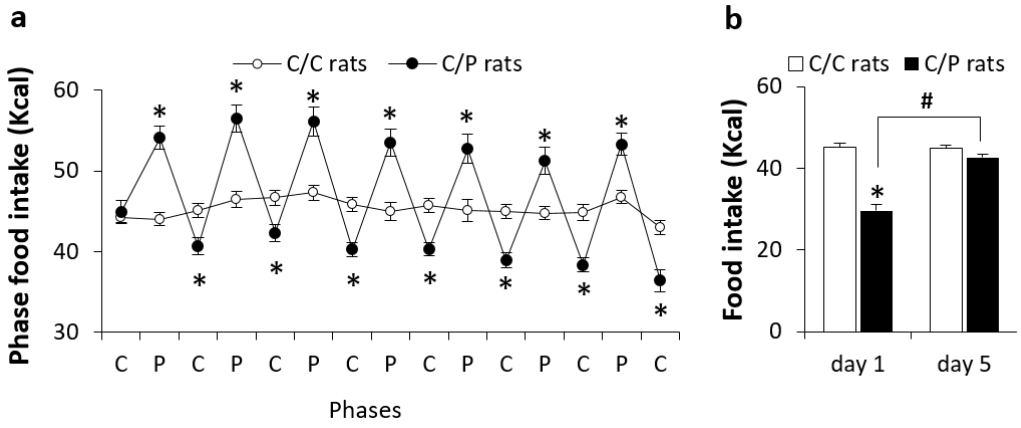

Figure 14. Calorie food intake of C/P rats during intermittent access to palatable food. a) Data represent the average daily food intake during the C (5 days of standard food) and P (2 days of palatable food) phases in C/P rats (n =23) compared to controls (n =22). * = P < 0.05, Fisher's PLSD *post hoc* test *vs* controls. **b)** Food intake of standard food of C/C and C/P rats the first and the last day of palatable food withdrawal. * = P < 0.05, Fisher's PLSD *post hoc* test vs control day1, # = P < 0.05, Fisher's PLSD *post hoc* test vs C/P group day1.

A two way repeated measure ANOVA revealed systematic overeating in C/P rats exposed to palatable food, followed by systematic undereating when re-exposed to standard chow pellets (*group*: $F_{(1,43)} = 26.83$, P =<0.001) and *group*: $F_{(1,43)} = 24.18$, P =<0.001, respectively) compared to C/C rats. The decrease of standard food intake in C/P rats, defined as "negative contrast" [156] was

particularly clear-cut the first day of standard food reintroduction, whereas, on the 5th day of standard food access, both groups of rats showed similar calorie intake (Figure14b).

In order to further analyze excessive chocolate-flavored food intake, we observed that consumption of palatable food, measured on the 7th cycle, was significantly increased within the first two hours of availability. This large food intake in C/P rats within a short period of time (2hours only) represented 49% ± 6.7 of their daily intake, while the amount of chow pellet consumed by control rats only represented 20.5% ± 2.7 of their daily intake, hence confirming the binge-like eating behavior exhibited by C/P animals (Figure 15a).

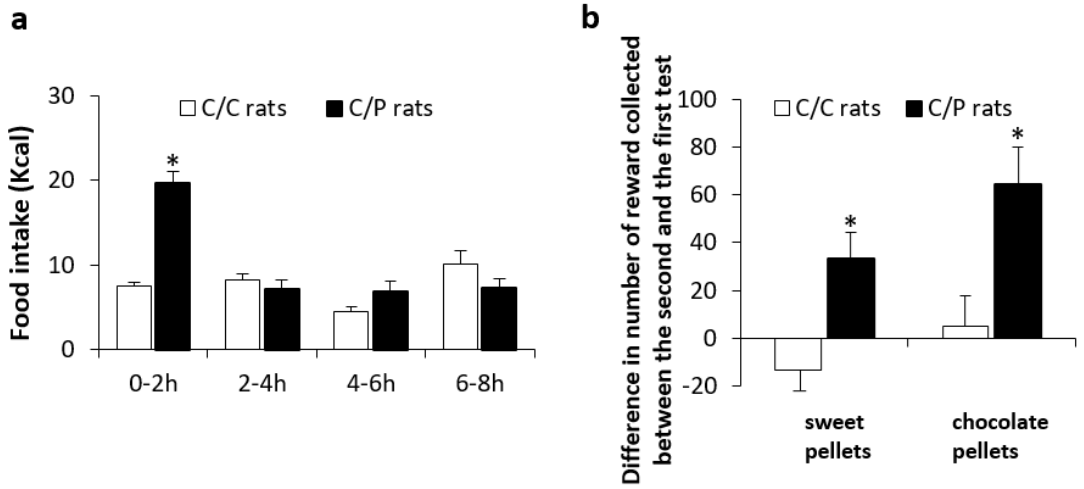

Figure 15 Binge-like behavior and compulsive food eating in rats. a) Calorie intake of C/C and C/P rats the first day of palatable food availability measured every two hours (two way repeated measures ANOVA: *group*: $F_{(1,43)} = 21.4$, $P < 0.001$; *hour*: $F_{(3,129)} = 14.67$, $P < 0.001$; *group*hour*: $F_{(3,129)} = 14.6$, $P<0.001$). * = P≤ 0.05, Fisher's PLSD *post hoc* test vs controls **b)** Compulsive food eating measured as the difference between reward collected the last and the first session of the test for each kind of pellets (two way repeated measures ANOVA: *group*: $F_{(1,43)} = 19.6$, $P < 0.001$; *pellets*: $F_{(1,43)} = 13581.8$, $P = 0.048$; *group*pellets*: $F_{(1,43)} = 0.256$, $P = 0.615$).C/C rats, n = 22; C/P rats, n = 23. * = P≤ 0.05, Fisher's PLSD *post hoc* test vs respective control group.

Basal rat impulsivity, measured with the 5-choice serial reaction time task procedure before the beginning of the feeding protocol (C/C rats = 0.283 ± 0.024, C/P rats = 0.286 ± 0.035, P= 0.938 Student's *t-test*), was compared with binge-like behavior in C/P rats. No significant correlation between these two variables has been found suggesting that, at least in this model, higher impulsivity is not associated with a vulnerability to develop a binge-like behavior (Pearson's correlation: R^2=0.104, P = 0.306), in contrast to former reports on cocaine intake [337].

4.2.1.1 Compulsive food eating

However, in line with the excessive palatable food intake observed in C/P rats (Figure 14a), we report an increased compulsive-like food seeking and taking in these animals at the end of the procedure. Indeed, whereas the C/C rats collected similar amount of sweet and chocolate pellets during the two testing sessions, C/P rats collected a significantly increased amount of food pellet rewards (with concomitant foot shock punishments) upon the second test session (Figure 15b). Noteworthy, C/P did not show this compulsive-like behavior for chocolate flavored pellets only, but for sucrose pellets as well, hence suggesting that chocolate pellet withdrawal triggered in C/P rats an indiscriminate sweetened taste compulsive seeking behavior. Nevertheless, a further analysis revealed a significant positive correlation for chocolate pellets (Pearson's correlation: R^2=0.392, P = 0.0013) but not for sucrose pellets (Pearson's correlation: R^2=0.037, P = 0.379) in C/P rats. The same analysis for C/C animals showed no correlation for both sucrose (Pearson's correlation: R^2=0.018, P = 0.550) and chocolate-flavored pellets (Pearson's correlation: R^2=0.0012, P = 0.879) (Figure 16).

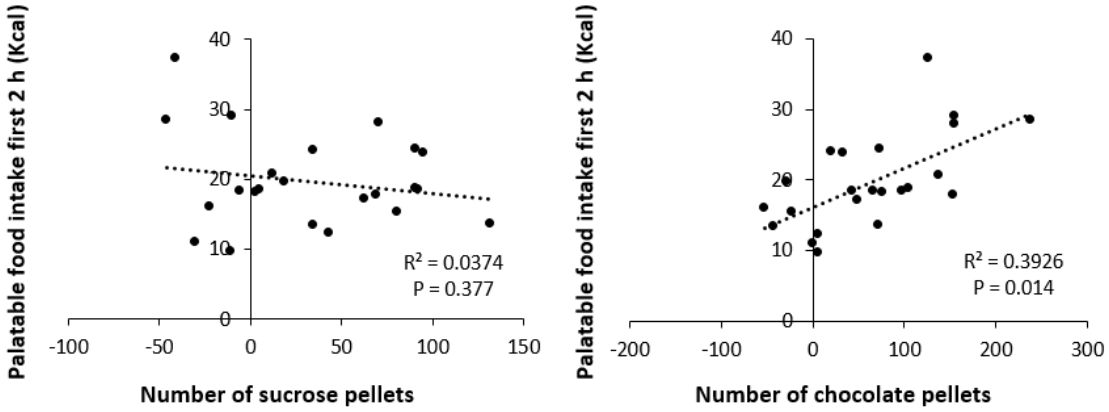

Figure 16. Correlation between binge-like behavior and compulsivity for sucrose and chocolate pellets in C/P rats.

