Università degli Studi di Cagliari

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Ciclo XXIX

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Settore scientifico disciplinare di afferenza
BIO/14-Farmacologia

Presentata da: *Dott. Roberto Collu*

Coordinatore Dottorato: *Prof. Antonio Argiolas*

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Esame finale anno accademico 2015 – 2016
Tesi discussa nella sessione d'esame marzo – aprile 2017


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Chapter 1. Introduction

1.1. The regulation of food intake

Eating is a complex behavior in which peripheral physiological signals are combined with central neural processes and psychological experiences to finely regulate energy homeostasis. A key point when considering feeding behavior is the relationship between the homeostatic and the hedonic regulation of appetite. The homeostatic control of appetite is mediated by a biological need to maintain a general body energy equilibrium, while, the hedonic control is based on sensory pleasure rather than biological needs and hence mediated by specific reward pathways. Hunger and satiety are psychological experiences that physiologically drives, respectively, the motivation to consume food and the consequent state of satiety in which feeding is inhibited (Blundell, 1991). Key peripheral components involved in the regulation of eating behavior are the gustatory system and the gastrointestinal tract along with pancreas, liver, muscle and adipose tissue. All these components are directly and bidirectionally connected with the brain through neural connections provided by the autonomic nervous system, hormones or metabolites.

1.1.1. *The homeostatic regulation of food intake*

The hypothalamus is the key brain region involved in the central modulation of feeding behavior (Farr *et al.*, 2016). Located under the thalamus, it forms the ventral part of the diencephalon and plays an important role in the regulation of metabolism by mediating numerous physiological processes, e.g. food intake, body temperature, circadian rhythms, sexual behavior, reproduction and emotional behaviors. It is composed by

several distinct nuclei that integrate endocrine, neural and metabolic signals from the periphery. It includes the arcuate nucleus (ARC), the paraventricular nucleus (PVN), the ventromedial nucleus (VMN), the dorsomedial nucleus (DMN) and the lateral nucleus (LH) (Williams *et al.*, 2001). All hypothalamic nuclei are in close correlation with many important areas of the brain (*e.g.* cerebral cortex, mesolimbic system, autonomic nervous system) creating a complex network of anatomical connections. Such a network processes signals of a different nature and influences food intake and energy balance through anorexigenic and orexigenic circuits (Morton *et al.*, 2006). The hypothalamus includes two different centers involved in the regulation of feeding: the "*hunger center*" composed by the LH, which electric stimulation and lesion cause, respectively, an increase of feeding and a decrease in food and water consumption, and the "*satiety center*" composed by the VMN and PVN, which electric stimulation and lesion produce, respectively, anorexia and hyperphagia/obesity (Hetherington and Ranson, 1942).

The ARC is located at the base of the 3rd ventricle, is not isolated from the circulation and has therefore an advantageously position to integrate different peripheral signals controlling food intake. It represents the processing center for the primary integration of hunger and/or satiety signals from the periphery (Funahashi *et al.*, 2000), and its lesion in mice causes obesity and hyperphagia (Olney, 1969). Once encoded, peripheral signals generate a response for different neurons localized in other hypothalamic nuclei, defined as *second order neurons*. Two different *first order neurons* expressing neuropeptides are present in this nucleus. The first is composed by orexigenic neurons, which secrete the neuropeptide Y (NPY) and the agouti-related protein (AgRP), which stimulation leads to an increase of food intake. The second population of neurons

includes the anorectic neurons secreting pro-opiomelanocortin (POMC) and cocaine- and amphetamine-regulated transcript (CART), which stimulation inhibits food intake. These two neuronal populations are influenced by hunger and satiety signals and regulated by a reciprocal inhibition relationship: when one is activated the other one is inhibited and *vice versa*. NPY is a 36 aminoacid neuropeptide belonging to the family of pancreatic polypeptides that possesses powerful orexigenic effects (Parker *et al.*, 2002). It is widely distributed throughout the central nervous system (CNS) with high concentrations in the hypothalamic appetite-regulating nuclei, particularly within the ARC where most of NPY is derived (Allen *et al.*, 1986). Local NPY injection in the hypothalamus nuclei of rats increases food intake and reduces energy dissipation while inducing lipogenic enzymes in the liver and white adipose tissue (Stanley *et al.*, 1986; Billington *et al.*, 1991). Furthermore, the direct administration of NPY in the 3rd ventricle of rats causes a persistent increase in food consumption, effect that is still evident after several days from its administration (Flynn *et al.*, 1999). The hyperphagic effect of NPY is mediated by specific NPY receptor subtypes. To date, six receptor subtypes (Y1-Y6) have been cloned and well characterized (Lin *et al.*, 2005).

AgRP is a 132 aminoacid protein released from the same synaptic terminals secreting NPY that acts as endogenous antagonist for melanocortin 3 and 4 receptors (MC3 and MC4) in the PVN (Baker *et al.*, 1995) and hence stimulates food intake by inhibiting the anorectic action of the α-melanocyte stimulating hormone (α-MSH). AgRP causes hyperphagia when intracerebroventricularly (ICV) administered (Hagan *et al.*, 1998) or when induced in mice (Ollmann *et al.*, 1997), and NPY/AgRP neurons ablation in young mice decreases food intake and body weight (Bewick *et al.*, 2005). POMC is a pro-hormone that thanks to the activity of a pro-convertase gives rise to a number of

hormones including corticotropin (ACTH) and, following further proteolytic cuts, the α-MSH (a potent inhibitor of caloric intake that acts through the MC3 and MC4 receptors) (Kalra *et al.*, 1999; Yang and Harmon, 2003).

CART is a neuropeptide found in several brain areas that in humans is encoded by the CARTPT gene and in animals has the same effect of cocaine and amphetamine. It acts as an anorectic mediator and is involved in complex peptide signalling mechanisms that regulate feeding behavior (Thim *et al.*, 1998). In particular, ICV administration of CART decreases food intake in rodents (Kristensen *et al.*, 1998), and CART deficient (knockout, KO) transgenic mice do not exhibit significant alterations in feeding behavior or body weight when a normal diet is administered (Asnicar *et al.*, 2001).

As mentioned above, the LH nucleus belongs to the hypothalamic "*hunger center*". It is composed by two distinct and homogeneously distributed populations of neurones synthesizing orexigenic peptides, such as orexin and MCH, which are able to stimulate appetite and reduce metabolism. Two MCH receptors, *Mchr1* and *Mchr2*, have been cloned in humans, whereas only the *Mchr1* has been identified in rodents. *Mchr1* KO mice display increased energy expenditure, locomotor activity and are resistant to diet-induced obesity (Chen *et al.*, 2003). Against these findings, MCH injection into the lateral ventricle of rats leads to an increase in food intake while fasting stimulates the expression of *Mch* mRNA (Qu *et al.*, 1996). Those neurons project to different important brain structures involved in regulating motivational states, such as the ventral tegmental area (VTA). The MCH-containing neurons project to the cortex, brainstem and spinal cord (Bittencourt *et al.*, 1992). In addition, these neurons receive projections from the first order neurons located in the ARC that regulates its activity: NPY/AgRP neurons stimulate the activity of these neurons while POMC/CART inhibit it. In the

VMN, identified as the "*satiety center*", neurons producing peptides involved in appetite regulation have not been identified to date. The VMN is therefore considered to act as LH inhibitor and as release station of orexigenic and anorectics fibers of the hypothalamic regulation system. Neuroimaging clinical studies have shown an increased signal in the VMN after oral administration of a high amount of glucose (Matsuda *et al.*, 1999).

The PVN is of crucial importance in the release of orexigenic signals because it secretes a large amount of neuropeptides. The most important are the corticotropin-releasing hormone (CRH) and thyrotropin releasing hormone (TRH), both with anorectic activity. TRH reduces appetite and food intake and stimulates thyroid axis (Kow and Pfaff, 1991). Microinjections of NPY and AgRP into the PVN stimulate feeding (Kim *et al.*, 2000). The perifornical hypothalamus seems to be mainly involved in orexigenic circuits and, together with the supraventricular area, takes part to the determination of the sense of hunger and satiety before meals. The sense of hunger is associated with a low dopaminergic tone in the perifornical area, while the sense of satiety after a meal coincides with higher dopaminergic activity. Hunger and satiety are phenomena coupled with the conduct of activities/supervision in the day-night cycles: the suprachiasmatic nucleus is involved in the circadian regulation of appetite.

The modulation of these central circuits is the result of a complex interaction between afferent signals which arise at the peripheral level and are classified into hunger and satiety signals. Among the hunger signals, ghrelin is a 26-amino acid peptide hormone secreted by the parietal P/D1 cell of the stomach and to a lesser extent by the pancreas. It was identified by Kojima and collaborators in 1999 and is involved in the release of the growth hormone and in feeding behavior. It has been shown that the blood levels of

this peptide are greatly increased before starting each meal and are drastically reduced in the post-prandial phase, thus highlighting one of its key role in the initiation of food intake. Once produced, ghrelin reaches the ARC via the bloodstream and interacts with its receptors (GH-R) on neurons releasing NPY/AgRP (Kamegai *et al.*, 2001; Cowley *et al.*, 2003) and on orexin fibres in the LH (Toshinai *et al.*, 2003). This activation leads to a release of NPY and AgRP, which on one hand stimulate the PVN involved in metabolic functions and on the other hand induce the release of MCH and orexin by LH. Ghrelin also stimulates food intake when directly injected into the PVN (Shrestha *et al.*, 2004). The final result is the increase in food consumption and the decrease of metabolism.

As opposed to signals of hunger, our body produces satiety signals that are mediated by leptin and insulin. Leptin is produced by adipose tissue in quantities proportional to the availability of energy reserves. Rodents lacking leptin (ob/ob mice) or the leptin receptor (OB-R, db/db mice and Zucker fa/fa rats) are obese and hyperphagic. Lack of leptin is rare in humans where it may cause severe obesity that can be ameliorate by peripheral leptin administration (Farooqi *et al.*, 1999). Leptin crosses the blood brain barrier and binds to its receptor (OB-R) in the hypothalamus (Faouzi *et al.*, 2007). These receptors are located in the two populations of neurons in the ARC that decreases the activity of AgRP/NPY thereby inhibiting their stimulating action towards MCH and orexin and increases activity of neurons secreting POMC/CART with anorectic actions. All these factors are responsible for the production of satiety signals that have as their ultimate goal the inhibition of food intake. The OB-R is generally expressed in the hypothalamus but mostly in the ARC, VMN, DMN and LH. Leptin over-expression in the ARC, PVN and VMN results in reduced food intake (Bagnasco *et al.*, 2002).

Insulin is a peptide hormone produced by the pancreas. Insulin receptors are widely distributed in the brain, but principally in hypothalamic nuclei involved in the regulation of food intake. It has an anorectic effect when administered ICV or directly into the VMN, an effect that is reversed by insulin antibodies (McGowan *et al.*, 1992). This hormone is also implicated in the recruitment of alleviating food hunger and together with the regulating glucose metabolism represents a neural network responsible for the long-term control of energy balance and body weight stability. Circulating insulin rises in response to a glucose load and, like leptin, is an indicative sign of fat mass availability. The mechanism through which insulin inhibits food intakes is not fully known, even though administration of insulin into the 3rd ventricle of fasted rats increases ARC POMC mRNA expression and decreases food intake. This anorexigenic effect of insulin is blocked by melanocortin antagonists (Benoit *et al.*, 2002).

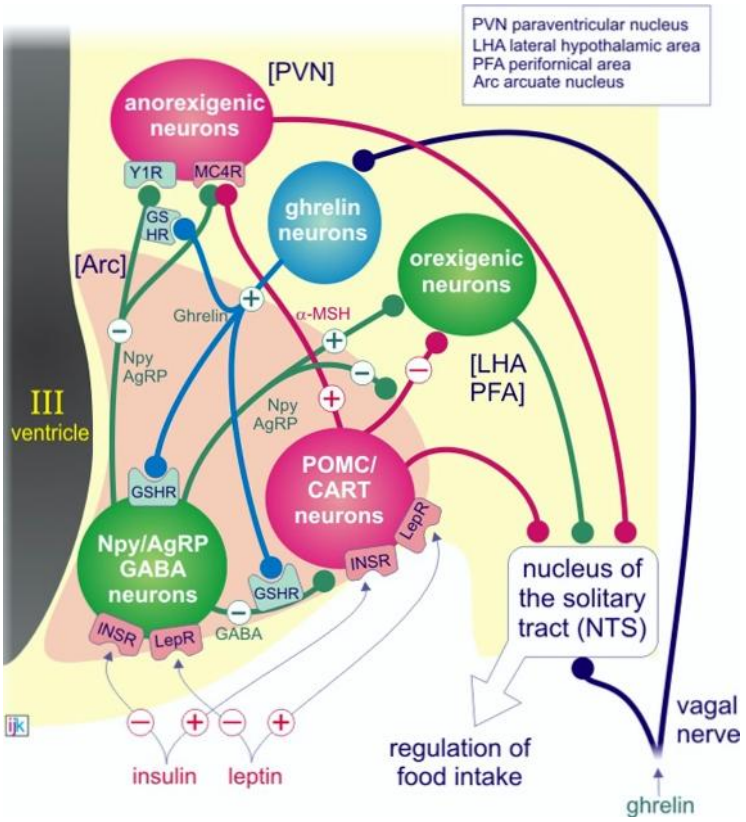


Figure. 1: Representation of the regulation of food intake in the hypothalamus (internet)

1.1.2. *The hedonic regulation of food intake*

The homeostatic control of feeding behavior is primarily based on the regulation of energy balance by increasing food consumption following a depletion of energy stores. In contrast, hedonic regulation of feeding is based on the reward aspects associated with food intake and sometimes can override the homeostatic control by increasing the motivation to consume palatable food even in the absence of real energy necessity. Motivation and reward responses have been studied particularly in the context of substance abuse disorders such as drug addiction (Berke and Hyman, 2000; Laakso *et al.*, 2002). However, a growing number of clinical and pre-clinical studies supports the theory that both drugs and food rewards shared common neural pathways to mediate motivated behaviors (Saper *et al.*, 2002; Lutter and Nestler, 2009). It is well established

that most drugs of abuse act on the mesolimbic dopaminergic pathways increasing the release of dopamine (DA) from neurons originating in the VTA and projecting to neurons located in the nucleus accumbens (NAcc), prefrontal cortex (PFC), amygdala (Amy) and hippocampus (Hipp) (Wise and Rompre, 1989; Fields *et al.,* 2007). Drug-induced activation of the VTA dopaminergic transmission could be mediated either by direct action on dopaminergic neurons or indirectly through the inhibition of GABAergic interneurons in the VTA (Nestler, 2005). It has been demonstrated that food stimulates similar responses within the mesolimbic dopaminergic rewarding pathway being responsible for the pleasurable feelings associated with the ingestion of food. In particular, high palatable foods produce a strong release of DA in the shell of the NAcc (Martel and Fantino, 1996; Di Chiara and Bassareo, 2007). According to this, Parkinson's disease (PD) patients, with a degeneration of dopamine-containing neurons in the midbrain, consume less food than healthy patients (Nirenberg and Waters, 2006). Moreover, administration of DA receptor agonists increase the consumption of palatable food in PD patients and in healthy people (Dagher and Robbins, 2009).