4.2.2 Evidence for altered genes expression in the brain of rats exposed to alternate access to palatable food

At the end of behavioral analyses, we sought to investigate whether the expression of genes involved both in food intake and mood regulation could be modified after intermittent exposure to palatable food. In the first condition, we processed the brains of C/P rats exhibiting excessive palatable food intake for 4 hours (i.e. binging phase). In the second condition, we processed the brains of C/P rats undergoing a 4-hour withdrawal of palatable food (i.e. withdrawal phase). In both conditions, C/P brains were compared to C/C brains extracted under similar timing conditions.

Several brain regions were targeted, as well as several genes, which expression is summarized in Table 2.

		Withdrawal phase			Binging phase		
Stucture	Gene	C/C rats	C/P rats	P value	C/C rats	C/P rats	P value
BLA	*Crh-r1*	1.00 ± 0.079	0.774 ± 0.052	**0.024***	1.00 ± 0.06	1.274 ± 0.169	0.173
	Crh-r2	1.00 ± 0.063	1.097 ± 0.154	0.575	1.00 ± 0.184	1.294 ± 0.247	0.388
	Gr	1.00 ± 0.034	0.873 ± 0.050	**0.050***	1.00 ± 0.076	0.850 ± 0.052	0.119
	LepRb	1.00 ± 0.089	0.672 ± 0.063	**0.005***	1.00 ± 0.101	0.711 ± 0.085	**0.046***
	Crtc1	1.00 ± 0.044	0.807 ± 0.022	**0.001***	1.00 ± 0.044	0.770 ± 0.031	**0.019***
	Bdnf	1.00 ± 0.088	0.706 ± 0.042	**0.006***	1.00 ± 0.106	0.707 ± 0.045	**<0.001***
LH	*Orx*	1.00 ± 0.062	0.707 ± 0.074	**0.005***	1.00 ± 0.183	1.144 ± 0.314	0.9495
HIP	*LepRb*	1.00 ± 0.076	1.219 ± 0.103	0.101	1.00 ± 0.183	1.719 ± 0.206	**0.023***

Table 2. Relative mRNA levels of genes measured in the brain of C/C and C/P rats. Rats of both groups were sacrificed the first day of palatable food withdrawal (C/C rats n=15, C/P rats n=15) or the first day of palatable food access (C/C rats n=7; C/P rats n=8). * = P ≤ 0.05, Student's *t-test*. BLA: basolateral amygdala; LH: lateral hypothalamus; HIP: hippocampus; *Crh-r1*: corticotropin-releasing hormone receptor1; *Crh-r2*: corticotropin-releasing hormone receptor2; *Gr*: glucocorticoid receptor; *LepRb*: leptin receptor b; *Crtc1*: CREB-regulated transcription coactivator 1; *Bdnf*: brain-derived neurotrophic factor; *Orx*: orexin.

In brief, C/P rats (sacrificed during withdrawal) had significantly lower levels of *Crh-r1* and *Gr* transcripts relative to control rats in the basolateral amygdala (BLA). Conversely, when C/P rats were sacrificed during binging, no change was observed in the expression of these genes. These results indicate that in the BLA the expression of these stress-related genes is influenced by the negative emotional state induced by palatable food withdrawal. In rodents, leptin signaling and CRTC1 pathway have been both involved in learning and memory processes and anxiety-like behavior. Therefore, we also measured in the BLA the expression of *LepRb*, *Crtc*1 and *Bdnf*, which is a CRTC1 downstream regulated gene. The transcription of these genes was strongly reduced in C/P rats, both during palatable food withdrawal and overeating, suggesting that recurrent cycles of dieting and binging have detrimental effect on leptin and CRTC1 neural pathways.

Lateral hypothalamus (LH) is a crucial structure in the regulation of food intake because it connects hypothalamic nuclei involved in the control of energy homeostasis and brain structures of the reward system. In the LH of C/P rats, we observed a downregulation of *Orexin* when rats were exposed to palatable food withdrawal, which is in line with the reported reduced food intake. Finally, in the hippocampus, we found an increased expression of *LepRb* in C/P rats only, sacrificed in the binge eating phase. Overall, these findings indicate that alternate exposure to palatable food alters physiological neurotransmission and affect the brain response to stress and food intake-regulating hormones.

4.2.3 *Crtc1* ko mice exhibit exacerbated compulsive- and binge-like behaviors for palatable food

Wild-type and *Crtc1* mutant female mice exhibited a high preference for palatable food. In line with the rat model, mice significantly reduced standard food intake, right after palatable food withdrawal (Figure 17 a), while respective control groups displayed quite regular food intake over time (Figure 17 b).

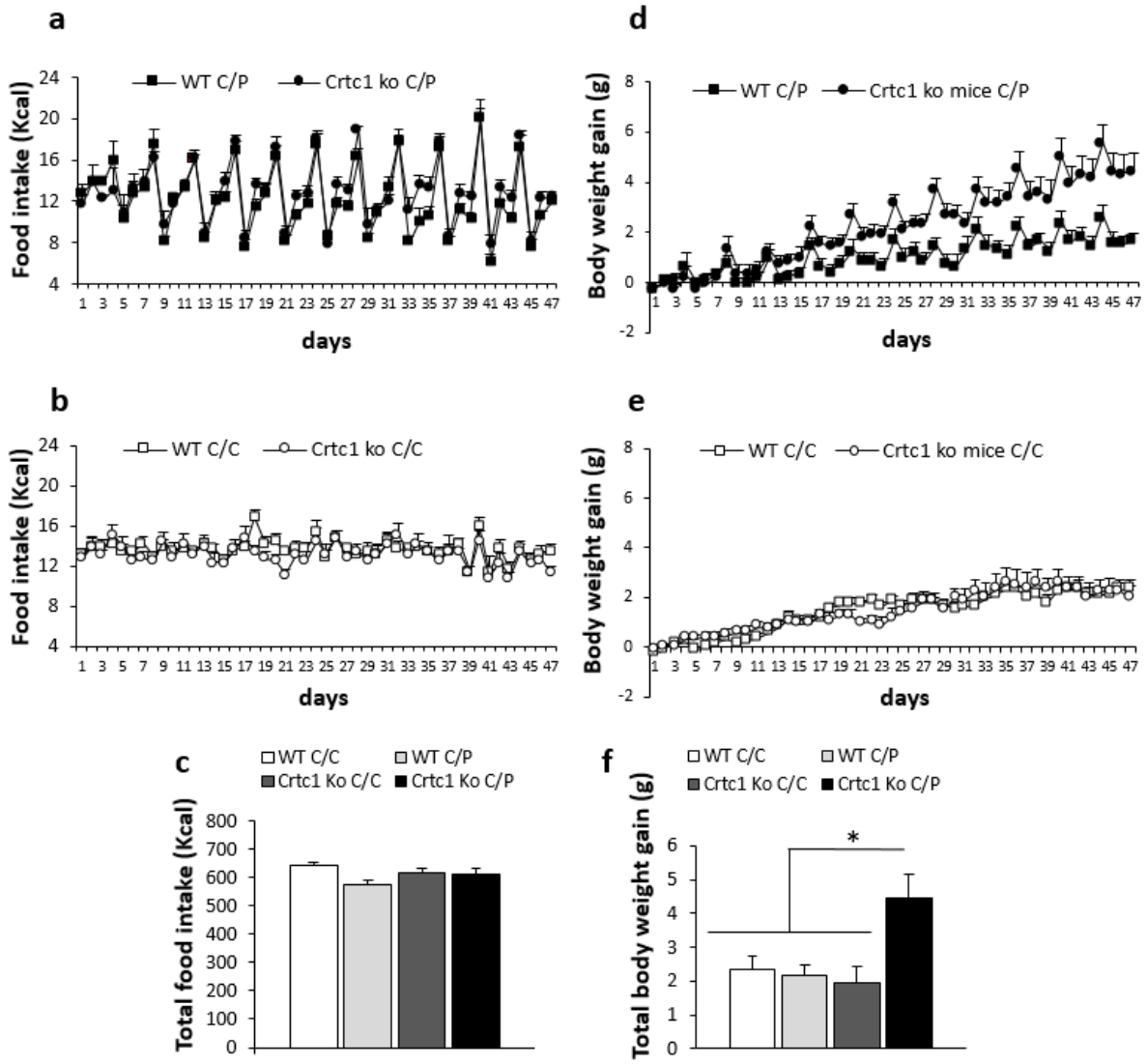

Figure 17. Food intake and body weight gain of WT and *Crtc1* ko female mice fed with intermittent access to palatable food. a) Food intake (Kcal) of wild-type (n = 10) and *Crtc1* ko mice (n = 10) submitted to the feeding procedure. **b)** Food intake (Kcal) of wild-type (n = 8) and *Crtc1* ko mice (n = 8) fed with standard food. **c)** Total food intake (kcal) at the end of the feeding procedure. **d)** Body weight gain (g) of wild-type (n = 10) and *Crtc1* ko female mice (n = 10) submitted to the feeding procedure. **e)** Body weight gain (g) of wild-type (n = 8) and *Crtc1* ko mice (n = 8) fed with standard food. **f)** Body weight gain (g) at the end of the feeding procedure. * = P ≤ 0.05, Bonferroni *post hoc* test.