As might be expected, prolonged activation of the mesolimbic system by drugs of abuse leads to cellular and molecular adaptations that are thought to be involved in the aberrant motivation to obtain drug-related rewarding effects observed in addicted patients (Nestler *et al.*, 2005). Interestingly, similar neuroadaptations have been described in the limbic regions of rodents exposed to highly palatable foods. For example, mice exposed to a high-fat diet for 4 weeks showed decreased levels of active CREB in the striatum (Lutter and Nestler, 2009). These findings are consistent with the evidence that decreased CREB activity in the ventral striatum increases the preference for both a sucrose solution and morphine (Barrot *et al.*, 2002).

The mesolimbic system is in close connection with the hypothalamus since many of the molecules involved in the regulation of the homeostatic feeding are also active in the limbic regulation of its hedonic component (Monteleone and Maj, 2013; Meye and Adan, 2014). For example, VTA dopaminergic neurons express both the GH-R and OB-R. It has been demonstrated that activation of the GH-R in this region increases the release of DA in the shell of the NAcc (Abizaid *et al.*, 2006; Jerlhag *et al.*, 2012). The increased ghrelin signal in the VTA affects the consumption of highly palatable food as well as the motivation to obtain a solution containing sucrose in rodents (Egecioglu *et al.*, 2010; Skibicka and Dickson, 2011). On the contrary, activation of the OB-R produces a decrease of the rewarding properties of food (Figlewicz *et al.*, 2006). Furthermore, receptors for orexin and NPY are also widely expressed in the dopaminergic neurons of the VTA and their activation increases the release of DA in the NAcc (Korotkova *et al.*, 2006).

Other non-dopaminergic systems also importantly contribute to the reward value of food. Serotonin (5-HT), for example, has a widely recognized influence on feeding and mood as well as important modulatory effects on reward circuits and motivated behaviors (De Deurwaerdère *et al.*, 1998; Pessia *et al.*, 1994). In particular, 5-HT directly influence, via $5HT_{2C}$ receptors, POMC arcuate neurons increasing the release of α-MSH but also of β-endorphin, which binds to opiate receptors (Heisler *et al.*, 2002). Given the wide distribution of opioidergic neurons and receptors in many regions of the brain, such as the hypothalamus, a key role of opiates in feeding and food reward related pathways has been demonstrated (Kelley *et al.*, 2002). Moreover, opioid receptor antagonists such as naloxone block the effects of AgRP in increasing feeding (Hagan *et al.*, 2001).

1.2. Anorexia nervosa

1.2.1. *Definition and classification*

Anorexia Nervosa (AN) is the most severe eating disorder with the highest mortality rate compared to any other mental illnesses (Arcelus *et al.*, 2011; Smink *et al.*, 2012). It is a chronic and disabling psychiatric pathology characterized by altered body image perception associated with modified patterns of feeding and weight-control behaviors such as excessive dieting and extreme physical hyperactivity (Smink *et al.*, 2013). AN affects significantly more females than males, typically has its onset during adolescence and is accompanied by other psychiatric conditions like depression, anxiety and substance abuse (Kaye *et al.*, 2004; Blinder *et al.*, 2006; Hudson *et al.*, 2007). According to the 5th edition of the Diagnostic and Statistical Manual of Mental Disorders (DSM-V), diagnostic criteria include: "*a persistent restriction of energy intake which leads to a significantly low body weight compared to that which would be expected for a given age or developmental stage*", "*an intense fear of gaining weight or becoming fat*", "*a disproportionate influence of weight and shape on one's self-evaluation and a disturbance in the way that changes in weight are experienced"* (APA, 2013). AN can be further classified by the strategy used to achieve and maintain low body mass index. The DSM-V specifies two subtypes of AN that differ in the mechanisms by which body weight loss is achieved. Patients who regularly engaged in bingeing episodes followed by purging behavior (*Binge-Eating Type or Purging Type*), such as self-induced vomiting, abuse of laxatives, diuretics or enemas, are distinguished from those who primarily lose weight through significant reductions in caloric intake (*Restricting Type*), whether by fasting or excessive exercise. Moreover, anorexic

patients can display mixed subtypes and present transition from one type to another (Polivy *et al.*, 2002).

1.2.2. *Epidemiology*

AN is a common pathological condition with a lifetime general population prevalence of 0.2-0.9 % (Smink *et al.*, 2012; Nagl *et al.*, 2016). Recent changes in the diagnostic criteria, such as elimination of amenorrhea as a criterion, will likely lead to higher rates of reporting (Mustelin *et al.*, 2016). Females are approximately affected by AN ten times more than males, although the disorder is likely under-diagnosed and under-recognized in men (Hoek, 2006). Typically diagnosed during adolescence (15-21 yrs), it has been suggested (but not definitively proved) that as in BN the annual incidence of AN may be gradually increased over the past several decades (Theander, 1985; Polivy *et al.*, 2002). Methodological variations and differences in the treatment setting complicate calculations, but current estimates suggest that the incidence of AN is approximately 4-8 per 100,000 person-years in the general population, with a much higher rate, nearly 110 per 100,000 person-years in females aged 15-19 (Lucas *et al.*, 1999). AN is rarely diagnosed after the mid-20's, and frequently patients suffer AN for many years with frequent relapse conditions (Carter *et al.*, 2004; Hudson *et al.*, 2007). Mortality rates in the literature range from 5 to 15 %, sometimes as high as 20 %, indicating that AN patients are at significant risk for premature death compared to the average population. The severe medical complications associated with anorexia are the most common causes of death (Sullivan, 1995). Suicidality in AN has been linked to comorbid depression, with one study reporting 81 % patients attempting suicide during

a depressive episode. Other personality features that conferred increased risk include high levels of anxiety, impulsivity and dysregulated affect (Bulik *et al.*, 2005).

1.2.3. *Medical complications*

The severe state of emaciation and malnourishment in AN inevitably leads to an increased risk for serious medical and metabolic complications. Patients affected by AN develop a long series of health problems and changes in the physiology of different regulator systems that contribute to the general altered medical condition and may lead to dramatic consequences (Sullivan, 1995).

Gastrointestinal complications

Complications of the gastrointestinal system are frequently reported in anorexic patients and usually reverted after refeeding and restoration (Mitchell *et al.*, 2006). Gastric emptying is often delayed, producing nausea and early satiety. Potentially fatal rapid gastric dilatation is a rare refeeding problem. Slow colonic transit and constipation are common, causing bloating which exacerbates body image problems if not treated (Chial *et al.*, 2002).

Renal and biochemical complications

Binge-purge type of AN confers a higher risk of electrolyte disturbance and metabolic alkalosis derived from the abuse of laxatives and diuretics. Hypophosphetimia, increased liver enzymes, hypokalemia and hypomagnesaemia are common abnormalities and can occur secondary to a starvation state as well as to chronic episodes of restriction (Winston, 2012).

Hematological complications

Abnormalities of red and white cells or platelets can occur in patients with AN, but are typically the result of malnutrition and are reversible after weight restoration. Anemia is reported in a third of patients; plasma volume depletion may mask an anemia that becomes apparent after rehydration (Caregaro *et al.*, 2005). Serum hematinics are usually normal, although ferritin can be high from circulating volume contraction with subsequent red cell breakdown and ferritin storage (Kennedy *et al.*, 2004). Neutropenia can develop in patients with AN, which may result in neutropenic sepsis. Thrombocytopenia is seen in 5-10 % of patients with AN (Hütter *et al.*, 2009).

Dermatological and dental complications

Dermatological signs such as xerosis, acne, dermatitis, alopecia and brittle nails as well as dental complications are very common and can be secondary to either metabolic disturbances or frequent vomiting, which exposes teeth, esophagus and digits to caustic gastric acid (Mitchell *et al.*, 2006).

Muscular and skeletal complications

Muscular and skeletal complications, such as significant reductions in bone size and mineral density, premature osteopenia, osteoporosis and increased rates of bone fractures, are among the most common complications in AN. Up to 90 % of anorexic patients have reduced bone mineral density, while 38 % are osteoporotic (Grinspoon *et al.*, 2000). All these alterations are difficult to revert with weight restoration and persist even after physiological weight achievement (Bachrach *et al.*, 1991; Herzog *et al.*, 1993).

Cardiological complications

Cardiovascular problems are among one of the most common causes of death in AN. Patients affected by AN frequently present abnormalities in physiological parameters such as arrhythmias and bradycardia (Galetta *et al.*, 2003), chronic hypotension and impaired orthostatic responses, sodium and fluid compartment derangements as well as low cardiac output, reduced ventricular mass and increased peripheral vascular resistance (Katzman, 2005; Casiero *et al.*, 2006).

Endocrine complications

Severe hypoglycemia is a common and often asymptomatic condition in AN and may be due to infection or liver dysfunction or be induced by excessive exercise, with fatal outcomes (Mattingly and Bhanji, 1995). Thyroid abnormalities are common with low triiodothyronine, normal-to-low thyroxine and thyroid-stimulating hormone levels and a reduced rate of peripheral thyroxine to triiodothyronine conversion. These alterations can cause hypotension, impaired orthostatic responses and hypothermia (Roche *et al.*, 2005). Primary and secondary amenorrhea are common in patients with AN. Menstrual abnormalities such as the loss of gonadotropin-releasing hormone pulsatility with increased follicle-stimulating hormone:luteinizing hormone (FSH:LH) ratio. Insufficient gonadotrophin release for ovulation is followed by an insufficient luteal phase for normal menstruation. Moreover, inadequate adipose tissue reduces the production of estrogen. As menstruation requires maintenance of normal or near-normal weight, it is an important physiological and psychological milestone in recovery (Usdan *et al.*, 2008).

1.2.4. *Psychiatric comorbidities*

In addition to the medical complications previously described, anorexic patients typically present a huge range of psychiatric comorbidities that influence the progression of the pathology. The most common co-occurring comorbid conditions are major depressive disorder, anxiety, obsessive-compulsive disorder and substance abuse disorder (O'Brien *et al.*, 2003; Halmi *et al.*, 1991). In some cases, those traits may constitute a distinct co-morbid psychiatric diagnosis, but in most cases they may represent critical illness-maintaining factors influencing the effectiveness of treatment, the very low rate of complete recovery and the frequent relapse episodes (Carter *et al.*, 2004; Bergh *et al.*, 2013). Whether psychological symptoms are the cause or the consequence of a starvation state is still difficult to understand. Classic studies examining the impact of severe caloric restriction on human behavior have shown that some AN psychological symptoms can be improved following a weight restoration period (Meehan *et al.*, 2006). However, other studies have found no relationship between affective symptoms and weight recovery (Mattar *et al.*, 2012). It is not yet clear whether the high rate of comorbidities in AN is a result of a shared etiology or a pre-morbid state that contributes to its onset, or whether depression is a phenomenon consequent to starvation. The high rate of comorbidity has driven several trials testing antidepressant drugs in AN, which yielded disappointing results but had the merit to focus the clinical interest on the role that different neuronal systems may have in the control of emotional circuits in AN (Claudino *et al.*, 2006). A large proportion of anorexic patients are diagnosed with a formal comorbid anxiety disorder, most commonly obsessive-compulsive disorders, generalized anxiety disorder and social phobia (Pollice *et al.*, 1997; Kaye *et al.*, 2004). As in depression, however, the exact

relationship with personality features remains unclear. In addition, it has been suggested a marked association between the development of eating disorders and the use of drugs like marijuana, steroids and inhalants and, to a lesser extent, nicotine, alcohol, cocaine, heroin, methamphetamine, ecstasy and hallucinogens (Pisetsky *et al.*, 2008; Fouladi *et al.*, 2015).

1.2.5. *Neurobiology*

Although the etiology of AN is still not fully understood, it is assumed to be a complex and multifactorial disorder, with numerous risk factors including genetic, biological, endocrine and social factors. It remains unclear, however, how these factors interact over time to predispose, trigger and maintain the anorectic behavior. When acutely ill, subjects with AN commonly display neuroendocrine and metabolic disturbances accompanied by psychiatric comorbidity exaggerated by malnutrition (Kaye *et al.*, 2005). Determining whether such symptoms are a cause or a consequence of altered feeding behavior in AN is still actually a critical issue.

Eating disorders involve a complex genetic component, with an estimated heritability between 50 and 83 % (Bulik and Tozzi, 2004; Bulik *et al.*, 2007). In recent years, different genetic association human studies have focused their attention on genes regulating appetite, food intake and body weight, as well as on serotonin- and dopamine-related genes (Trace *et al.*, 2013). Moreover, alterations of neurotransmitters (DA, 5-HT, NE), neuropeptides (opioids, neuropeptide-Y, peptide YY), and peripheral peptides (vasopressin, oxytocin, ghrelin, leptin) regulating eating behavior and energy homeostasis have been found in acutely ill anorexic patients and in patients at follow-up

(Monteleone, 2011; Avena and Bocarsly, 2012). Such alterations may contribute to the persistence of AN symptoms and to relapse and chronicity.