Despite this "yo-yo dieting" observed in C/P mice compared to C/C controls, by the end of the feeding procedure, we did not observe any difference in the total amount of calorie ingested (WT C/C = 642.81 Kcal ± 10.8; *Crtc1* ko C/C = 619.14 kcal ± 15.9; WT C/P = 573.76 Kcal ± 18.2; *Crtc1* ko C/P = 611.47 kcal ± 22.4) (Figure 17 c). However, whereas WT C/C and *Crtc1* ko C/C mice had comparable weight along the procedure, an observation in agreement with previous experiments reported in *Crtc1* ko females, *Crtc1* ko mice exposed to intermittent access to palatable food exhibited a drastic increase in body weight gain compared to WT animals (Figure 17 d, e). Accordingly, at the end of the feeding procedure, only the *Crtc1* ko C/P group displayed a statistically significant weight gain compared to the three other groups (WT C/C = 2.36 g ± 0.37; *Crtc1* ko C/C = 1.93 g ± 0.53; WT C/P = 2.19 g ± 0.30; *Crtc1* ko C/P = 4.46 g ± 0.7) (Figure 17 f).

Again, we measured palatable food consumption in two hour-intervals in all these mice. In contrast to rats' observations, mice did not exhibit binge-eating behavior. Even though *Crtc1* ko C/P mice seem to eat more the first two hours of palatable food availability, great individual variability was seen among mice and statistical analysis did not show any difference between groups in each two-hour intervals (two way repeated measures ANOVA: *group*: $F_{(3,32)} = 2.28$, P= 0.097; *hours*: $F_{(3,96)} = 0.74$, P= 0.532; *group*hours*: $F_{(9,96)} = 1.13$, P= 0.349). Although this binge-eating-like measure requires to be confirmed, total food intake (24 hours) of the same day of palatable food access attested again that calorie intake of WT and *Crtc1* ko C/P mice was higher than that of control groups (Figure 18a, b).

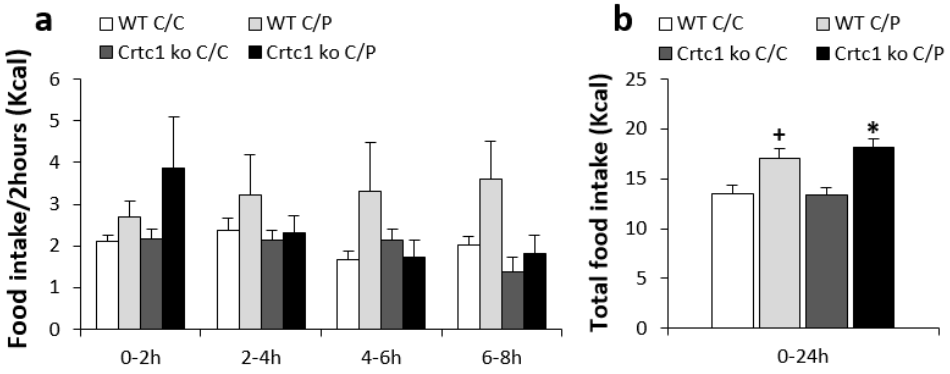

Figure 18. Binge-like behavior in mice. a) Calorie intake (Kcal) of C/C and C/P mice the 10th cycle of palatable food exposure. **b)** Total food intake over 24-hours (one way ANOVA: group: $F_{(3,32)} = 7.29$, $P < 0.001$). WT C/C: n = 8; WT C/P: n = 10; *Crtc1* ko C/C: n= 10; *Crtc1* ko C/C: n= 8. **+** = P≤0.05, Bonferroni *post hoc* test vs WT C/C, * = P≤0.05, Bonferroni *post hoc* test vs *Crtc1* ko C/C.

Emotional disturbances, such as anxiety and depressive-like states, could strongly affect food consumption and, therefore, we performed a measure of anxiety-like behavior in the elevated plus maze to see whether the intermittent exposure to palatable food could increase the basal anxiety of

Crtc1 ko mice. Total distance moved in the maze was not different between WT C/C (1408.7 cm ± 75.3) and WT C/P mice (1340.0 cm ± 63.5). Like rats, C/P wild-type mice did not show a strong increase in anxiety in this test. However, *Crtc1* ko C/P mice (793.5 cm ± 75.8) moved significantly less than *Crtc1* ko C/C mice (1107.4 cm ± 96.3) and WT mice (Figure 19). No difference was found in the distance moved on the opens arms (WT C/C = 330.8 cm ± 69.8; *Crtc1* ko C/C = 236.47 cm ± 45.9; WT C/P = 246.16 cm ± 23.8; *Crtc1* ko C/P = 214.59 cm ± 45.8) and in the time spent on the open arms (WT C/C = 81.6 sec ± 19.8; *Crtc1* ko C/C = 69.6 sec ± 15.9; WT C/P = 69.5 sec ± 10.0; *Crtc1* ko C/P = 84.7 sec ± 19.7) among the four groups.

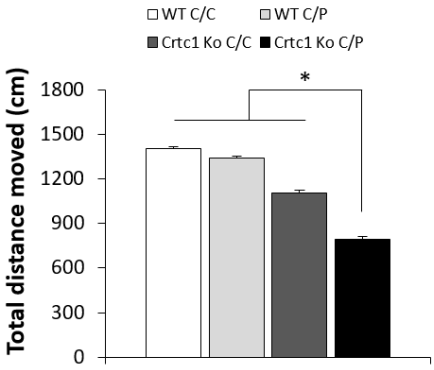

Figure 19. Anxiety-like behavior in the elevated plus maze. Total distance (cm) travelled in the maze. WT C/C: n = 8; WT C/P: n = 10; *Crtc1* ko C/C: n= 10; *Crtc1* ko C/C: n= 8. One way ANOVA: *group*: $F_{(3,32)} = 11.13$, $P < 0.001$. * = $P \leq 0.05$, Bonferroni *post hoc* test.

The light/dark box is also frequently used to test anxiety-like behaviors in mice. Unlike the elevated plus maze in which mice avoid open arms, in this procedure the aversive factor is represented by the strong bright light used to illuminate the light compartment. During the first day of habituation, WT mice (regardless of their diet) were more active than *Crtc1* ko mice and spent more time in the light aversive compartment (WT C/C = 221.0 s ± 25.9; *Crtc1* ko C/C = 112.8 s ± 27.6; WT C/P = 187.8 s ± 19.9; *Crtc1* ko C/P = 94.8 ± 28.2, Figure 20a). The following day, all mice increased their exploratory behavior as reflected by the increased time spent in the light side of the box, but the reduced exploration persisted in mutant mice (WT C/C = 293.5 s ± 43.3; *Crtc1* ko C/C = 167.6 s ± 35.9; WT C/P = 356.0 s ± 41.3; *Crtc1* ko C/P = 179.0 s ± 41.9) (Figure 20b). The amount of palatable food consumed in the bright side of the box during the 15 min of test, reflected reward seeking and taking behavior despite aversive conditions, an acknowledged definition of compulsive-like behavior. Interestingly, *Crtc1* ko C/P mice exhibited a drastic increase in chocolate-flavored food pellets consumption compared to the three other groups (WT C/C = 2.5 mg/s ± 1.6; *Crtc1* ko C/C = 49.0 mg/s ± 18.9; WT C/P = 17.0 mg/s ± 9.5; *Crtc1* ko C/P = 255.0 mg/s ± 4.7), indicating that *Crtc1*

deficient mice exhibit a severe proneness to develop compulsive-like behavior for palatable food (Figure 20c).

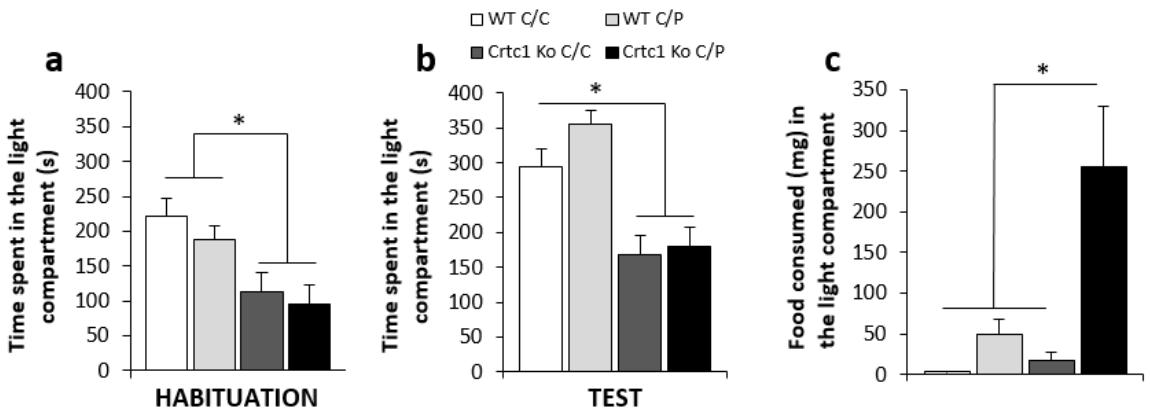

Figure 20. Compulsive food eating of female mice under intermittent access to palatable food. a) Time (s) spent in the bright side of the maze during 15 min of habituation (one way ANOVA: *group*: $F_{(3,32)} = 5.72$, $P < 0.0029$). b) Time (s) spent in the bright side of the maze during 15 min of test (one way ANOVA: *group*: $F_{(3,32)} = 5.58$, $P < 0.0034$).d) Food consumed (mg) in the light compartment (one way ANOVA: *group*: $F_{(3,32)} = 10.33$, $P < 0.001$). WT C/C: n = 8; WT C/P: n = 10; *Crtc1* ko C/C: n= 10; *Crtc1* ko C/C: n= 8.* = $P \leq 0.05$, Bonferroni *post hoc* test vs WT C/C.

5 DISCUSSION

5.1 ANIMAL MODELS OF OBESITY AND DEPRESSION

Obesity and depression are both complex, multifaced and polyfactorial diseases. Genetic background can in part explain obesity predisposition but nutritional and life-style changes are certainly the most important causes [338-340]. Intriguingly, modifications that occurred in the human environment, which have led to nutritional changes, reduced physical activity and inadequate sleep, are recognized as crucial factors contributing not only to obesity epidemic, but also to the progression of psychiatric diseases, including major depression [341, 342]. According to the multifactorial nature of obesity many models of obesity have been developed to mimic the influence of genetic and environmental components on energy homeostasis. These models have provided key insights into the central and peripheral pathways regulating food intake and body weight. At the beginning of obesity research, the study of spontaneous single gene mutations has played an important role in the understanding of biological underpinnings of energy balance. Spontaneous mutations that alter leptin signaling have been described both in the mouse and in the rat. In the mouse the most important are the mutation of the gene for leptin, which characterizes the *ob/ob* mouse (obese mouse), and that of the leptin receptor carried by the *db/db* mouse (diabetic mouse) [30, 343]. Equivalent models are also found in rats. Zucker rats (also named fa/fa rats) and Koletsky rats present mutations in the extracellular domain of the leptin receptor that block the externalization [344] or the expression of the leptin receptor [345], respectively. In addition to progresses made in the analysis of obesity through these models, further and consistent improvements in this field have been obtained with planned genetic manipulations. Gene targeting technologies have allowed to overexpress or suppress particular genes supposed to be involved in energy balance regulation. Obesity in mice has been induced by inactivation of several genes, including those coding for POMC [346], melanocortin receptors MCR_3 and MCR_4 [347], serotonin receptor 5-HT_{2C} [348] and STAT3 [349], which is involved in the intracellular signaling of leptin. Noteworthy, these knockout techniques, resulting in constitutive gene deletion throughout the entire organism and throughout development, may lead to phenotypes that are difficult to evaluate. The most important caveats are the development of compensatory mechanisms to the lack or overexpression of the targeted gene, and the influence of the background phenotype. Genetic manipulation limited to particular brain structures/organs or to specific stages of development will help to define more precisely the role of the multiple factors that contribute to energy homeostasis.

Other animal models have been of great utility for shedding light on the effect of environmental factors on obesity. Two factors in particular have been manipulated to induce obesity in rodents: diet and stress. A large body of literature reports the negative effects of high-fat or cafeteria-style diets on the development of obesity-like profile, strongly suggesting the adverse consequences of frequent chronic exposure to processed/industrialized diets in humans [350, 351]. Importantly, some of these procedures have identified high-fat vulnerable and resistant animals (for example diet-induced obese (DIO) and diet-resistant (DR) rats) that allow deeper investigation on individual characteristic predisposing to obesity [352]. Stress also heavily impacts body weight. However, stress, depending on its nature and duration, can have opposite effects on food consumption and body weight. Indeed, physical stressors such as cold exposure, foot shocks, tail pinch and physical restrain seem to reduce food consumption, whereas social stress, like that obtained in specific behavioral paradigms (trauma witness model, resident intruder or social defeat test), would enhance palatable food consumption and preference [353]. Curiously, despite the growing body of evidence suggesting that psychological stress is more effective in promoting food intake, most animal models aiming at investigating the role of stress on metabolic processes focus on physical stressors [354]. It is also important to note that besides the nature of the stressor, the duration of the stressful event can also differently affect food consumption and body weight.

With chronic exposure to stress also being a strong cause for depression, both eating and mood disorders appears to be closely intermingled. While studying behavioral response to chronic stress might represent an interesting approach to investigate the cellular and molecular underpinnings of both hyperphagia and depression as comorbid pathologies, one has to keep in mind that these two brain pathologies might be correlational, at best, and delineating the precise link of one to the other, as a cause or a consequence, remains quite intricate.

Depression and obesity are both heterogenic pathologies, composed of a wide range of physical and psychological symptoms, that are not systematically reported in depressive patients. These dissimilarities make the modeling of depression in animals a real challenge. From this it follows that all models do not have complete face validity, thus being impossible to reproduce in the same animal all the symptoms and endophenotypes of depression.

Crtc1 ko mice represent a genetic model of obesity and depression, in which, at least in males, both phenotypes seem to develop at the same time. These mice exhibit spontaneously different behavioral and molecular endophenotypes characterizing depression, but do not manifest clear signs of anhedonia. With regards to obesity, one important advantage is that *Crtc1* ko mice develop

obesity on standard food. Thus, it is possible to investigate homeostatic impairments of energy balance separately from hedonic dysregulation induced by recurrent consumption of palatable food.

However, *Crtc1* mutant mice, being constitutive knockout mice may have developed compensatory mechanisms that are worriedly identified. Moreover, the hyporeactivity showed by these mice in response to a novel environment and their aggressive behavior limit the implementation of some behavioral tests.

5.2 CRTC1 DEFICIENCY AND ENERGY BALANCE

Previous research conducted in our laboratory has demonstrated that, in the mouse, CRTC1 deficiency determines a series of behavioral changes that are reminiscent of classical symptoms of depression. In more detail, *Crtc1* ko mice manifest dramatic reduction of social-related behavior, aggressiveness, altered emotional response to a novel environment and depressive-like behavioral despair. These behaviors are accompanied by changes in monoamine turn over and in the expression of CREB-regulated genes in the prefrontal cortex and hippocampus. In parallel, the lack of CRTC1 affects energy balance regulation and produces obesity. Because of the anorexigenic activity demonstrated by serotonin [355], we first considered the possibility that the obesity of *Crtc1* ko mice could originate from their serotonergic impairment. We therefore compared the obese profile of our mice with mice in which the serotonergic transmission was altered through genetic manipulation of the serotonin transporter or of different receptors. It has been shown that the suppression of the serotonin transporter (5-HTT) reduces brain serotonin content and determines late onset obesity, which is not due to hyperphagia but rather to reduced energy expenditure [356]. Other approaches targeting serotonin receptors indicated that mice lacking functional 5-HT_{2C} receptors exhibited hyperphagia and late onset obesity [357, 358], whereas genetic inactivation of 5-HT_6 receptor was protective against high fat diet induced obesity [359]. The comparison of the obese phenotype of *Crtc1* mice with those induced by manipulated serotonin transmission indicated that CRTC1 deficiency has more detrimental effects on energy balance than that due to the serotonin impairment. Therefore, aiming at elucidating the mechanisms underlying CRTC1 deficiency-induced obesity, we looked at possible alterations in the homeostatic and hedonic regulation of food intake, as well as, in energy expenditure.