In the past years, several studies have raised the hypothesis of an alteration in the levels of monoamines, such as 5-HT, DA and norepinephrine (NE) that could contribute to the pathophysiology of AN. The 5-HT neuronal system is involved in the modulation of motor activity, mood, obsessive behaviors, impulse control, reward and appetite. Different pre-clinical studies showed inhibitory 5-HT effects on feeding behavior suggesting 5-HT as one of the factor that could promote anorexic satiety (Tallett *et al.*, 2009). According to this, the anti-obesity drug sibutramine inhibits the reuptake of 5-HT (Heal *et al.*, 1998). Moreover, selective 5-HT reuptake inhibitors have been used in clinical practice for the treatment of AN, although with no significant positive effects (Holtkamp *et al.*, 2005). Serotonin disturbances could contribute towards the deregulation of appetite and compulsive and anxiety-related behaviors (Blundell, 1984; Lucki, 1998). Considerable physiologic and pharmacologic studies suggest that significant alterations of 5-HT activity occur during the ill state and after recovery from AN, and deregulation of the 5-HT system has been revealed in AN patients. Indeed, anorexic patients in the ill state show a significant reduction in the cerebrospinalfluid (CSF) concentration of the 5-HT metabolite 5-HIAA when compared to control (Kaye *et al.*, 1984, 1988). Interestingly, these reduced concentrations are reverted after recovery from AN and become even higher than normal concentrations, more or less about 50% higher than CSF 5-HIAA levels found in the ill state (Kaye *et al.*, 1991). More recently, neuroimaging studies have been conducted to characterize 5-HT_{1A} and 5-HT_{2A} receptors in ill and recovered AN patients compared to control people. Bailer and colleagues (2007) reported that ill AN individuals have increased 5-HT_{1A} binding in

the subgenual, mesial temporal, orbital frontal and raphae brain regions, and the prefrontal, lateral temporal and parietal regions. Conversely, recovered AN patients have been reported to have diminished and increased binding potential for the 5-HT_{2A} and the 5-HT_{1A} receptors, respectively (Bailer *et al.*, 2007; Galusca *et al.*, 2008).

The dopaminergic system is largely related to the serotonergic system and has been implicated in both the etiology and the course of the disorder. DA is involved in the regulation of a wide array of physiological functions such as cognition, sleep, motor activity, mood, learning, as well as in feeding and reward. Malfunctioning of the DA system has been implicated in different psychiatric diseases like schizophrenia, depressive disorder, attention deficit disorder and substance use disorders. DA dysfunction has been suggested to predispose to AN by acting on the rewarding drive associated with food intake, and also by the potential involvement in personality traits associated with AN. A clinical study showed that AN patients were more anhedonic than those suffering from other eating disorders (Davis and Woodside, 2002) indicating a disturbance in the reward circuits of AN patients. Reduced CSF homovanillic acid (HVA) concentration, the major metabolite of DA in humans, occurs in malnourished individuals with AN in the ill state which returns to normal levels following weight restoration (Kaye *et al.*, 1984). However, a more recent study undertaken by the same investigators reported significantly lower CSF HVA levels in individuals recovered from AN when compared to healthy controls (Kaye *et al.*, 1999). Individuals with AN have altered frequency of functional polymorphisms of DA D2 receptor genes that might affect receptor transcription and translation efficiency (Bergen *et al.*, 2005). Frank and collaborator (2005) used the positron emission tomography (PET) imaging to assess D2/D3 receptor function in recovered AN patients and reported a significantly

increased [^{11}C]raclopride receptor binding in the antero-ventral striatum compared to control participants. This study thus provided support to the possibility that AN is associated with decreased intrasynaptic DA concentration or increased D2/D3 receptor density and suggested this alteration as a possible contributor to the disturbed reward processing exhibited in AN (Frank *et al.*, 2005).

NE has been long studied in relation to its involvement in food consumption and its function appears to be altered in AN patients. Yet, only very few association studies have explored its components. Its action is very complex as NE is known to either increase or decrease eating and the outcome depends on the target site in the CNS. In the PVN of the hypothalamus, NE infusions produce hyperphagia, while infusions in the perifornical hypothalamic produce hypophagia (Leibowitz *et al.*, 1984). Kaye *et al.* (1984) found normal CSF NE levels in underweight and weight-restored AN patients, but lower levels in long-term weight recovered patients (Kaye *et al.*, 1984). An association has been found for a polymorphism in the region located in the 5′ promoter of the NE transporter protein, SL6A2 (NETpPR) and AN (Urwin *et al.*, 2002); however, this finding were not replicated in a following analysis (Hu *et al.*, 2007).

Different pre-clinical findings showed that all the three major classes of opioid receptors are involved in the regulation of the hedonic aspects of food intake. This involvement is thought to be driven by endogenous opioids mainly acting on the mu-opioid receptor, but also on the delta-opioid receptor (Bodnar, 2015). Interestingly, rewarding mechanism seem to be strongly affected and activated by rigorous exercise in AN patients (Bergh and Södersten, 1996). An alteration of the opioid signalling connected with feeding-related reward mechanisms has been proposed to play a key role in AN. It has been hypothesized that a dysfunction in systems regulating opioid

functions predisposes patients to develop an addiction to exercise and to food restriction driven by dieting. In this condition, starvation and exercise are means to compensate for diminished reward related to consumption. It is interesting to note that preliminary clinical trials with the opioid antagonist naltrexone have shown some positive effects on weight gain (de Zwaan and Mitchell, 1992; Marrazzi *et al.*, 1995). A genome-wide linkage analysis of AN patients identified a region of chromosome 1 (1p33-36) which contains the genes encoding for the delta opioid receptor (OPRD1), the serotonin receptor 1D (HTR1D) and hypocretin (orexin) receptor 1 (HCTR1), to be associated with a risk of developing AN (Grice *et al.*, 2002). Such association was stronger when the sample population was divided into restrictive and binge/purge AN subtypes. Moreover, different association studies found an association for single-nucleotide polymorphism**s** SNPs in the gene encoding for the delta opioid receptor with the development of AN (Bergen *et al.*, 2003). Delta opioid receptor signalling is believed to contribute to the activity of dopaminergic neurons in the VTA, affecting reward mechanisms in the mesolimbic circuit. So far, no association studies investigating the development of AN and the genes encoding mu and kappa opioid receptors have been performed.

Appetite and satiety regulating hormones have also been studied in the context of the neurobiology of AN. As mentioned above, leptin and ghrelin are two peripheral hormones involved inthe control of food intake whichtransmit important information on the energy status to the brain. A deranged leptin signalling system has been found in AN (Monteleone *et al.*, 2000; Holtkamp *et al.*, 2006). In particular, AN patients classically present low leptin plasma levels, a paradoxical finding attributable to low body fat levels. Ghrelin levels are typically elevated in AN patients, a likely

compensatory response to prolonged starvation, and central resistance to its effects has been postulated as explanation for the severe caloric restriction. Interestingly, ghrelin has also been involved in chronic stress-related changes in feeding and body weight homoeostasis (Lutter *et al.*, 2008), and leptin has been found to be decreased in mice subjected to chronic mild stress (Lu *et al.*, 2006). Emerging data support the notion that leptin and ghrelin can also modulate the brain reward system and, consequently, the hedonic aspects of food intake (Lutter and Nestler, 2009).

The reward system plays an important role as it integrates 'liking' (pleasure/palatability) and 'wanting' (appetite/incentive motivation) perceptions associated with food intake. Dysfunction in the reward circuits seems to be one mechanism accountable for the development of AN (Davis and Carter, 2009). From this perspective, AN could be considered as a dependency syndrome, leading to food intake-related dysphoria that would promote a vicious cycle of decreasing eating to avoid the dysphoric consequences of food consumption (Kaye, 2008). Different brain imaging studies have shown structural and functional alterations in areas that contribute to reward processing that may predispose subjects to the onset as well as to relapse (Frank *et al.*, 2013). For example, people with AN have decreased gray matter in several brain regions, including those involved in reward processing (Van den Eynde *et al.*, 2012; Titova *et al.*, 2013).

1.2.6. *Treatments for anorexia*

The development of novel pharmacological treatments for AN that may sustain and improve weight gain, reduce symptoms and treat associated psychopathologies, represents the ultimate goal of scientific research in the field. Several medications have been tested so far in different experimental trials. However, despite some promising

results, there are currently no medications approved by the FDA for the treatment of AN.

Among the different targets, serotonin has been identified as a possible protagonist in medical therapies, given its hypothesized role in the pathogenesis of AN and in the associated comorbid psychiatric disorders. Most recent clinical trials have focused on serotonin reuptake inhibitors (SRIs), with findings from earlier trials with tricyclic antidepressants yielding generally disappointing results (Crisp *et al.*, 1987). The earliest study pointed out no significant changes inclinical outcomes in 31 patients compared to the placebo group (Attia *et al.*, 1998). A different trial study conducted in 35 patients who had completed treatment found that one year later the number of patients still taking the medication presented marked clinical improvements in the anxiety component. The clinical effect observed in these weight-restored patients is consistent with the established difficulty of treating mood and anxiety symptoms in underweight patients (Kaye *et al.*, 2001) but contrasts with those obtained in a more recent study using fluoxetine in weight restored patients (Walsh *et al.*, 2006). This study evidenced no significant differences in clinical effects compared to placebo at 1-year follow-up. Other SSRI's, including citalopram and sertraline, have also been investigated with variable results, suggesting a possible role for their use in the management of depression and anxiety in AN although with no significant effect on weight gain (Santonastaso *et al.*, 2001; Fassino *et al.*, 2002).

Medications targeting the dopaminergic system have also been long investigated in AN. Treatment with typical antipsychotics has shown not significant effects on AN core symptoms or weight (Vandereycken, 1984). Atypical anti-psychotics, olanzapine in particular, have shown some promising effects on weight gain and other AN associated

symptoms (Barbarich *et al.*, 2004). Different published placebo-controlled studies using olanzapine found significant differences in the body mass index and in obsessive symptoms as well as a trend in body weight improvement (Powers *et al.*, 2002; Brambilla *et al.*, 2007).

Open label trials have investigated the effects of other atypical antipsychotics, including quetiapine and risperidone, and generally found modest improvements in body weight and in AN psychological traits (Powers *et al.*, 2012). Other medications have been tested in AN, which include antihistamines, zinc supplementation, human growth hormone, naltrexone and lithium. Zinchas been shown to support weight gain, and to improve mood and anxiety symptoms in AN patients (Katz *et al.*, 1987; Birmingham *et al.*, 1994). At the moment, the main stay of AN treatment is psychotherapy, and several different types have been investigated so far, including cognitive-behavioral therapy, interpersonal therapy, dialectical behavioral therapy, cognitive analytical therapy, family based therapy and specialist supported clinical management (Treasure *et al.*, 2010).

1.3. The endocannabinoid system

The endogenous cannabinoid system is an ubiquitous lipid signalling system that plays a crucial role in a plethora of physiological functions in all vertebrates (De Petrocellis *et al.*, 2004). The word "cannabinoid" generally refers to endogenous, exogenous, synthetic and phytoderivative compounds acting on the "endocannabinoid system" (ECs). The neuronal ECs includes: (a) endogenous ligands, like N-arachidonoylethanolamine (anandamide), 2-arachidonoylglycerol ether, 2-arachidonoyl glyceryl ether (noladinether), O-arachidonoyl ethanolamine (virodhamine) and N-arachidonoyl dopamine, (b) the cannabinoid receptors and (c) corresponding biosynthesis and degradation pathways, (d) the endocannabinoid transporter across membranes (Di Marzo, 2009). The ECs is densely distributed in different brain areas involved in the regulation of motor control, cognition, emotional responses, motivated behavior and homeostasis, but also in the periphery, being one of the most important modulators of the autonomic nervous system, the immune system and microcirculation. This complex system is integrated in helping to control fundamental processes at both the central and peripheral nervous system and its regulation and dysregulation might counteract disturbances of such functions.

1.3.1. *Cannabinoid receptors*

The effects of endocannabinoids are primarily mediated by two principal types of cannabinoid receptors, the cannabinoid type 1 receptor (CB1R) and the cannabinoid type 2 receptor (CB2R), which differ in tissue distribution and signalling mechanisms (Pertwee *et al.*, 2010). Both CB1Rs and CB2Rs belong to the big family of G-protein-

coupled receptors (GPCRs), which primarily couple to G proteins of the G_i and G_0 classes (Plasse, 1991). These receptors consist of seven transmembrane domains connected by three extracellular and three intracellular loops, an extracellular N-terminal tail and an intracellular C-terminal tail. Both cannabinoid receptor types are coupled to similar transduction systems which actions are relevant to the role of cannabinoidsas modulators of neurotransmitter release (Schlicker and Kathmann, 2001) and short-term synaptic plasticity (Wilson and Nicoll, 2001). Their activation was initially reported to cause inhibition of the adenylate cyclase (cAMP) formation through its coupling to G_i proteins (Devane *et al.*, 1988; Howlett *et al.*, 1990), resulting in a decrease of the protein kinase A-dependent phosphorylation processes. However, additional studies evidenced that cannabinoid receptors were also coupled to certain voltage-dependent channels, resulting in the inhibition of Ca^{2+} influx through N- (Mackie and Hille, 1992), P/Q- (Twitchell *et al.*, 1997) and L-type (Gebremedhin *et al.*, 1999) calcium channels, as well as in the activation of inwardly rectifying potassium conductance and A currents (Mackie *et al.*, 1995; Childers and Deadwyler, 1996). Further research also described the coupling of CB1Rs and CB2Rs to the mitogen-activated protein kinase cascade, the phosphatidylinositol3-kinase, the focal adhesion kinase, the ceramide signalling and the nitric oxide production (Derkinderen *et al.*, 1996; Bouaboula *et al.*, 1997; Howlett *et al.*, 2002). Finally, recent studies revealed that under certain conditions, the CB1R can also stimulate the formation of cAMP by coupling to the Gs protein (Felder *et al.*, 1998).