Overall, our findings confirmed and extended the former observations of Altarejos and coworkers [303]. Briefly, we observed hyperphagic obesity in *Crtc1* ko males, but not in females, in which the late onset obesity seems more attributable to abnormalities in the peripheral metabolism.

Male hyperphagia was not caused by increased preference for hedonic foods, but rather, to impairments occurring in the homeostatic regulation. Indeed, molecular analysis revealed that the expression of genes coding for orexigenic and anorexigenic factors was different in *Crtc1* ko males compared to controls. We found, like Altarejos et al., reduced expression of *Cart* in obese old males. However, transcript levels of this gene were not changed in young not obese males suggesting that *Cart*, despite being a CREB-regulated gene, is not critical in the obesity onset of *Crtc1* mutant males. This interpretation is in agreement with a former study reporting that *Cart* null mice develop mild obesity only after high-fat diet exposure and thus, are less prone to gain weight than *Crtc1* mutant mice [360]. Thus, the obesity of *Crtc1* mutant males seems rather to depend on the dysregulation of multiple homeostatic factors as attested by the changes in gene expression that we discovered in their arcuate nucleus.

With regards to the hedonic control of food intake, we did not find differences in the preference and in the self-administration of saccharine of WT and CRTC1-deficient mice. However, to rule out the possibility of an increased motivation for hedonic food in knockout mice, other measurements should be performed in operant cages, such as the determination of a motivational threshold in a progressive ratio schedule. Unfortunately, the relative hypoactivity of *Crtc1* mutant mice in operant conditioning procedures represents a limitation in this type of investigations.

One important outcome of our experiments was the discovery that *Crtc1* ko males are more vulnerable to develop obesity than female mice. The reasons for this less severe and delayed obesity of female mice is due to a lack of hyperphagia and an enhanced daily spontaneous locomotor activity. A possible explanation of these differences may come from the role of sex hormones in energy homeostasis. Luteinizing hormone, released by anterior pituitary upon the secretion of the hypothalamic gonadotropin-releasing hormone, controls the production of estrogens in both sexes [361, 362]. It is well known that luteinizing hormone and estrogens reduce eating and facilitate energy expenditure through locomotor activity. Their influence on energy balance is, however, gender dependent, being more pronounced in females than in males [363]. Therefore, it can be speculated that luteinizing hormone and estrogens may render *Crtc1* ko females less vulnerable to obesity.

Contradictory results about circulating levels of luteinizing hormone were reported in *Crtc1* ko mice. Our group did not find alterations in the plasmatic level of this hormone in *Crtc1* ko males and females, compared to their respective controls [304]. By contrast, others authors reported a strong reduction of luteinizing hormone in *Crtc1* null females [303]. With regards to estrogens, it has been proven that they regulate energy homeostasis, mainly through their alpha receptors (ERα), acting

both at central and peripheral levels. Peripherally they control fat distribution and thermogenesis [364], whereas centrally, they increase leptin and insulin sensitivity [365], enhance the expression of *Bdnf* and *Pomc* [366], reduce the orexigenic potency of ghrelin [365], and inhibit the effect of NPY and MCH in the hypothalamus [367, 368]. The higher ERα expression in the hypothalamus of WT female mice, compared to males, was associated with reduced visceral fat deposition, whereas targeted disruption of ERα in the VMH led to obesity in female mice [369, 370]. Until now, no data are available on estrogens levels in *Crtc1* mutant mice, as well as on central and peripheral distribution of ERα. Specific investigations on this issue may help to understand whether the protective effect of estrogens in *Crtc1* ko females may counteract the effect of CRTC1 deficiency.

In agreement with this observation, human studies investigating the association of a *CRTC1* polymorphism (rs6510997C>T) and body mass index in the general and psychiatric population, have found a protective effect of this polymorphism in particular in premenopausal women taking contraception pills [305, 306]. Authors of these works have hypothesized that this protective effect may be the results of the convergence of estrogens, leptin and CRTC1 pathways. They suggested that the presence of this polymorphism associated with higher estrogen levels would facilitate the anorexigenic action of leptin and protect against obesity. These finding are in apparent contradiction with animal studies, because they are based on the assumption that this polymorphism would confer a gain of function. Nevertheless, the comparison between human and animal data remains complicated because we do not measure the real effect of human polymorphism on the biological activity of CRTC1.

The second most relevant finding of our study was the discovery of an altered circadian activity in CRTC1 deficient male mice. As reported, they displayed enhanced locomotion and food consumption during the resting phase of the light cycle. CREB /CRE transcriptional pathway, which is also regulated by CRTC1, is long-time known to be activated by photic inputs [371, 372] CREB phosphorylation was found to be under the control of SCN master clock [373, 374], and the CREB/CRE pathway regulates the expression of some core clock genes [375, 376]. Since CREB-regulated gene transcription requires CREB phosphorylation and is also strongly activated by CRTC1, the lack of this CREB coactivator might contribute to dysregulate the normal expression of clock genes.

So far, only a few data have been published on a possible involvement of CRTC1 in master clock entrainment. In one article, Sakamoto and colleagues have demonstrated that in the SCN, CRTC1 translocation from the cytoplasm to the nucleus varied as function of clock time, being highest during the day and lower during the early night [377]. This effect was specific for CRTC1,

because CRTC2 did not show any variation. Interestingly, the raise of CRTC1 nuclear translocation coincided with the time peak of CREB-mediated gene expression [378]. In the same work, it was shown that the transcription of *Per1*, a core clock gene whose promoter is regulated by the CREB/CRE pathway [379], was increased *in vitro* in HEK 293 cells over-expressing *Crtc1* [377]. In addition to what was observed by Sakamoto et al., another group has reported the participation of CRTC1 in the process of clock-resetting, which occurs after a light phase shift (jet lag). The circadian system, like all other physiological systems, is maintained stable by buffering mechanisms that impede sudden inappropriate Zeitgeber stimuli to affect the function of the SCN master clock. More precisely, it has been observed that CRTC1 in the SCN, in addition to induce *Per1* transcription, increases the expression of *Sik1*. SIK1 (salt-inducible kinase 1) is a kinase belonging to the AMPK family which is able to phosphorylate CRTC1 blocking its nuclear translocation with a negative feedback mechanism (see Figure 10). As a result of SIK1 induction, the expression of *Per1* lowers and the clock re-setting slows down. In this way, CRTC1-SIK1-driven inhibition of *Per1* allows to maintain a balance between clock entrainment and the prevention of an internal dysynchrony of the master clock with peripheral clocks [380].

The aforementioned observations define a role of CRTC1 in clock entrainment and, the circadian abnormalities that we observed in *Crtc1* ko males support this role. However, the effective participation of CRTC1 in the entrainment of circadian clock remains to be proven *in vivo*. Further behavioral and molecular experiments should be performed in the future to address this issue. Behaviorally, it would be important to explore the spontaneous activity of *Crtc1* Ko mice under altered light conditions, notably during a dark/dark cycle (24 hours of obscurity), during a light/light cycle (24 hours of light) and eventually after a light pulse to possibly show a more rapid clock-resetting. With regards to molecular experiments, the analysis of clock gene expression in the SCN would be very informative about the precise role of this CREB coactivator in the synchronization of the master clock.

The discovery of abnormalities in the master clock of *Crtc1* ko mice would support the rationale for investigating possible circadian perturbations in the release of hormones, notably corticosterone and leptin. It has long been known that in depressive patients, the acrophase of corticosterone occurs early in the morning, rather than in the afternoon. A similar result in our mice would allow us to establish a cause-effect interaction between circadian dysregulation and their depressive phenotype. Likewise, disrupted daily leptin fluctuation may support the hypothesis that *Crtc1* ko mice develop leptin resistance and obesity as a consequence of a loss of circadian

rhythmicity. Moreover, the effect of corticosterone on leptin functioning and the regulation exerted by leptin on the HPA axis, highlight how much detrimental could be their circadian disruption.

Sleep disorders, caused by shift work, are associated with deficits in brain serotonin transmission, depressive symptomatology and body weight gain. Our experiments have point out that during the resting phase *Crtc1* ko males are more active and eat more than WT mice. However, at the moment we do not know whether they do not sleep because they are hungry or whether the eating occurs as an activity induced by insomnia. Help in answering this question could come from the analysis of sleep duration and structure of mutant mice.

CRTC1, although mainly expressed in the brain, is also found in some peripheral tissues like the pancreas. Since obesity of *Crtc1* ko mice is accompanied by insulin resistance, it would be interesting to know whether its lack may affect pancreatic circadian clock and insulin release.

A peculiar feature of *Ctrc1* ko mice is their insensitivity to the antidepressant effect of fluoxetine [302]. Intriguingly, in a mouse model in which depression is induced by prenatal exposure to glucocorticoids, reduced circadian oscillation of clock genes in the brain anticipated the onset of depressive-like behaviors, which were not rescued by fluoxetine treatment [381]. This observation supports the hypothesis that circadian perturbations may be at the origin of depressive symptoms exhibited by *Crtc1* ko mice and may influence their response to antidepressants.

The contribution of disrupted circadian rhythmicity to the depressive and obese phenotype of *Crtc1* mutant mice may be further investigated by verifying whether procedures able to reset the master clock may rescue their behavioral abnormalities. There is growing evidence that calorie restriction and timed meals are able to entrain the circadian clock [382]. Therefore, one possibility would be to limit food access of *Crtc1* ko mice to the dark phase of the cycle and determine whether this procedure reduces body weight gain and the severity of depressive symptoms.

In summary, our work adds new evidence to the possible involvement of CRTC1 in the circadian clock entrainment, and suggests that the development of obesity and the depressive-like behaviors of *Crtc1* ko males may be due, at least in part, to a misalignment of physiological functions with the circadian clock. The reason why *Crtc1* ko females do not manifest neither circadian alterations nor hyperphagic obesity requires further specific investigations.

5.3 INTERMITTENT ACCESS TO PALATABLE FOOD IN RATS: a model of binge eating

According to the DSM V, binge eating is an abnormal and compulsive eating pattern, defined as the consumption of an amount of food that is significantly larger than most individuals would eat under the same period of time (≤ 2hours) [383]. This unusual food intake is always coupled with a sense of feeling out of control. Binge foods are calorie dense foods rich in carbohydrates or fats or a combination of the two, usually with high hedonic value. In the U.S., binge eating afflicts approximately 5% of adults [19], and 78% of individuals with binge eating, which do not adopt compensatory behaviors to lose weight, are overweight or obese [384]. Binge eating behavior and depression are strongly comorbid and higher rates of suicide attempts have been reported in binge eaters relative to general population [385, 386].

Nowadays, the large availability and consumption of highly palatable foods is believed to heavily contribute to the emergence of binge eating. Indeed, due to their ability to induce positive feelings by stimulating the reward system [80], palatable foods are often binged. In patients with eating disorders, episodes of overeating are typically followed by periods of dieting which tend to avoid excessive weight gain. This altered eating pattern has long-term consequences because there is substantial evidence that recurrent cycles of palatable food overeating and dieting increase the risk of developing compulsive palatable food consumption (binge eating) [387-389]. Consequently, different laboratory rat models, mimicking the alternation of palatable overeating and dieting, have been developed to study binge eating. In most of them, palatable food overeating was stimulated by food restriction or by stress induction [390-393]. In our work, we reproduced a model proposed by Cottone and colleagues in which rats are neither stressed nor food deprived [394]. Rather, in this model, binge eating is induced by intermittent access to palatable food. This is an important procedural aspect, because both stress and food deprivation are potent confounding elements. Indeed, they could affect experimental results being able to influence the physiology of several neurotransmitters and hormones (e.g. leptin, insulin and ghrelin) involved in the homeostatic and hedonic regulation of food intake.

In our hands, rats submitted to intermittent access to palatable food, spontaneously developed a typical “yo-yo” eating pattern. Compared to control rats, C/P animals significantly overate palatable food when available, and dramatically reduced their consumption of standard food on the following day. With both groups exhibiting similar weight gain across the procedure, it could be argued that this “yo-yo dieting” is no more than a homeostatic control of feeding, with

satiety thresholds reached after binging phases triggering a reduced motivation for feeding afterwards. This assumption is most likely correct, at least at the beginning of the experiment, but our demonstration emphasizes the long-term consequences of this diet cycling on the stress response (see discussion in Rossetti et al. Addiction Biology, 2014). Hence, we rather suggest that a common spiraling distress (defined as a progressive dysregulation of the brain reward function and concomitant development of counter adaptive processes within the brain stress system) may grow with repeated cycles of binging and dieting, producing an allostatic state that drives further consumption, and ultimately compulsive intake.

Of note, we and others using this intermittent access model did not observe body weight gain in C/P rats. This is because the total number of calories consumed during the entire feeding protocol by C/P rats was equivalent to that consumed by control rats. However, when looking at the average amount of kcal consumed in the single C and P phases (see Figure 13) it is clear that C/P rats take fewer calories than control rats during the standard food introduction. Thus, one might wonder whether the binge-like eating develops because of the self-imposed restriction that occurs prior to palatable food access. A study performed to elucidate this point showed that binge eating still develops even when undereating does not occur on the previous day [395]. In addition, the maintenance of a relatively constant body weight by rats submitted to this feeding protocol reflects what is observed in patients that associate binge eating to compensatory behaviors (like for instance in bulimia nervosa), and shows that body weight gain can be dissociated by the development of a compulsive eating behavior.

Since binge-like behavior is not only a daily food overconsumption but rather an abnormally high food intake in a short time window, we measured palatable food intake in 2-hour intervals and we found that C/P rats ate around 50% of their daily food amount in the first two hours of palatable diet availability. This result corresponds to what was already reported by Corwin et al. in a similar model of binge eating [396] and support the validity of our model.

The cyclic pattern of palatable food consumption consisting in recurrent periods of abstinence and relapse observed in binge eaters, has raised the idea that binge eating may be considered as a chronic relapsing condition similar to drug addiction [397]. In binge eaters, like in drug addicts, compulsive food consumption is associated with periods of abstinence (withdrawal). This self-restriction is characterized by a strong desire for palatable food (craving) and negative emotional states (anxiety, depressive symptoms), which in turn lead to new episodes of binge eating. In the case of drug addiction, a shift from positive to negative reinforcement is hypothesized to be

responsible for the transition from casual drug use to compulsive use (dependence) [398] A similar transition seems to occur in binge eaters in which stress and negative emotional states would induce palatable food relapse. In this vicious circle, palatable food would represent a sort of self-medication ("comfort food", as proposed by Dallman [399]) that allows temporary alleviation of discomfort induced by palatable food withdrawal [400].

According with this theory, binge eaters exhibit high anxiety, depressive symptomatology and elevated stress reactivity. Our findings are in agreement with this fact showing that intermittent exposure to palatable food affects the response to stress. When comparing plasmatic corticosterone in C/P and control rats following an acute stress, we found lower corticosterone release in C/P rats after seven cycles of alternation, but not after three cycles.

These outcomes indicate that: 1) changes in stress reactivity are progressive and require repeated episodes of overeating and dieting to appear and 2) adaptations in the response of HPA axis may be a maladaptive response to chronic stress induced by palatable food withdrawal. Interestingly, a few other studies reported blunted corticosterone responses after alternate access to palatable food or sucrose solution [401, 402]. This apparent contradiction with rats maintained on high fat/high sugar diet in which increased endocrine response to stress was claimed [403, 404], seems to depend on the manner in which these palatable foods are consumed [401].

Furthermore, our results and those of others demonstrating blunted corticosterone responses in association with alternate palatable food overeating, coincide with measures done in binge eaters and addicted subjects [405-407] in which reduced plasmatic levels of cortisol have been associated with lower cardiac reactions to psychological stress. Collectively, the aforementioned observations support the concept that HPA axis stress-induced adaptations are crucial in sustaining palatable food overconsumption.

Behaviors induced by intermittent palatable food access in rats, such as palatable food overeating, hypophagia of standard food and withdrawal-induced anxiety were found to be mediated by amygdala. Iemolo et al. showed that the activation of CRH-CRH-R1 signaling in the central nucleus of amygdala and in the BLA has a pivotal role in regulating these behaviors [408] By contrast, the CRH-CRH-R2 pathway has been reported to have no or opposite effect on anxiety [409, 410]. According with the relevance of stress in inducing palatable food relapse, C/P rats sacrificed during withdrawal showed, relative to controls, decreased expression of *Crh-r1* and *Gr*. As expected, no change in the mRNA levels of *Crh-r2* was found in the BLA. Overall, our data support former

findings suggesting that stress response, mediated by CRH-CRH-R1 pathway, is a strong input for the maintaining of a binge eating behavior [411].