The CB1R is the most abundant and widely distributed GPCRs in the CNS, particularly expressed in brain regions responsible for movement (basal ganglia, cerebellum), memory processing (hippocampus, cerebral cortex) and pain modulation (certain parts

of the spinal cord, periaqueductal grey) as well as in the hypothalamus and the limbic system (De Petrocellis *et al.*, 2004). Besides the brain, the CB1R is also widely expressed in the peripheral nervous system and in almost all mammal peripheral organs and tissues including endocrine glands, salivary glands, leukocytes, heart, liver, urinary and gastrointestinal tracts (Borrelli *et al.*, 2015). Most CB1Rs are expressed in presynaptic terminals of both glutamatergic and gamma aminobutyricacid (GABA)-ergic neurons: by inhibiting these neurotransmitters release, CB1Rs activation protects the CNS from the possible over-activation or over-inhibition (Elphick *et al.*, 2001). One of the most important property of the CB1Rsis theirpreservation throughout evolution, e.g. human, rat and mouse CB1Rs have 97-99% amino acid sequence identity. The preservation of this ancient signalling system invertebrates and several invertebrates reflects the crucial functions played by the endocannabinoids in cell and system physiology (Howlett *et al.*, 1990; Herkenham *et al.*, 1991).

The CB2Rs, initially proposed to be mainly expressed in the periphery (Munro *et al.*, 1993; Galiegue *et al.*, 1995), are present principally in immune cells such as monocytes, macrophages, B- and T-cells, and for this reason have been long considered immune peripheral receptors involved in immunological functions. More recently, the CB2R has been described also in different peripheral organs and cells including the spleen, tonsils, thymus gland, mast cells and keratinocytes (Iannotti *et al.*, 2013), and in the gastrointestinal system (Izzo, 2004). In contrast to the CB1R, the expression of the CB2R in the brain is very low, restricted to specific neuronal cells and becomes abundant in activated microglia and astrocytes (Mackie, 2005; Atwood *et al.*, 2010).

Although CB1Rs and CB2Rs are now well known and characterized, increasing evidence support the existence of additional cannabinoid receptor subtypes in both the

brain and periphery (De Petrocellis and Di Marzo, 2010). For example, two G-protein-coupled receptors, GPR55 and GPR119, have been proposed as novel potential cannabinoid receptors (Baker *et al.*, 2006), but these receptors have not been completely cloned and characterized yet (Fride *et al.*, 2003; Ryberg *et al.*, 2007). There is also clear evidence that the transient receptor potential cation channel vanilloid type 1 (TrpV1) is modulated by the endocannabinoids, in particular by AEA (Chávez *et al.*, 2010; De Petrocellis *et al.*, 2010).

1.3.2. *Endogenous ligands*

The identification of the CBRs immediately put forward the hypothesis of the existence of their endogenous ligands, which were later defined "endocannabinoids" (Di Marzo *et al.*, 1994). Endocannabinoids are lipophilic neuromodulators that in contrast to other brain chemical signals are not stored in vesicles, but produced "on demand" (only when necessary) from their membrane cells lipid precursors in a receptor-dependent manner and then released from neurons in response to several physiological and pathological stimuli (Piomelli, 2003). After release, they act as retrograde messengers in GABAergic and glutamatergic synapses, as well as modulators of postsynaptic transmission, interacting with other neurotransmitters. The first endogenous ligand of CB1Rs and CB2Rs identified in 1992 was the N-arachidonoyl-ethanolamine (AEA), later called anandamide (Devane *et al.*, 1992). Three years later, a second agonist was isolated from the canine gut and turned out to be a common intermediate in phospholipid and triglyceride metabolism, i.e. 2-arachidonoyl-glycerol (2-AG) (Mechoulam *et al.*, 1995; Sugiura *et al.*, 1995). This class of molecules includes other endogenous ligands such as 2-arachidonoyl-glyceryl ether or noladin ether (2-AGE), O-arachidonoyl-ethanolamine

(virodhamine), N-arachidonoyl-dopamine (NADA) and oleamide (OA). Moreover, two related compounds, i.e. N-oleoylethanolamine (OEA) and N-palmitoylethanolamine (PEA), are part of this "extended" ECs. Although these two latter molecules lack strong affinity for either CB1Rs or CB2Rs, they are synthesized by the same class of enzymes of AEA (Ahn *et al.*, 2008).

1.3.3. *Metabolism*

Endocannabinoids are enzymatically metabolized through multiple pathways by different enzymatic systems. The principal mechanism for the synthesis of AEA involves the cleavage and hydrolysis of a minor phospholipid precursor, the N-acyl phosphatidylethanolamine (NAPE). This precursor can be synthesized by the enzyme N-acyltransferase (NAT), which catalyzes the transfer and formation of AA from phosphatidylcholine to the phosphatidylethanolamine. This enzyme activity is dependent on the presence of high concentrations of Ca^{2+} and is regulated by cAMP, which enhances its activity by the phosphorylation mediated through the cAMP-dependent activity of protein kinase A (Cadas *et al.*, 1996; Piomelli, 2003). Different pathways have been described for the conversion of NAPE into N-acylethanolamines (NAEs), like AEA. The principal pathway is carried out by a phosphodiesterase of the phospholipase D (PLD) family, N-acyl phosphatidylethanolamine-specific phospholipase D (NAPE-PLD), that converts NAPEs into phosphatidic acid (PA) and NAE (Okamoto *et al.*, 2007). Moreover, NAPE can also be deacylated to lyso NAPE by phospholipase A2 family members (Sun *et al.*, 2004) and by alpha-beta hydrolase domain containing protein 4 (Abhd4; Simon and Cravatt, 2006) resulting in the formation of glycerophospho-N-acylethanolamine (GP-NAE), a substrate for

glycerophosphodiester phosphodiesterase 1 (GDE1) which catalyzes its hydrolysis to glycerol-3-phosphate and NAE (Simon and Cravatt, 2008). An additional multistep pathway that generates NAE from NAPE involves the phospholipase C (PLC) and mediates the conversion of NAPE in diacylglycerol (DAG) and phospho-NAE, subsequently dephosphorylated by different phosphatases (Liu *et al.*, 2006). Another synthetic pathway for AEA formation that does not involve NAPE as a precursor has been identified. In this pathway, the NAE hydrolyzing enzyme, fatty acid amide hydrolase (FAAH), acts as NAE synthase to directly couple AA and ethanolamine to form AEA (Arreaza *et al.*, 1997). This mechanism is activated in different conditions, e.g. hepatic damage, that are characterized by elevated concentrations of ethanolamine, necessary to drive the activity of the enzyme (Mukhopadhyay *et al.*, 2011).

The mechanism for the synthesis of 2-AG involves the hydrolysis of 2-arachidonoyl-containing DAG by diacylglycerol lipase (DAGL). Two isotypes of DAGL are expressed in mammals and known as DAGLα and DAGLβ. Both enzymes share a four loops transmembrane domain, coupled with a catalytic domain and a regulatory loop (Bisogno *et al.*, 2003). DAGLα has an additional, large C-terminal tail. Both isotypes are expressed in the brain; however, DAGLα is present in high density at perisynaptic regions of dendrites in many brain regions (Uchigashima *et al.*, 2007), which is consistent with a prominent role for this isoform in ECs-mediated regulation of synaptic plasticity. The DAG substrate for DAGL can be generated by the PLC family of enzymes, particularly by PLCβ and PLCγ isoforms. These enzymes act on phosphatidylinositol 4,5-bisphosphate (PIP2) to produce DAG and inositol triphosphate. AEA and 2-AG are rapidly deactivated by a two-step process that includes: (1) the re-uptake into the cells and (2) the subsequent hydrolysis by a specific enzymatic system.

The cellular re-uptake from the extracellular to the intra-cellular space is due to a membrane endocannabinoid transporter that is likely to take up both AEA and 2-AG. Once into the cell, AEA degradation is mediated by different hydrolyzing enzymes: a FAAH, a lysosome-localized fatty acyl amide hydrolase with an acid optimum (NAAA) (Tsuboi *et al.*, 2005) and a recently identified FAAH-2 localized in lipid droplets (Kaczocha *et al.*, 2010). Inactivation of 2-AG is mainly induced by the enzyme monoacylglycerol lipase (MAGL). Both FAAH and MAGL are widely distributed within the CNS (Dinh *et al.*, 2002).

1.3.4. *Physiological and pharmacological effects*

The presence of ECS in vertebrates, mammals, and humans implies a role in several physiological processes, including appetite, cancer, cardiovascular diseases, fertility, immunefunctions, memory, neuroprotection, and pain modulation (Ligresti *et al.*, 2009; Maccarrone *et al.*, 2010).

Analgesia

The analgesic effects of cannabinoids are the most studied and described properties of these compounds. It is believed that endocannabinoids take part in a natural analgesia system in the body, mediated by the CB1Rs in the brainstem (Walker *et al.*, 1999). The anatomical distribution of CB1Rs coincides with brain areas involved in the transmission and modulation of nociceptive signals from the periphery to the brain (Hohmann, 2002). It has been shown an increased endocannabinoids tone in the brain stem after different painful stimuli, and administration of CB1R antagonists produces hyperalgesia, while administration of CB1R agonists produces analgesia (Welch and

Stevens, 1992). In addition, the ECs participates in the perception of the spinal processing of pain and peripheral nerve endings. The main mechanism involved in the analgesic control seems to be the pulse modulation descendant inhibitors from the trunk to the spinal cord, as a result of a presynaptic blocking of the release of GABA from the neurons located in the brain stem areas involved in nociception (RVM-rostral ventromedial medulla; PAG-midbrain periaqueductal gray).

Motor activity

Endogenous, natural or synthetic cannabinoids produce an inhibition of motor activity in a dose-dependent "triphasic" effect (Crawley *et al.*, 1993; Romero *et al.*, 1995). In particular, THC is able to reduce the spontaneous motor activity when administered at very low doses, at intermediate doses it produces an increase while at higher doses it induces catalepsy in mice. The administration of the CB1R antagonist SR141716 reverts these effects producing hyperlocomotion. The high density of CB1Rs in brain regions strongly involved in the motor control like the basal ganglia and cerebellum confirms their involvement in psychomotor modulation. Furthermore, patients suffering from different neurological disorders and related animal models show altered CB1R expression and function in the above mentioned areas (Consroe *et al.*, 1998). These motor effects appear to be attributable to the inhibitory action of cannabinoids on the release of GABA and glutamate levels in the presynaptic terminals of the basal ganglia and cerebellum (Romero *et al.*, 2002).

Learning and memory

The hippocampus, crucially involved in learning and memory processes regulation, constitutes one of the brain areas with the highest density of CB1Rs. Numerous experimental evidence have confirmed that the use of cannabis induces marked changes in the short-term memory similar to those seen in Alzheimer's disease characterized by hippocampal degeneration. It is thought that in this area endocannabinoids inhibit the release of several neurotransmitters such as GABA, glutamate and acetylcholine (Davis *et al.*, 2002), and that the reduction of glutamate release at levels lower than those required for activation of N-methyl-D-aspartate (NMDA) receptors is responsible for the short-term memory reduction observed in cannabis intoxications, being these receptors strongly involved in memory formation. Endocannabinoids were shown to promote the removal of unpleasant memories due to the inhibitory effect at the level of the Amy (Marsicano *et al.*, 2002).

Antiemetic

The ECs has an important role in the control of nausea and vomiting, processes principally regulated by two different brain areas: the bulb and the area postrema. The vomiting center can be stimulated by impulses coming from the digestive tract. Different studies have demonstrated that vomiting inhibition produced by exposure to cannabinoids is due to the activation of CB1Rs, effects that can be observed both in the areas involved in vomiting and nausea as well as in the peripheral nervous system following activation of CB1Rs in the small intestine (Van Sickle *et al.*, 2001; Coutts and Izzo, 2004). Two different clinical trials using cannabinoids as antiemetic

confirmed that it may be useful in the treatment of nausea and vomiting following chemotherapy (Tramèr *et al.*, 2001).

Neuroprotection

The ECs may also play an important neuroprotective role in conditions of acute neuronal injury, head trauma, epilepsy and chronic neurodegenerative diseases such as amyotrophic lateral sclerosis, Alzheimer's, Parkinson's and Huntington's diseases (Glass, 2001; Pryce *et al.*, 2003). Furthermore, endocannabinoids act by reducing astrocyte and oligodendrocyte death rate, a mechanism that could explain at least in part the reported neuroprotective effects of these compounds (Molina-Holgado *et al.*, 2002). Yet, mechanisms underlying the protective properties of cannabinoids can be different. Acute brain damage causes the release of excitatory aminoacids such as glutamate, that through interaction with NMDA receptors produces the massive entry of Ca^{2+} into the cell inducing neuronal death for cytotoxicity. Endocannabinoids are produced in response to this massive increase of Ca^{2+} concentration, and by activating the CB1Rs they lead to the reduction of glutamate release and, therefore, to a reduced neuronal damage. It has been demonstrated that different cell damage conditions lead to increased levels of endocannabinoids in organs and tissues as well as in the brain (Hansen *et al.*, 2000). Accordingly, classical cannabinoids possess antioxidant properties independent on the activation of CB1Rs (Marsicano *et al.*, 2002).

1.3.5. *The endocannabinoid system and the regulation of eating behavior*

Although the appetite stimulating properties of cannabis are known for centuries, it was only recently that the orexigenic properties of its main psychoactive compound, Δ^9-THC, were clearly demonstrated (Williams *et al.*, 1998). During the last years, a huge body of evidence supported the implication of the ECs in the regulation of eating behavior and energy balance through both central and peripheral mechanisms (Coutts and Izzo, 2004; Di Marzo and Matias, 2005). In the brain, the ECs appears to control food intake through activation of CB1Rs mainly at two functional levels, i.e. the hypothalamus and the mesolimbic system, where CB1Rs are widely expressed (Matias and Di Marzo, 2007) and strategically positioned to influence the regulatory circuits controlling energy homeostasis (Cota *et al.*, 2003).

The endocannabinoid system and the homeostatic regulation of eating behavior

The ECs in the hypothalamus modulates feeding integrating satiety and orexigenic signals (Higgs *et al.*, 2003). CB1Rs are widely expressed in the hypothalamus and in other important brain areas involved in the control of eating behavior (Herkenham *et al.*, 1990), and their activation produces profound effects on the expression and release of orexigenic and anorexic signals (Childers and Breivogel, 1998). Several studies have analyzed the relationship and interaction between the ECs and different endogenous messengers involved in the regulation of food intake. Cota and colleagues (2003), for example, have demonstrated that CB1R mRNA is co-expressed with mRNA encoding for neuropeptides, such as CRH, cocaine- and amphetamine regulated transcript (CART), melanin concentrating hormone (MCH), and orexin/hypocretin, all involved in the modulation of food intake in the hypothalamus (Cota *et al.*, 2003).

It has been demonstrated that AEA and the synthetic CB1R agonist CP-55,940 significantly increase NPY release, an effect that is blocked by the CB1R antagonist AM251 which by itself inhibits the release of NPY (Anand *et al.*, 1951). Moreover, Poncelet and collaborators (2003) found that rimonabant prevent NPY hyperphagia, and that the ability of NPY to stimulate feeding is abolished in CB1R KO mice, although rimonabant is as effective in reducing food intake in NPY-KO as in wild-type (WT) mice (Poncelet *et al.*, 2003).

Mice deficient in FAAH have reduced levels of CART-immunoreactive nerve fibers and terminals in several hypothalamic nuclei, that return to control (WT) levels after rimonabant administration (Osei-Hyiaman *et al.*, 2005). Moreover, in fasted rats, rimonabant significantly reduces the increase in ghrelin levels, while plasma levels of ghrelin in rimonabant-treated fed rats were 35 % lower than in vehicle-treated rats (Cani *et al.*, 2004).

Accordingly, through the use of the patch-clamp recording technique, Kola and colleagues (2008) showed that ghrelin is able to inhibit the excitatory synaptic inputs in the PVN and that this effect can be abolished by AM251 or by the inhibition of endocannabinoids synthesis (Kola *et al.*, 2008). These authors also showed that both THC and ghrelin increases the activity of the AMP-activated protein kinase (AMPK), a key enzyme in the regulation of appetite and metabolism, in the hypothalamus of WT mice compared to CB1R-KO mice. In addition, pre-treatment with rimonabant was able to abolish the stimulatory effect of ghrelin on AMPK activity and to reduce the hyperphagic effect (Tucci *et al.*, 2004).

Feeding stimulated by intra-hypothalamic injection of ghrelin is blocked by pre-treatment with rimonabant, suggesting that the expression of ghrelin-induced

hyperphagia is dependent on an intact ECs (Verty *et al.*, 2003). In sub-anorectic rats, ICV doses of melanocortin receptor 4 agonist or oxytocin and rimonabant work synergistically to suppress feeding (Verty *et al.*, 2004 a, b). The anorexigenic effects of rimonabant and naloxone are synergistic (Kirkham *et al.*, 2001), and blockade of CB1Rs attenuated opioid-induced increase of food intake (Verty *et al.*, 2003).

Interestingly, it has been consistently reported that pharmacological manipulation of this important neuromodulator system through systemic and local administrations of exogenous (i.e. THC, CP55940, WIN 55212-2) and endogenous cannabinoids (i.e. AEA, 2-AG) increases food consumption both in humans and laboratory animals (Hollister, 1971; Williams *et al.*, 1998; Koch, 2001; Cota *et al.*, 2003). In agreement, different studies demonstrated that Δ^9-THC and endocannabinoids as well as FAAH inhibitors increase the effort to obtain food and the consumption of food when locally infused into several areas of the forebrain, including the hypothalamic nuclei (Anderson-Baker*et al.*, 1979; Jamshidi and Taylor, 2001) and the shell of the NAcc of satiated rats. Several evidence have confirmed that these hyperphagic effects are mediated by the activation of CB1Rs since they are blocked by specific CB1R antagonists such as rimonabant and AM251 (Williams and Kirkham, 1999; Kirkham *et al.*, 2002; Soria-Gómez *et al.*, 2007).

The endocannabinoid system and the hedonic regulation of eating behavior

Several evidence support the hypothesis that the ECs represents a key control over hedonic aspects of eating behavior. CB1Rs are expressed in several areas of the mesolimbic system, where they appear to play an important role in brain motivation and reward processes (Gardner, 2005). Acting as retrograde messengers, endocannabinoids

are able to modulate the excitatory and inhibitory signals that control the dopaminergic neurons (Wilson *et al.*, 2001) through the activation of CB1Rs located on the presynaptic axon terminals of GABAergic and glutamatergic neurons in the VTA and NAcc (Melis *et al.*, 2004). The final effect on dopaminergic VTA neurons will depend on the level of activation of these signals (Lupica and Riegel, 2005). Different studies, conducted both in humans and animals, have shown that CB1R agonists increase food intake, and the consumption of highly palatable food in particular (Foltin *et al.*, 1986). Low doses of THC increase the intake of highly palatable food in rats after both central and peripheral administration (Koch and Matthews, 2001). Another evidence of the important role of the endocannabinoids in the hedonic component of food intake is highlighted by the ability of CB1R agonists like CP 55,940 to facilitate the intake of palatable food when administered in the hindbrain (Miller *et al.*, 2004). Moreover, DA inhibits endocannabinoid biosynthesis in the limbic forebrain (Patel *et al.*, 2003), administration of 2-AG into the shell of the NAcc produces hyperphagia (Kirkham *et al.*, 2002) and AEA, through activation of the mesolimbic dopaminergic system, participates in the signalling of brain reward processes (Solinas *et al.*, 2006). Finally, direct parabrachial infusions of 2-AG and the FAAH inhibitor AA-5HT in rat stimulate the consumption of highly palatable food without affecting the intake of standard food (Dipatrizio and Simansky, 2008). In contrast, CB1R antagonists diminish food intake, preferentially by reducing the consumption of highly palatable food. For example, rimonabant reduces more effectively the consumption of highly palatable food compared to standard food the preference for sucrose and the reinforcing properties of chocolate (Arnone *et al.*, 1997; Simiand *et al.*, 1998). South and collaborators (2007) have demonstrated that administration of AM251 in mice given a choice between one

diet rich in fat and one diet poor in fat, selectively reduces the consumption of food rich in fat (South *et al.*, 2007). Using a supercharger model characterized by a continuous access to standard laboratory food and limited access to a highly palatable food, it was shown that rimonabant (injected for 7 days) and AM251 (injected for 15 days) specifically reduce the consumption of highly palatable food (Mathes *et al.*, 2008). In addition, microinjection of AEA in the medial region of the NAcc shell increases two-fold the number of positive reactions of "liking" aroused from taking oral sucrose without altering negative ones produced by consumption of bitter quinine (Mahler *et al.*, 2007). It was also demonstrated that CB1R-KO mice consume less sucrose than WT mice (Wiley *et al.*, 2005). All these data suggest that cannabinoid agonists act to facilitate intake of highly palatable foods although, when given a choice between diets, effects are not limited to palatable food but also extend to normal chow (Foltin *et al.*, 2007; Scopinho *et al.*, 2011).

1.3.6. *The endocannabinoid system and anorexia nervosa*

The ECs has a crucial role in the modulation of energy balance playing important regulatory actions in the control of food consumption and energy expenditure through central and peripheral mechanisms (Di Marzo and Matias, 2005; de Kloet and Woods, 2009; André and Gonthier, 2010; Maccarrone *et al.*, 2010). Growing evidence support a role of the ECs also in the mesolimbic regulation of the hedonic and rewarding component of feeding behavior (Di Marzo *et al.*, 2009). In recent years, both clinical and preclinical evidence have led to the hypothesis of a correlation between a dysfunction of the ECs and AN.

Human studies

A possible involvement of the ECs in the pathogenesis of AN has been recently supported by a study of Monteleone and collaborators (2005) which reported increased plasma levels of AEA in underweight women with restricting AN and binge eating disorder, but not bulimia nervosa (Monteleone *et al.*, 2005). On the contrary, no significant changes in the circulating concentration of the other endocannabinoid 2-AG were detected among groups of patients examined. Moreover, in agreement with previous studies (Monteleone *et al.*, 2002), authors showed significantly decreased levels of circulating leptin in AN patients and proposed that this hypoleptinemia may be an important factor underlying the excessive physical activity, one of the hallmarks of AN. Furthermore, AEA levels were significantly and inversely correlated with plasma leptin concentrations in both healthy controls and anorexic women, in line with the previously reported negative modulation of endocannabinoids production by leptin (Di Marzo *et al.*, 2001), suggesting that increased levels of AEA are secondary to a leptin deficiency. Finally, given the role of the endocannabinoids in regulating the brain reward system, authors also suggested that increased AEA levels could represent an adaptive response aimed to mediate the rewarding aspects of the aberrant eating behavior of AN and BED patients (Monteleone *et al.*, 2005). However, a limitation of this work was to have not verified whether peripheral levels could reflect CNS levels.

Using the CB1R-specific radioligand [^{18}F]MK-9470, Gerard and colleagues (2011) investigated by PET imaging the *in vivo* CB1R availability in female AN patients in comparison with age matched healthy volunteers. They found a general CB1R density increase in cortical and subcortical brain areas of anorexic patients, which has been explained as a consequence of a long-term compensatory mechanism up-regulated in

response to underactive endocannabinoid signalling under the anorectic conditions. The authors also found an increase of CB1Rs in the insular cortex of both AN and BN patients, a key area involved in the integration of interoceptive and gustatory information, reward and emotional processing (Gerard *et al.*, 2011).

Few years before, significantly higher levels of CB1R mRNA in the blood were found in AN patients compared to healthy controls, further supporting the notion of impaired endocannabinoid signalling in EDs (Frieling *et al.*, 2009). No differences in CB2R mRNA expression have been found between groups examined, in line with previous findings (Monteleone *et al.*, 2005). The authors assumed that the observed upregulation of CB1 mRNA may compensate for otherwise impaired endocannabinoid signalling that could be due to reduced receptor sensitivity. However, reduced peripheral CB1R mRNA levels were found in a mixed sample of AN and BN patients with self-injurious behavior compared to patients without this behavior and healthy controls (Schroeder *et al.*, 2012). Genetic association studies between the ECs and AN demonstrated significant associations between polymorphisms of the gene encoding the CB1R and the gene encoding FAAH in AN and BN (Monteleone *et al.*, 2009). Functional polymorphisms in the gene encoding the orphan G protein-coupled receptor GPR55 (recently reported to have an affinity for endocannabinoids) may represent a risk factor for AN (Ishiguro *et al.*, 2011), although other studied have shown no association with AN (Müller *et al.*, 2008).

Animal studies

Evidence from different preclinical studies also support the hypothesis of a dysregulation of the ECs in animal models of AN. For example, Casteels and

collaborators (2013) investigated for the first time the *in vivo* changes in cerebral CB1R binding in the activity-based anorexia (ABA) model of AN using a small animal PET technique. Similar to human studies, ABA rats showed increased CB1R availability in all cortical and subcortical areas that normalized to baseline after weight gain (Casteels *et al.*, 2013). Lewis and Brett (2010) attempted to reverse some of the typical traits of the ABA paradigm by the manipulation of the ECs with both the natural CB1R/CB2R agonist THC and the endocannabinoid uptake inhibitor (S)-N-oleoyl-(10-hydroxybenzyl)-20-ethanolamine (OMDM-2). They found that THC (0.5 mg/kg) administration was able to increase food intake, while not affecting body weight and excessive running wheel activity. Besides restoration of food consumption, THC-treated AN mice also displayed high mortality rates, possibly as a consequence of the hypothermic effect of THC and/or of a compromised thermoregulatory system in food-restricted animals. A limitation of this study was the use of only one dose of THC which leaves unexplored putative biphasic or triphasic responses that could help in the identification of therapeutic doses. Furthermore, the authors tried to increase the activity of the ECs also by the administration of the AEA re-uptake inhibitor OMDM-2 that has been previously shown to increase brain levels of both AEA and 2-AG (de Lago *et al.*, 2005). Notably, they found that OMDM-2 was not able to reverse weight loss despite the increase in food intake at 1 and 3 mg/kg doses. Additionally, no significant changes in RWA or survival were found in OMDM-2-treated compared to vehicle-treated mice (Lewis and Brett, 2010). On the contrary, Verty and collaborators (2011) showed for the first time the effectiveness of THC administration in attenuating the weight loss associated with ABA development in female rats. More specifically, they found that subchronic THC treatment, at the dose of 2.0 mg/kg/day, transiently stimulated chow

intake and significantly reduced weight loss with a moderate effect on running wheel activity. The ability of THC to delay AN progression was significantly amplified when high-fat palatable food was introduced in conjunction with THC treatment. The combination of HFD and THC treatment led to a profound positive effect on energy balance with reductions in energy expenditure, lipolysis and thermogenesis (UCP1) (Verty *et al.*, 2011). The discrepancies between the above studies could reflect the fact that administration of cannabinoids produces contrasting results in rats and mice as also seen in other experimental animal models and procedures (Berrendero and Maldonado, 2002; Valjent *et al.*, 2002; Patel and Hillard, 2006).

1.4. Animal models of anorexia

Animal models of AN represent a very important tool to understand the pathophysiological factors involved in the onset and development of this psychiatric pathology. Even though AN models can only provide some of the principal pathological symptoms, none of these models could be considered a true replica of the human disease. For example, psychological aspects, such as obsessing over body weight and shape, cannot be assessed in animals. Yet, these models proved to be very useful for studying other and more identifiable behavioral traits (Casper *et al.*, 2008). AN models include the following aspects: adolescent onset, predominance in females, decreased food intake and body weight, increased physical activity and changes in the neuroendocrine function which strongly mimic the human condition.