A peculiar behavior of rats under cycled palatable food exposure is the spontaneous reject of standard food upon its reintroduction. This "negative contrast" effect seems to not depend on a homeostatic regulation, but rather on hedonic devaluation of less attractive foods [156]. The ability of hypothalamic neurons expressing orexin to stimulate the reward system is well known and the expression of this peptide neurotransmitter in the LH has been seen to increase in rats fed a fat-rich diet [412] and in mice on high-fat diet with a history of caloric restriction [225]. Consistent with the standard food hypophagia observed in diet-cycled rats, the expression of *Orx* in the LH was reduced in C/P rats under withdrawal compared with control rats. Unlike animals fed with high-fat diets, C/P rats sacrificed after palatable food access did not express higher levels of *Orx* than controls.

Leptin, CRTC1 and BDNF are related factors (see discussion of Article 1) with pleiotropic effects in the brain. Down-regulated levels of mRNA transcripts for *LepRb*, *Crtc1* and *Bdnf* were observed in the BLA of C/P rats independently of the time of sacrifice. This result is particularly interesting because it supports a role of CRTC1/BDNF and leptin pathways in binge eating. The notion that BDNF and leptin may be involved in the etiology of eating disorders is not new. Several human studies have shown abnormal plasmatic levels of leptin and BDNF in patients with eating disorders [413-416]. Both leptin and BDNF have many biological functions in the brain, including homeostatic and hedonic control of energy balance, mood regulation and cognitive improvements. Such a fact implies that dysregulation of their function may lead to diversified effects able to sustain palatable food consumption.

It is worth to note that, in our hands, diet-cycled rats, did not manifest clear signs of anxiety in the Elevated Plus Maze (EMP) and Open Field (OF) tests, in contrast to the reports by Cottone and colleagues [394, 417]. Two factors may have contributed to this result. Firstly, control rats in the Cottone studies showed unusual exploratory behaviors in the open arms with consequently, significantly reduced exploration time for C/P rats in comparison, whereas these exploratory behaviors match with the usual data reported in the literature. Secondly, we demonstrated that blunted corticosterone levels appear only after 7 to 8 cycles of dieting and binging, while similar corticosterone release was observed in C/C and C/P animals after 3 weeks of intermittent access to palatable food only. Since we testes rats in the EPM apparatus after 5 weeks of feeding procedure, it is likely that the altered behavioral response to stress remained undetectable in C/P animals.

However, after a 2-month period of intermittent access to palatable food, C/P rats developed a clear-cut compulsive-like behavior for chocolate-flavored food pellets. The transition towards compulsivity has been hypothesized to reflect a shift from a positive to a negative reinforcement [418] According to our working hypothesis, we expected C/P rats to develop specifically a higher responding for chocolate pellets despite electrical foot shocks. Surprisingly, these rats exhibited indiscriminate craving for both sucrose and chocolate-flavored pellets. We suggest that C/P rats developed a sort of addiction for sweetened food, in which a down regulation of brain reward systems that subserve appetitive responses to rewards and a concomitant amplification of brain stress (aka "anti-reward") systems concur to elicit anxiety-like disorders and cognitive deficits responsible for the loss of control over palatable food intake after repetitive cycles of dieting and binging.

Experimentally, we measured compulsive behavior in conditioning operant cages as a perseverative responding despite adverse consequences (shock punishments). However, compulsivity can also be conceptualized and measured as rigidity or inflexibility in behavior. In such case, animal procedures measuring reversal learning or attentional set-shifting can be used [419]. A current hypothesis of B. Everitt and T. Robbins suggests that the compulsive behavior observed in drug addicts, and in binge eaters as well, may originate from brain adaptations ultimately reducing the inhibitory control exerted by cortical structures over behavioral responses, hence triggering stereotypic stimulus-response behaviors orchestrated by the striatum [420]. While cortical dysfunctions in serotonergic, GABAergic and glutamatergic neurotransmission have been involved in the lack of inhibitory control [421], ventral and dorsal striatal dopamine circuits would be responsible for the persistence of stimulus-response habits. Furthermore, the formation of associative memories linking palatable food and environmental cues processed by the amygdala and hippocampus, may contribute to strengthen these stereotypic behaviors and perpetuate, in a sort of automatic response, palatable food overeating. Therefore, from this perspective, the loss of control over palatable food of binge eaters may be conceptualized as a "cognitive" deficit.

In agreement with this theory, it has been shown that obese people and binge eater show cognitive impairments [422]. The hippocampus has long been known to be a relevant structure in modulating cognitive functions and recent investigations about leptin point toward a role of this adipokine in mechanisms of synaptic plasticity that are fundamental in learning and memory processes [423]. Indeed, it has been observed that both increase and decrease of leptin in the brain affect learning and memory performance and LTP in the hippocampus [424]. According to these

findings, we observed upregulation of *LepRb* in the hippocampus of C/P rats sacrificed just after the binge eating episode suggesting that leptin-induced cognitive impairments may be beyond their compulsive overeating.

Impulsivity is a personality construct that can be defined as a predisposition toward, rapid unplanned reactions [425, 426]. Like compulsivity, impulsivity may occur when the top-down inhibitory control is lost. In our work, we measured impulsivity as the propensity to make premature responses in the 5-CSRTT. No correlation was found between basal impulsivity and compulsivity or binge measures in C/P rats. This result contrasts with what was observed for cocaine addicted rats and seems to suggest that impulsivity is not a vulnerability maker of compulsive overeating [337]. Nevertheless, it has to be reminded that impulsivity is a multifaceted construct that appears under different forms. In our experiment, we used the 5-CSRTT that measures a form of motor impulsivity based on the number of premature responses during a 5-sec period of imposed abstinence. Further development with a 7-sec time window would be relevant to discriminate impulsive behaviors not screened with the 5-sec time window [337]. Additionally, impulsivity can also be measured as preference for small immediate rewards versus larger but delayed one (Delayed Discount test) or as the inability to control and stop a response that has already been initiated (Stop Signal Reaction Time test) [427]. Since it has been observed that some forms of compulsivity are associated with specific impulsivity traits, we should assess impulsivity of our rats in other behavioral tests before ruling out the possibility that this personality trait represents a vulnerability factor for developing binge eating-like behavior.

A very recent study of binge eating in rats [428], based on intermittent presentation of milk chocolate, has tested the efficacy of some drugs on the reduction and/or suppression of this eating behavior. Nalmefene, R-baclofen, SB-334867 (orexin-1 antagonist) and the CNS stimulant lisdexamphetamine, recently approved by the FDA in the US as binge eating treatment [429], were found capable to selectively decrease palatable food consumption without affecting standard chow intake. As a further exploration of the predictive validity of our model, it would be important to verify whether the same pharmacological treatments are effective in blocking compulsivity.

In conclusion, we confirm that the intermittent access to palatable food is a valid and reliable model for inducing binge eating-like behavior in rats. In fact, it allows to reproduce most of behavioral, metabolic and molecular alterations observed in human eating disorders characterized by compulsive overeating. Moreover, its predictive validity will permit to perform additional translational oriented investigations.

Among the relevant outcomes emerged from the use of this rat model, the observation that leptin signaling and CRTC1/BDNF pathway are strongly perturbed in cycled rats and possibly involved in binge eating behavior, is remarkable. A first step toward a better characterization of the role of CRTC1/BDNF in binge eating has been done in the present study by submitting *Crtc1* ko mice to an alternate palatable food exposure.

5.4 COMPULSIVE OVEREATING OF *Crtc1* KO MICE

Impulsivity and aggressiveness are personality traits that frequently emerge in the frame of mood disorders and in particular in depressive patients with a propensity to suicidal attempts [430, 431]. In a former article, we reported that *Crtc1* ko mice manifested reduced serotonin in the brain and, as a component of their depressive like behavior, strong aggressiveness towards their littermates. In fact, when tested in a resident-intruder test, *Crtc1* ko males and lactating females engaged in serious attacks against the intruder mouse [302]. Aggressiveness, which results from the combination of compulsive and impulsive traits, is related to an impairment of the cortical inhibitory control. Monoamine transmission and serotonin in particular, is considered to be fundamental in this behavioral regulation. Cognitive flexibility is required for an appropriate decision-making process and deficits in the serotonergic transmission were found to inhibit reversal learning in animal studies [432]. Nevertheless, the role of serotonin in reversal learning is complex as revealed by opposite effects obtained with antagonists targeting different type of its receptors [433, 434].