One of the most used and validated rodent paradigm of AN is the ABA model, which reproduces key aspects of the human pathology, in particular body weight loss, physical hyperactivity and reduced food intake. Routtenberg and Kuznesof in 1967 discovered time that the simultaneous combination of food restriction and physical exercise is able to produce starvation and death in rodents. In this experimental paradigm, animals (rats or mice) have free access to a running wheel in combination with restricted feeding schedules (1-2 h/day access to food). When presented simultaneously, these two factors determine a progressive increase in running wheel activity associated to a dramatic reduction of body weight and food consumption (Routtenberg and Kuznesof, 1967). This is an unusual consequence, since the increase in energy expenditure occurs at the same moment when food consumption is limited. These aspects are clinically relevant, since some anorexic patients often use excessive exercise with the aim to control body

weight and maintain it as lower as possible. In addition, ABA anorexic like animals also present other typical clinical traits reported in anorexic patients, such as decreased leptin and increased ghrelin concentrations in plasma, as well as an alteration and cessation of the estrous cycle in females (Adan *et al.*, 2011). Stress-based animal models of AN have also been used because stress can induce weight loss and contribute toward loss of appetite. These models include different physical stressors, such as tail pinching, cold swimming with or without food deprivation and direct brain stimulation (Shimizu *et al.*, 1989). However, these models have several disadvantages, as following acute stress administration animals can be physically harmed. Dietary restriction can also be used as AN model even if, unlike the situation in patients with this disease, food restriction is involuntary. However, many changes in neuroendocrine functions found in AN patients can be mimicked by this model, thus validating its use (Avraham *et al.*, 2001).

There are also some genetic models of AN, the most commonly used being the anx/anx mouse. These mice exhibit spontaneous anorexia and are characterized by decreased food intake behavior, reduced stomach size and body weight, inability to regulate food intake, reduced serum leptin levels and hypothalamic degeneration (Johansen *et al.*, 2003). Other genetic models of AN include gene KO models for the evaluation of the contribution of specific genes to the phenotype of AN, such as BDNF, tyrosine hydroxylase, d-opioid receptor, 5-HT receptors, or M3 muscarinic receptors (Kim, 2012).

1.5. Aim of the study

Based on the increasing knowledge on the functional relationship between altered ECs signalling and development of AN, the aim of this thesis was to deeply investigate the possible involvement of the ECs in the pathogenesis of this dramatic eating disorder. Using the widely validated ABA model of AN, we initially aimed to reproduce in young adolescence female rats the typical pathological phenotype that best represents the disease: body weight loss, physical hyperactivity, endocrine dysregulation. During the first phase of the study, identified as the *biochemical study*, we investigated in ABA rats whether the ECs signalling becomes dysregulated in specific regions of the brain involved in the modulation of homeostatic and hedonic aspects of eating behavior, as well as mood and cognition (i.e. Frontal and Pre-Frontal Cortex, Nucleus Accumbens, Caudato Putamen, Amygdala, Hippocampus and Hypothalamus). For this purpose, ABA female rats displaying a manifested AN were compared to control animals and the following parameters were measured: (a) body weight, food intake, physical activity, leptin, ghrelin and corticosterone plasma levels; (b) central levels of the principal endocannabinoids precursor arachidonic acid (AA); (c) central levels of the most studied endocannabinoids AEA and 2-AG, as well as of the ethanolamide OEA and PEA; (d) the expression of the CB1R by means of [^{3}H]CP-55,940 quantitative autoradiographic assay. A possible dysregulation of the ECs signalling was also assessed after a period of restoration from AN in order to evaluate whether any alteration still persist after weight recovery.

Since no effective pharmacological interventions for the treatment of AN are currently available and frequent relapses are typical of the human disease, in the second phase of

the study, identified as the *pharmacological study*, we evaluated whether cannabinoid-based treatments were able to provide positive effects by acting on behavioral, neurochemical and/or hormonal altered parameters. Therefore, we tested the effect of: (a) the natural CB1R/CB2R agonist Δ^9-tetrahydrocannabinol (0.5 and 0.75 mg/kg/day, IP); (b) the synthetic CB1R agonist CP-55,940 (0.03 and 0.06 mg/kg/day, IP), and (c) the CB1R inverse agonist/antagonist rimonabant (0.15 and 0.3 mg/kg/day, IP). All pharmacological treatments were conducted during the *ABA relapse* phase, a 2nd cycle of AN designed to reproduce a relapse representation of the disease. In order to specifically create a control group of the two variables combined in the ABA model (restricted feeding and access to the running wheel), in this set of experiments we investigated the effect of the tested drugs not only in *ABA* and *Control* animals, but also in a group of animals undergoing the same restricted feeding schedule of *ABA* rats, but with no access to the running wheel (*Restricted*), and in a group of rats fed *ad libitum* with free access to the running wheel (*Exercise*). During this phase, plasma leptin, ghrelin and corticosterone concentration were measured in each experimental group both in a basal condition and after drug administration.

Chapter 2. Materials and methods

2.1. Animals and housing

Experimentally naïve Sprague-Dawley female rats (Envigo, Italy) weighing between 125 and 150 g (50 days old) at the beginning of the experiment were used. Animals were housed in a climate-controlled animal room and maintained on a reversed 12/12 h light/dark cycle (lights on at 12:00 a.m. and off at 12:00 p.m.) with 21 ± 2 °C temperature and 60 % humidity. Animals were fed with standard rat chow (3 % kcal from fat, 61 % kcal from carbohydrate, 16 % kcal from protein, 20 % moisture, containing 2.9 kcal·g^{-1}, Safe, France) throughout the entire duration of the study with water available *ad libitum*. All procedures and experiments were carried out in an animal facility according to Italian (D.L. 26/2014) and European Council directives (63/2010) and in compliance with the approved animal policies by the Ethical Committee for Animal Experiments (CESA, University of Cagliari) and the Italian Department of Health (286/2016). All possible efforts were made to minimize animal pain and discomfort and to reduce the number of experimental subjects.

2.2. Drugs

Δ^9-tetrahydrocannabinol (Δ^9-THC) (THC Pharm, Germany), CP-55,940 (Tocris Bioscience, Bristol, UK) and SR 171416A (rimonabant) (National Institute on Drug Abuse, NIH, Baltimore, MD, USA) were dissolved in a vehicle containing 2% Tween 80, 2% ethanol, saline and administered intraperitoneally (IP) in a volume of 1 mL·kg^{-1}.

2.3. Apparatus

Experimental chambers consisted of standard polycarbonate cages [48 (h) x 32 (w) x 47 (d) cm] or of cages equipped with running wheels (35 cm in diameter, 11 cm in width). Each running wheel was associated to a manually operated lock and connected to a digital magnetic revolution LCD counter which continuously monitored activity wheel revolutions (1 wheel revolution equals 1.1 meters) (Ugo Basile, Italy).

2.4. Experiment 1: biochemical study

As illustrated in figure 2, the ABA protocol designed for biochemical studies consisted of the following four phases: *Habituation* (3 days), *Baseline* (5 days), *ABA induction* (6 days), *Recovery* (7 days).

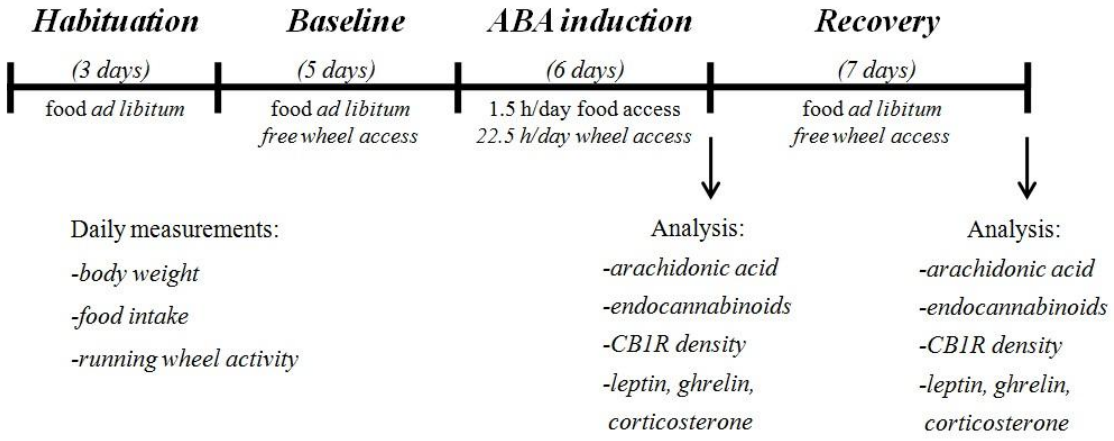


Figure. 2: Schematic representation of the experimental design.

After a 7-day period of acclimation to the housing conditions, animals were divided into two groups, homogeneously matched for initial body weight, and individually housed in standard home cages (sedentary rats) or in cages equipped with a running wheel (running rats). In order to allow animals to acclimatize to the new individual cages and

to avoid possible neophobia-induced reactions to the wheel, during the first 3 days of the *Habituation* phase, all running wheels remained locked. Body weight and food intake were monitored every day at the same time before the onset of the dark phase. Then, during the successive *Baseline* phase (5 days), all running wheels were unlocked thus giving running rats the possibility to access the running wheel in every moment of the day. Measures of body weight, food intake and running wheel activity (RWA) were detected daily in order to obtain a stable baseline. In both these initial phases chow and water were available *ad libitum* for all experimental animals.

2.4.1. *ABA induction*

Following the *Baseline* phase, experimental groups were created. The two groups of animals with or without access to the running wheel (sedentary and running rats), were divided in two experimental groups. Running rats became the *ABA* group (1.5 h/day access to food, free access to the wheel) and the sedentary rats became the *Control* group (24 h/day free access to food, no access to the wheel). During the 6-day period of the *ABA induction* phase (or until animals lost a maximum of 25 % of their initial baseline body weight), *ABA* animals were subjected to a temporal, but not quantitative, restricted feeding schedule consisting in a free access to a pre-weighed quantity of food for 1.5 h/day only starting at the onset of the dark phase and with running wheels locked to prevent running from competing with eating. At the end of this feeding period, food was removed and re-weighed in order to record the amount of food consumed in that period of time. For the remainder of the day (22.5 h), *ABA* animals had no access to food but free access to the running wheel. During this phase, *Control* animals were no subjected to a restricted schedule, i.e. continued to have free access to food. Body

weight, 24 h food intake (for *Control* animals only) and RWA (for *ABA* animals only) were monitored daily 30 min before the 1.5 h/day feeding period. On Day 6 of the *ABA induction* phase, part of the experimental animals were sacrificed before the onset of the dark phase, and the brains and plasma were rapidly collected and differently processed for AA and endocannabinoids analysis, CB1R density evaluation, and plasma measurements.

2.4.2. *Recovery phase*

At the end of the *ABA induction* phase, the remaining animals started the recovery phase (7 days). In particular, on Day 6 of the *ABA induction* phase, after the 1.5 h/day access regimen, *ABA* animals underwent a restoration period during which they were allowed *ad libitum* access to food. *Control* rats continued to have an *ad libitum* access to food. During this phase, running rats (*ABA*) had unlimited access to the wheel (Dixon *et al.*, 2003; Ratnovsky and Neuman, 2011). Recovery from ABA was assessed by monitoring daily body weight, food intake and RWA 30 min before the start of the dark cycle. As previously described for the *ABA induction* phase, on the last day of the recovery phase (Day 7), all experimental animals were sacrificed at the end of the light phase, i.e. before the onset of the dark phase, and brains and plasma were rapidly collected and differently processed for AA and endocannabinoids analysis, CB1R density evaluation, and for plasma measurements.

2.4.3. *Plasma measurements*

Plasma concentration of leptin, ghrelin and corticosterone were evaluated using commercially available ELISA kits. Leptin and ghrelin plasma levels were measured

using ELISA kits (respectively Cat. EZRL-83K and Cat. EZRGRA-90K) from EMD Millipore Corporation (St. Charles, Missouri, USA), while corticosterone plasma levels were determined using ELISA kit (Cat. No. ADI-900-097) from Enzo Life Sciences (Lausen, Switzerland). Kits were based on a standard sandwich enzyme-linked immune-sorbent assay technology for specific molecules quantifications with minimal detecting concentration of 0.08 ng/mL for leptin, 8 pg/mL for ghrelin and 27 pg/mL for corticosterone. For leptin and corticosterone, trunk blood was collected into K_3EDTA tubes, centrifuged at 3,000 x g for 15 minutes (4 ± 2 °C) and supernatant plasma was then stored at -20 °C for later analysis. For ghrelin, to avoid breakdown of this active molecule extremely unstable in plasma , trunk blood was collected into K_3EDTA tubes previously prepared by adding enough of the protease inhibitor AEBSF Hydrochloride (Te.Pro. Service, Cagliari, Italy, CAS 30827-99-7) to achieve a final concentration of 1 mg/mL. After collection, blood samples tubes were centrifuged at 3,000 x g for 15 minutes (4 ± 2 °C). Following centrifugation, supernatant plasma samples were transferred into separated tubes and ghrelin was acidified with diluted 0.05 N HCl. Small aliquots of plasma were then prepared and stored immediately at -20 °C for later analysis. Samples were subsequently processed according to kit protocol and instructions. Optical density absorbance of each sample was read with the appropriate filter (405 nm, 450 nm or 590 nm) in a microplate reader (Thermo Scientific Multiskan FC) after the final step of the kit procedure. Data obtained were analyzed using the Thermo Scientific SkanIt Software 3.0 for Multiskan FC.

2.4.4. *Arachidonic acid measurements*

After brains collection, selected brain areas were isolated according to the Paxinos and Watson atlas (1997) with a pre-cooled rat brain slicer using cold forceps and blades (Heffner *et al.*, 1980). Dissected brain regions were immediately disposed on an aluminum plate over dry-ice until completely frozen and then stored in a -80 °C freezer for later analysis. For tissue extraction, pre-cooled steel balls of 5 mm were added to pre-cooled tubes containing the tissue. A solution of 600 µL of MTBE and 50 µL of ACN/water (1:1, v/v) containing internal standards at a target concentration in the final volume (50 µL) of 1 ng/mL AEA-d_4, 125 ng/mL 2-AG-d_5, 3000 ng/mL AA-d_8, 2 ng/mL OEA-d_2; PEA-d_4, 12.5 ng/mL 1-AG-d_5, 2.5 ng/mL $PGF_{2\alpha}$-d_4, and 5 ng/mL for PGD2-d_4, PGE2-d_9, 5(S)-HETE-d_8, 12(S)-HETE-d_8, 20(S)-HETE-d_6 and TXB2-d_4, respectively, were added to each sample. Then, 400 µL of 0.1 M formic acid were added. Samples were homogenized with a Tissue Lyser (Qiagen, Hilden, Germany) for 60 s at 30 Hz, centrifuged for 15 min (4000 rpm, 4 °C) and then frozen for 10 min. The upper organic phase was recovered, evaporated under a gentle stream of nitrogen at 37 °C and reconstituted prior to analysis in 50 µL ACN/water (1:1, v/v). The aqueous phase was further analyzed for protein content determination made using the BCA method (bicinchoninic acid assay) and the measurements performed on a FLUOstar Galaxy (BMG Labtechnologies). The lipid extract was re-dissolved in 50 µl acetonitrile/water (1:1, v/v) and quantitative analysis was carried out by liquid chromatography-multiple reaction monitoring (LC-MRM). LC/MRM conditions were set as previously reported (Wenzel *et al.*, 2013) and AA levels were normalized to the corresponding protein content of the tissues and quantified by Analyst 1.6.2 software.

2.4.5. *Endocannabinoids measurements*

Brain regions were collected as previously described for brain areas isolation for AA measurements. The overall isolation procedure was carried out in a very short period of time in order to avoid *ex vivo* production/degradation of endocannabinoids. For extraction, tissues were later homogenized in 5 volume of chloroform/methanol/Tris-HCl 50 mM (2:1:1, v/v) containing 10 pmol of d^8-anandamide, d^4-palmitoylethanolamide, d^4-oleylethanolamide and d^5-2-AG as internal deuterated standards. Homogenates were centrifuged at 13000 g for 16 min (4 °C), and the aqueous phase plus debris were collected and extracted again twice with 1 volume of chloroform. The organic phases from the three extractions were pooled and the organic solvents evaporated in a rotating evaporator. Lyophilized extracts were resuspended in chloroform/methanol 99:1 by volume. The solutions were then purified by open-bed chromatography on silica. Fractions eluted with chloroform/methanol 9:1 by volume (containing AEA, 2-AG, OEA and PEA) were collected, the excess solvent was evaporated with a rotating evaporator, and aliquots were analyzed by isotope dilution liquid chromatography/atmospheric pressure chemical ionization/mass spectrometry (LC-APCI-MS) carried out under conditions allowing the separations of AEA, 2-AG, OEA and PEA. MS detection was carried out in the selected ion-monitoring mode using *m/z* values of 356 and 348 (molecular ion + 1 for deuterated and undeuterated AEA), 384.35 and 379.35 (molecular ion + 1 for deuterated and undeuterated 2-AG), 304 and 300 (molecular ion + 1 for deuterated and undeuterated PEA) and 330 and 326 (molecular ion + 1 for deuterated and undeuterated OEA). The amounts of endocannabinoids and related N-acylethanolamines were expressed as pmols/mg of tissue extracted (Bisogno *et al.*, 1997; Valdeolivas *et al.*, 2013).

2.4.6. *CB1R density analysis*

Animals were euthanized, brains were rapidly removed and frozen in an isopentane and dry ice bath and stored in a -80 °C freezer. Brain were then transferred to a cryostat (Leica C3050S) and 12-16 μm thick coronal sections were cut, mounted onto Superfrost Plus slides (BDH, Lutterworth, UK) and stored with desiccant at -20 °C until use. According to the Paxinos and Watson atlas (1997), brain areas analyzed were:

1. cingulate cortex 1 and 3 (Cg1 e Cg3) (AP: +3.2)
2. caudate putamen (CPu) (AP: +1.60)
3. nucleus accumbens core (Nacc Core) and shell (Nacc Shell) (AP: +1.60)
4. hippocampal Ammon's horn 1, 2, 3 (CA1, CA2, CA3) (AP: -2.14)
5. hippocampal dentate gyrus (DG) (AP: -3.14)
6. amygdala (Amy) (AP: da -2.14 a -3.14)
7. ventromedial hypothalamus (VMH) (AP: -2.14)
8. lateral hypothalamus (LH) (AP: -2.14)

CB1R distribution and density evaluation was performed in selected brain areas by quantitative [^{3}H]CP-55,940 autoradiographic binding as previously described (Castelli *et al.*, 2007). Tissue slides were gradually brought back to room temperature and then incubated at 37 °C for 2.5 h in 50 mM Tris-HCl (pH 7.4) containing 5 % bovine serum albumin (BSA) and 10 nM of [^{3}H]CP-55,940 (specific activity, 131.8 Ci/mmol, Perkin Elmer, Boston, MA, USA). In order to determine non-specific binding, adjacent brain sections were incubated in parallel in the presence of 10 μM unlabelled CP-55,940. Following incubation, slides were rinsed twice at 4 °C for 2 h in ice-cold Tris-HCl buffer (50 mM, pH 7.4) with 1 % BSA, once (5 min) with 50 mM Tris-HCl, dipped in ice-cold deionized water and then air dried. Dried tissue sections and slide-mounted

[^{3}H]-micro scales standards (ARC 0123B and 0123C, St. Louis) were placed in a Fujifilm BAS cassette with a BAS-5000 imaging plate. Resulting images were analyzed with the Fujifilm-BAS 5000 imaging system (AIDA, Raytest, USA), and optical densities were transformed into levels of bound radioactivity (fentomoles per milligram of protein) with gray values generated by co-exposed [^{3}H]-standards.

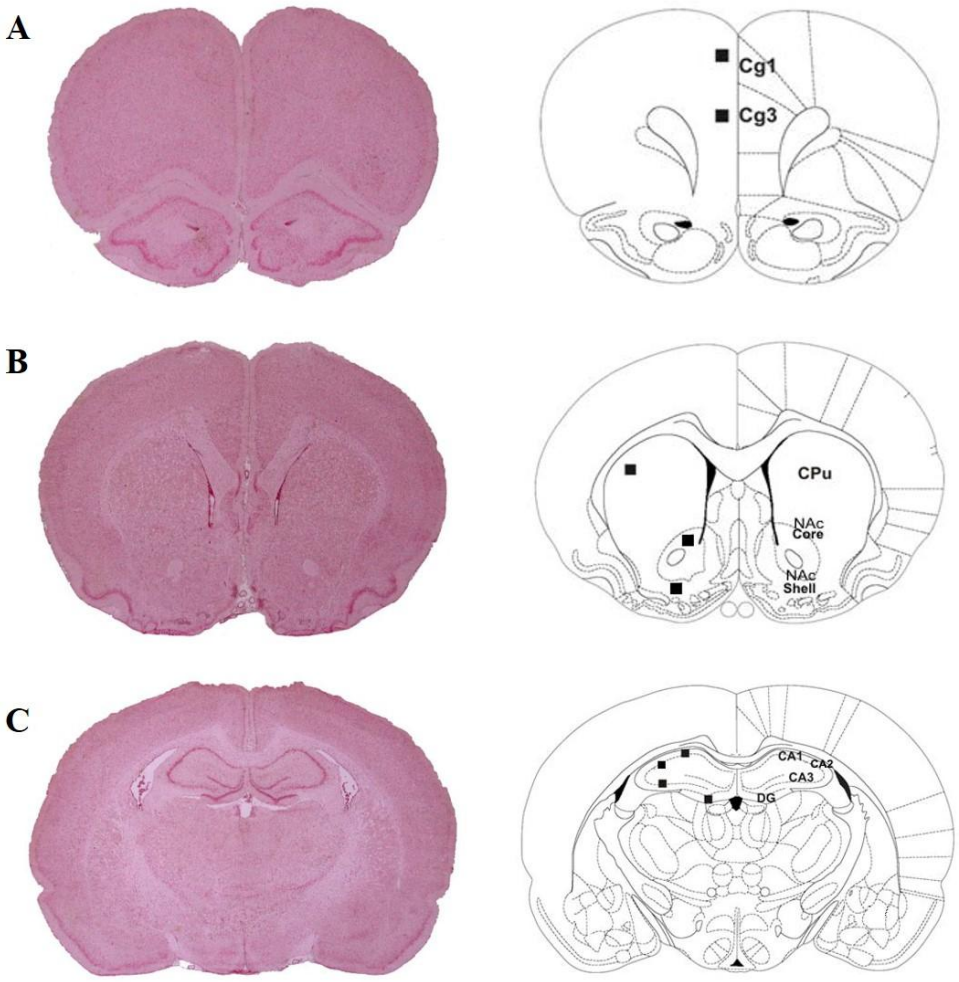


Figure. 3: Graphical and schematic representation of the brain areas studied.

2.5. Experiment 2: pharmacological study

As illustrated in figure 4, the ABA protocol for the second experiment consisted of five phases in the following order: *Habituation* (3 days), *Baseline* (5 days), *ABA induction* (6 days), *Recovery* (10 days), 2nd cycle of anorexia: *ABA relapse* (6 days).

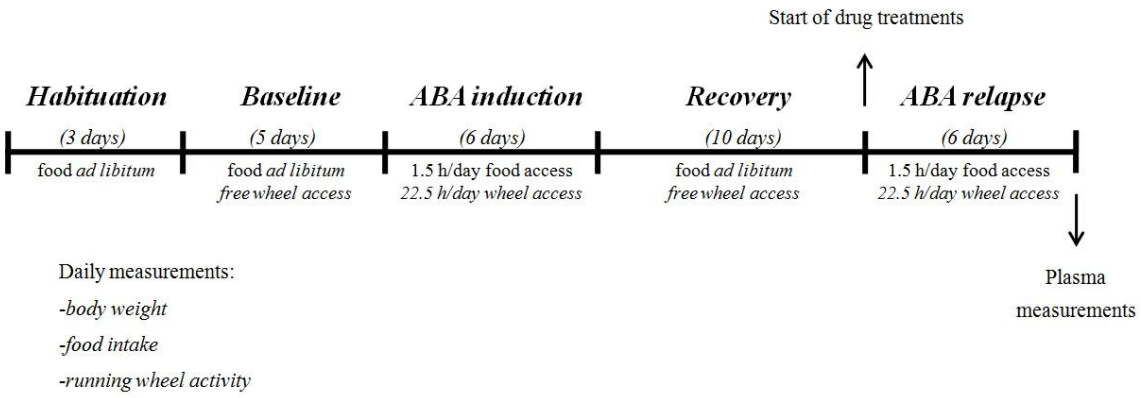


Figure. 4: Schematic representation of the experimental design.

After a 7-day period of adaptation to the housing conditions, animals were divided into two groups homogeneously matched for initial body weight, and individually housed in standard home cages (sedentary rats) or in cages equipped with a running wheel (running rats). In order to allow animals acclimatize to the new individual cage and to avoid possible neophobia-induced reactions to the wheel, during the 3 days of the *Habituation* phase, all running wheels remained locked and body weight and food intake were monitored every day time 30 minutes before the onset of the dark phase. For the entire duration of the *Baseline* phase (5 days), running wheels remained unlocked, i.e. animals housed in the activity cages (running rats) had free access to the wheel. Body weight, food intake and RWA were measured daily in order to obtain a

stable baseline. During both the *Habituation* and *Baseline* phases all animals had *ad libitum* access to chow and water.

2.5.1. *ABA induction*

Following the *Baseline* phase, the two initial groups of animals, with (*running* rats) or without (*sedentary* rats) access to the running wheel, were further divided into two subgroups homogeneously matched for body weight, food intake and RWA recorded during the previous *Baseline* phase. *Running* rats were divided into: *Excercise* (24 h/day free access to food, free access to the wheel) and *ABA* (1.5 h/day access to food, free access to the wheel). *Sedentary* rats were divided into: *Control* (24 h/day free access to food, no access to the wheel) and *Restricted* (1.5 h/day access to food, no access to the wheel) groups. During the 6-day period of the *ABA induction* phase (or until animals lost a maximum of 25 % of their initial baseline body weight), animals belonging to the food restricted groups, with (*ABA*) or without (*Restricted*) access to the running wheel, were subjected to a temporal, but not quantitative, restricted feeding schedule, being given free access to a pre-weighed quantity of food for 1.5 h/day only starting at the onset of the dark phase and with running wheels locked to prevent running from competing with eating. At the end of this feeding period, food was removed and re-weighed in order to record the amount of food consumed in that period of time. For the remainder of time (22.5 h), restricted animals had no access to food but free access to the wheel. During this phase, both groups belonging to the no restricted animals (*Excercise* and *Control*) continued to have free access to food. In all experimental groups, body weight and RWA were monitored daily 30 min before the 1.5 h feeding period. At the same time, daily 24 h food intake was measured in *ad libitum*-fed rats.

2.5.2. *Recovery phase*

At the end of the *ABA induction* phase, animals started the 10-day period of the *Recovery* phase during which all rats were allowed to feed *ad libitum*. In particular, on Day 6 of the *ABA induction* phase, after the 1.5 h/day access regimen, animals belonging to the food restricted groups (*ABA* and *Restricted*) underwent a restoration period during which they were allowed *ad libitum* access to food. Conversely, animals belonging to the no restricted groups (*Excercise* and *Control*) continued to have *ad libitum* access to food. During this phase, running rats had unlimited access to the wheel (Dixon *et al.*, 2003; Ratnovsky and Neuman, 2011). Recovery from ABA was assessed by monitoring body weight, food intake and RWA daily 30 min of the start of the dark cycle.

2.5.3. *2nd cycle of anorexia: ABA relapse*

At the end of the *Recovery* phase, animals started the six days of the 2nd cycle of anorexia (*ABA relapse*), during which all groups were exposed to the same conditions described for the *ABA induction* phase. In particular, restricted animals (*ABA* and *Restricted*) had access to food for 1.5 h/day only starting at the beginning of the dark phase, while no restricted animals (*Excercise* and *Control*) had free access to food. Access to the running wheel was blocked during the 1.5 h feeding period only. Pharmacological treatments started the last day of the recovery phase (Day 10) and animals from each experimental group (*Control*, *Excercise*, *Restricted*, *ABA*) were randomly allocated into three different groups according to the pharmacological treatment assigned (Vehicle, Dose 1, Dose 2) and treated with two different drug doses and corresponding vehicle once a day 30 minutes before the onset of the dark phase.