Given the role of serotonin in mood alterations, impulsivity and compulsive behavior, it is not surprising that this monoamine has been linked to the etiology of eating disorders. Different human polymorphisms of serotonin receptors (5-HT_{1A}, 5-HT_{2A}, 5-HT_3) and of serotonin transporters (5-HTT) have demonstrated a tight association between serotonin transmission and the development of compulsive eating behavior [435, 436]. Moreover, it has been reported that antidepressant increasing serotonin in the brain, such as SSRI and tricyclics, reduce the frequency and the severity of binge eating episodes [437, 438].

Linking together all the aforementioned observations and the fact that CRTC1/BDNF pathway was seriously affected in binging rats, we sought to test *Crtc1* ko mice in the alternate feeding procedure. In this part of the study, we used *Crtc1* ko females because we aimed to assess whether this feeding protocol could exacerbate their obese profile and trigger compulsive overeating.

Intermittent access to standard and palatable food triggered similar behavioral adaptations in rats and mice. All rodents overate the chocolate-flavored preferred food and restrained themselves

when returned on the standard diet. This change in food intake had strong effect on their daily body weight that, accordingly, increased or decreased. Despite this periodic change in daily food intake, all mice consumed a similar amount of kcal during the entire procedure. Surprisingly, only C/P *Crtc1* ko mice gained more weight at the end of the feeding protocol, relative to the others groups. Since body weight gain was not accompanied by hyperphagia, it seems plausible that obesity in *Crtc1* ko females develops, as already observed for standard food, as a consequence of reduced energy expenditure. However, a substantial difference in the progression of body weight can be noted between mutant females fed with standard food and those that had intermittent access to palatable food. Whereas the obesity of the former was strongly delayed, that of mutant females on intermittent access to palatable food appeared only after a month and a half, suggesting that the alternate feeding protocol exacerbated females' obesity.

Binge eating measure indicated that C/P *Crtc1* ko mice had the tendency to consume an important part of their daily food ration during the first two hours of palatable food availability.

Despite food intake variability observed in mice, *Crtc1* ko mice exhibited a binge eating pattern similar to that observed in rats and displayed, overall, an exaggerated hyperphagia compared to all other mice.

Intermittent access to palatable food elicited a clear-cut anxious profile in C/P *Crtc1* ko mice, with a strong reduction of their locomotor activity in the EPM compared to the three other groups. Strong inhibition of locomotion was also observed in both mutant groups in the light/dark box during habituation. When retest the following day in the same box, all mice increased their locomotion in the illuminated compartment showing that they were habituated to the novel environment, but the difference between groups remained similar to that observed the day before. Of note, despite the reduced exploration of the illuminated side of the box, C/P *Crtc1* ko mice consumed significantly more food than others mice in the aversive side of the chamber, exhibiting compulsive-like eating.

These data about the effect of intermittent access to palatable food in mice are very encouraging, and call for further development. They allow to suggest that: 1) mice, like rats, are sensitive to this feeding protocol and show similar changes in their feeding behavior, eating more when provided with palatable food and restraining themselves when re-fed with standard chow; 2) *Crtc1* ko mice are more vulnerable than WT mice in developing binge eating-like behavior that is also accompanied by increased anxiety and body weight gain.

In order to expand our knowledge on cognitive inflexibility as a cause of compulsive behavior in *Crtc1* ko mice, we should evaluate reversal learning in those mice. The T maze test, the Y maze test that allow to asses working memory, as well as the measure of reversal platform learning in the water maze test, would represent useful strategies. In addition, a detailed molecular analysis of *Crtc1* ko mice before and after the application of the alternate feeding protocol is required to explore the emergence of putative brain adaptations. Particular attention should be paid to the role of BDNF and leptin in amygdala and hippocampus to see whether they may be involved in the binge eating behavior exhibited by these mice. Measuring the functionality of the CRH-CRH-R1 pathway would be of similar importance.

In conclusion, *Crtc1* mutant mice may become an interesting model to evaluate binge eating behavior and to test new and exciting hypothesis.

6 CONCLUDING REMARKS AND PERSPECTIVES

Since their generation, *Crtc1* ko mice have shown to be a valuable model for investigating the participation of the CRTC1-CREB pathway in many cerebral physiological processes. CRTC1 deficiency affects these processes leading to two principal phenotypes, a depressive and an obese phenotype. In the last years, the intensive and valuable work of our group has revealed that *Crtc1* ko mice exhibit social avoidance, altered emotional responding, depressive-like behavioral despair and aggressiveness, associated with an antidepressant resistance. Concomitantly, investigations carried out in the frame of this thesis have shed light on their obese phenotype. The first important observation is that CRTC1 deficiency induces both phenotypes, at least in male mice, at the same time. This fact suggests that the concept of an overlap between depression and obesity is a realistic theory and that the alteration of genes that are at the beginning of complex and intricate brain pathways may affect apparently distinct physiological processes.

Crtc1 ko male mice develop obesity because of hyperphagia, which is related to a homeostatic gene dysregulation. Hyperphagia occurs mainly in the resting phase of the light cycle indicating that circadian perturbations may underlie the abnormal eating behavior of *Crtc1* ko mice. Given the high prevalence of binge eating behavior in the clinical cases of obesity and mental disorders comorbidity, *Crtc1* ko mice represent a relevant model for investigating this association. The characterization of the intermittent access to palatable food in rats, as a model of binge eating, has largely contributed to improve our knowledge about the behavioral and molecular adaptations that this compulsive eating behavior elicits. Using the same procedure in *Crtc1* ko mice, we obtained

preliminary but encouraging and exciting evidence about the possible involvement of CRTC1 in compulsive overeating. These findings, therefore, identify these mice as a suitable model for investigating the biological underpinning of binge eating behavior.

In the future, additional data on the biological effect of CRTC1 deficiency on obesity development should be collected. Further experiments concerning the homeostatic role of CRTC1 in food intake regulation should be focused on other hypothalamic structures, particularly on the VMH. The relevance of this structure is represented by its content in neurons expressing BDNF that, as a CREB-regulated factor, plays a crucial part in energy homeostasis. Moreover, viral approaches able to restore CRTC1 in discrete brain structures may help to expand our knowledge about the role of this coactivator in the regulation of energy balance and in particular in those brain processes leading to obesity. Of equal relevance are manipulations of the circadian rhythm of *Crtc1* ko mice. These experiments should shed light on the possibility that a misalignment of metabolic functions with the circadian clock may be a common cause of depression and obesity in these mice.

Furthermore, in an extent to confirm and expand this concept, a survey concerning circadian rhythmicity (able to investigate sleep habits and food timing) on people carrying different CRTC1 polymorphisms in the general and psychiatric population, may give us new elements to support this hypothesis. Yet, an important issue till now unexplored, is the possibility that a functional reduction of CRTC1 is responsible for the obesogenic effect of antipsychotics.

Additional investigations are also required to better elucidate the role of CRTC1 in compulsive eating behavior. The use of the rat model may be of great utility in establishing the biological significance of CRTC1/BDNF adaptations observed in the amygdala after recurrent cycles of binge eating and dieting. Finally, a pharmacological approach, based on those treatments currently used for binge eaters, would permit to further validate this model and test whether their action may restore physiological levels of CRTC1 and BDNF in the brain. As a complement of these studies, the hypothesis concerning the role of serotonin in compulsive overeating could be further assessed in *Crtc1* ko mice by enhancing serotonin transmission acting specifically (with selective agonist and antagonist) on the receptors that have been involved in this behavior.

Obesity is one of the most pressing health issue that we have to face today, and certainly, in the next several decades. The slow but constant effort made by scientists in understanding the biological underpinning of this pathological condition, hopefully, should allow to identify new

pharmacological targets and new efficient therapies. These progresses will be of high importance to treat those critical patients that do not respond to the current available medicines and possibly to prevent the development of other serious diseases associated to obesity. However, since obesity is preventable, comparable efforts should to be done to avoid its rise. Obesity tends to run in families, suggesting a genetic cause. However, family members share not only genes, but also diet and lifestyle. Diet and lifestyle are two major environmental factors closely associated with the prevalence of obesity. Based on this evidence, the initial attempt made to fight obesity was centered in reducing calorie intake and boosting physical activity. However, the poor results obtained until now attest that these measures, which targeted only the loss of weight, are not sufficient to fix the problem. Obesity is more than a nutritional disease and successful strategies to reduce it are probably those that take into account also its emotional and psychological dimension. The morbid association of obesity with mental disorders suggests that, for breaking the vicious circle linking overeating to negative mood, people should be helped in managing not only their weight but also their emotional health. In this perspective, people at risk to develop obesity, should be encouraged to accept healthy food and to do physical exercise but also to follow psycho-behavioral therapeutic programs aiming at reducing stress and anxiety, at promoting self-control and at strengthening self-esteem.

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