Treatments and drugs tested were the following: (i) the natural CB1R/CB2R agonist Δ^9-tetrahydrocannabinol (Δ^9-THC): vehicle, 0.5 and 0.75 mg·kg^{-1}, (ii) the synthetic CB1R agonist CP-55,940: vehicle, 0.03 and 0.06 mg·kg^{-1}, (iii) the CB1R inverse agonist/antagonist SR 171416A (Rimonabant): vehicle, 0.15 and 0.3 mg·kg^{-1}. Body weight, 24 h food intake (in *ad libitum* groups) and RWA were recorded daily just before drug administration.

2.5.4. *Plasma measurements*

The last day of the *ABA relapse* phase (Day 6) animals were euthanized and plasma was collected and processed following the same procedures previously described for experiment 1 (see above *biochemical study*). In all experimental groups, leptin, ghrelin and corticosterone concentrations were analyzed at the end of the 2nd cycle of anorexia in a basal condition (drug free animals) and after pharmacological treatments.

2.6. Statistical Analysis

Body weight, food intake and RWA are presented as mean ± SEM and were analyzed by Two-way analysis of variance (ANOVA) for repeated measures with Groups as a between-subjects factor and Time (Days) as within-subjects factor, and Time as a repeated factor. Within each experimental group, body weight, food intake and RWA from pharmacological treatment are presented as mean ± SEM and were analyzed by Two-way ANOVA analysis for repeated measures with the Treatment as a between-subjects factor and Time as within-subjects factor (Days), and Time as a repeated factor. Plasma levels are presented as mean ± SEM and were analyzed by One-way ANOVA

with groups as a between-subject factor. *Post-hoc* comparisons, when appropriate, were performed by Bonferroni test. Student's t-test was used to assess the statistical difference when two groups or conditions have being compared. In all cases, differences with a p value < 0.05 were considered significant.

Chapter 3: Results

3.1. Experiment 1: biochemical study

3.1.1. *ABA induction*

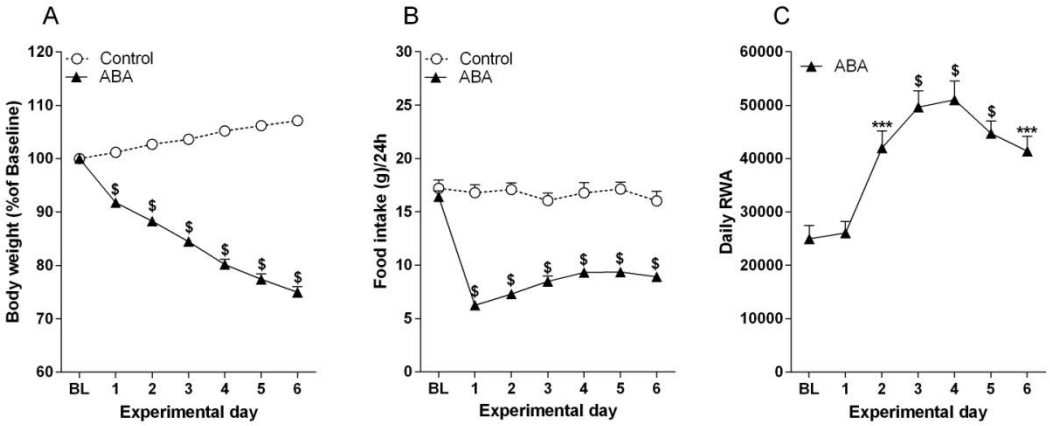


Figure 5. Body weight (A), food intake (B) and daily RWA (C) during the six days of the *ABA induction* phase. All data are presented as mean ± SEM.

As presented in figure 5, rats placed on a restricted-feeding schedule and given access to a running wheel showed a dramatic body weight loss during the six days of the *ABA induction* phase. Two-way ANOVA analysis revealed a significant Group x Time interaction effect [$F_{(6, 372)} = 418.9$, $p < 0.0001$]. Bonferroni *post-hoc* analysis showed that *ABA* animals significantly loss body weight compared to *Control* rats ($^{\$}$ $p < 0.0001$). Due to the restricted-feeding schedule (*ABA*: 1.5 h/day access to food; *Control*: free access to food), daily food intake was significantly lower in *ABA* animals compared to *Control* animals [Two-way ANOVA: $F_{(6, 180)} = 20.31$, $p < 0.0001$; Bonferroni *post-hoc* test: Days 1-6 $^{\$}$ $p < 0.001$]. As expected, *ABA* animals progressively increased the mean number of daily running wheel revolutions compared to the basal activity [One-

way ANOVA: $F_{(6, 209)} = 14.18$, $p < 0.0001$; Bonferroni *post-hoc* test: Days 2 and 6: *** $p < 0.01$, Days 3-5 $^{\$}$ $p < 0.0001$].

3.1.2. *Recovery*

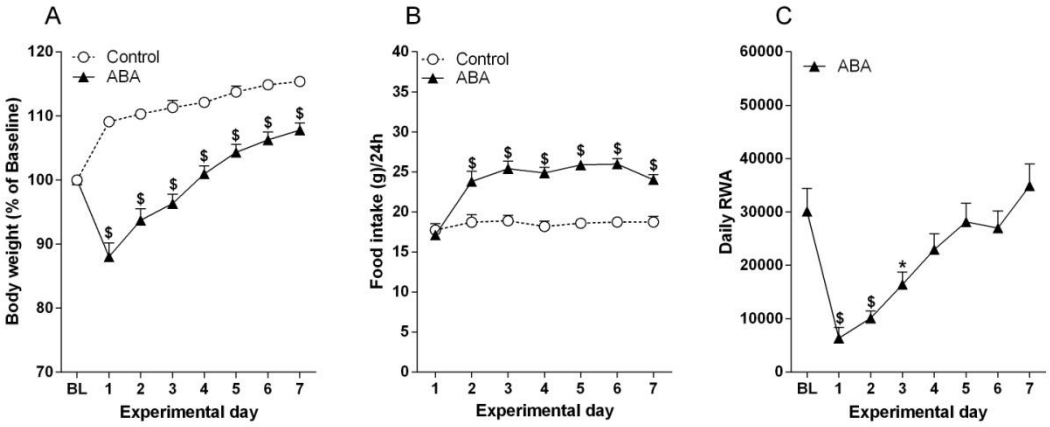


Figure 6. Body weight (A), food intake (B) and daily RWA (C) during the seven days of the *Recovery* phase. All data are presented as mean ± SEM.

At the end of the *ABA induction* phase, animals started the *Recovery* phase, during which food was available *ad libitum*, running wheels remained unlocked and the effect on body weight, food intake and RWA was monitored daily. As regards to body weight, Two-way ANOVA analysis showed a main significant Group x Time interaction effect [$F_{(7, 210)} = 40.49$, $p < 0.0001$]. Bonferroni *post-hoc* analysis revealed that *ABA* animals recovered to the basal body weight by Day 4, but from Days 4 to 7 they weighed significantly less than *Control* animals ($^{\$}$ $p < 0.0001$). During this 7-day restoration period, chow consumption was increased in *ABA* rats and Two-way ANOVA revealed a Group x Time significant interaction effect [$F_{(6, 180)} = 8.882$, $p < 0.0001$]. Bonferroni *post-hoc* test showed that *ABA* animals consumed significantly more chow than *Control* group for the entire duration of the *Recovery* phase ($^{\$}$ $p < 0.0001$). Noteworthy, after an

initial decreased physical activity in the first days of the restoration period, daily RWA progressively and significantly increased during the following days until reaching basal levels [One-way ANOVA: $F_{(6, 105)} = 12.52$, $p < 0.0001$; Bonferroni *post-hoc* test: Days 1-2 $^{\$}$ $p < 0.0001$, Day 3 * $p < 0.05$].

3.1.3. *Plasma measurements*

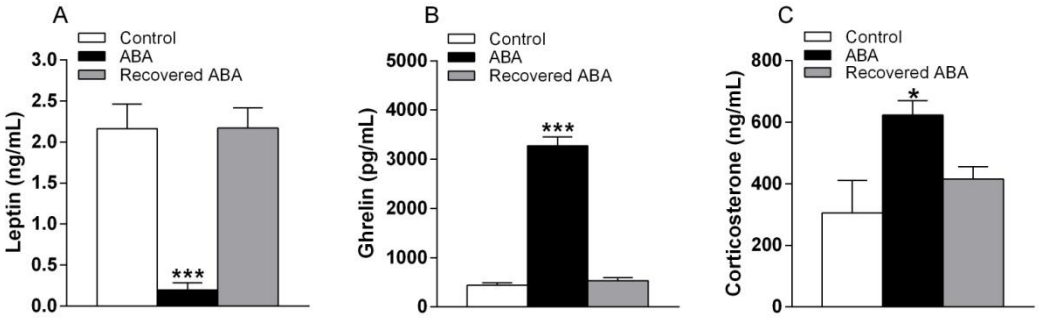


Figure 7. Basal leptin (A), ghrelin (B) and corticosterone (C) plasma levels measured at the end of the *ABA induction* and at the end of the *Recovery* phase. Data are expressed as the mean ± SEM.

As shown in figure 7, our data revealed significantly altered leptin, ghrelin and corticosterone levels in *ABA* animals at the end of the *ABA induction* and *Recovery* phases. In particular, *ABA* animals, with manifested AN, at the end of the *ABA induction* displayed significantly lower levels of leptin (*** $p < 0.001$), and higher levels of ghrelin and corticosterone compared to *Control* rats (*** $p < 0.001$, * $p < 0.05$, respectively). Interestingly, when looking at the following *Recovery* phase, the restoration regime of re-feeding was able to completely revert these altered levels, since leptin, ghrelin and corticosterone in *Recovered ABA* rats came back to *Control* group levels [One-way ANOVA: Leptin $F_{(2, 9)} = 15{,}13$, $p < 0.001$ - Ghrelin $F_{(2, 24)} = 95.08$, $p < 0.001$ - Corticosterone $F_{(2, 21)} = 5.233$, $p = 0.0143$].

3.1.4. *Arachidonic acid measurements*

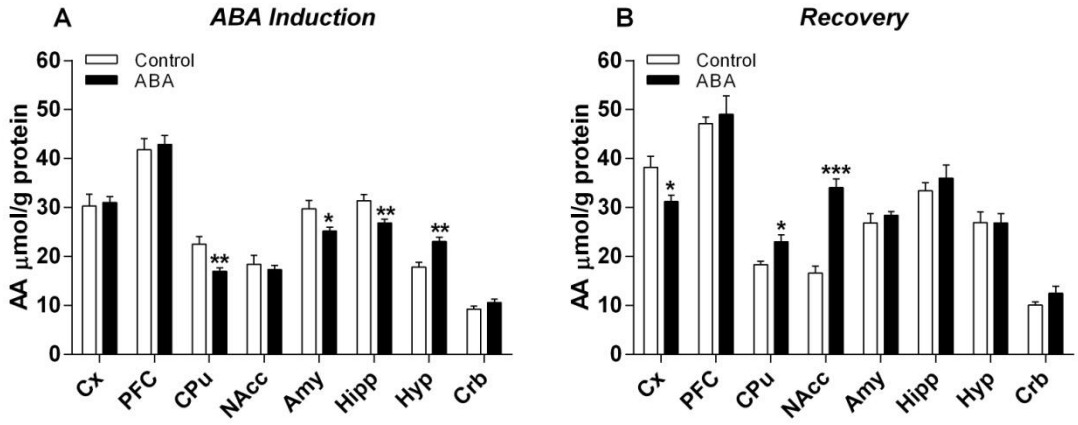


Figure 8. AA brain region levels in *ABA* and *Control* rats at the end of the *ABA induction* (A) and the *Recovery* (B) phases. Values are expressed as the mean ± SEM (n=8 each group). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ *vs* corresponding *Control* group.

As shown in figure 8, the combination of restrictive feeding with free access to the running wheel significantly affected AA brain tissue levels in *ABA* animals. In particular, at the end of the *ABA induction* phase, significantly reduced concentration of AA was found in the Caudato Putamen (CPu), Amy and Hipp of *ABA* animals compared to *Control* animals (unpaired Student's t test: CPu $t(14) = 3.194$, $^{**}p = 0.0065$; Amy $t(14) = 2.465$, $^{*}p = 0.0272$; Hipp $t(14) = 3.115$, $p = ^{**} 0.0076$). Interestingly, increased AA level was detected in the Hypothalamus (Hyp) (unpaired Student's t test: $t(14) = 3.998$, $^{**}p = 0.0013$). No significant difference was found in the other brain regions analyzed in this phase [unpaired Student's t test: Frontal Cortex (Cx) $t(14) = 0.2829$, $p = 0.7814$; PFC $t(14) = 0.3740$, $p = 0.7140$; NAcc $t(14) = 0.5351$, $p = 0.6010$; Cerebellum (Crb) $t(14) = 1.495$, $p = 0.1571$]. When looking at the end of the following *Recovery* phase, reduced AA levels previously detected in the CPu were completely reverted, and significantly increased in *ABA* rats compared to *Control* group (unpaired Student's t test: $t(13) = 2.993$, $p = 0.0104$). Moreover, significantly lower and

higher concentrations of AA were found in the Cx and in the NAcc, respectively, of *ABA* animals [unpaired Student's t test: Cx t(14) = 2.681, p = 0.0179; NAcc t(13) = 7.663, $p < 0.001$]. No significant difference was found in the other brain regions analyzed at the end of the *Recovery* phase [unpaired Student's t test: PFC t(14) = 0.4817, p = 0.6374; Amy t(14) = 0.7558, p = 0.4623; Hipp t(14) = 0.8144, p = 0.4291; Hyp t(14) = 0.01912, p = 0.0.9850, Crb t(14) = 1.519, p = 0.1510].

3.1.5. *Endocannabinoids measurements*

3.1.5.1. *Anandamide levels*

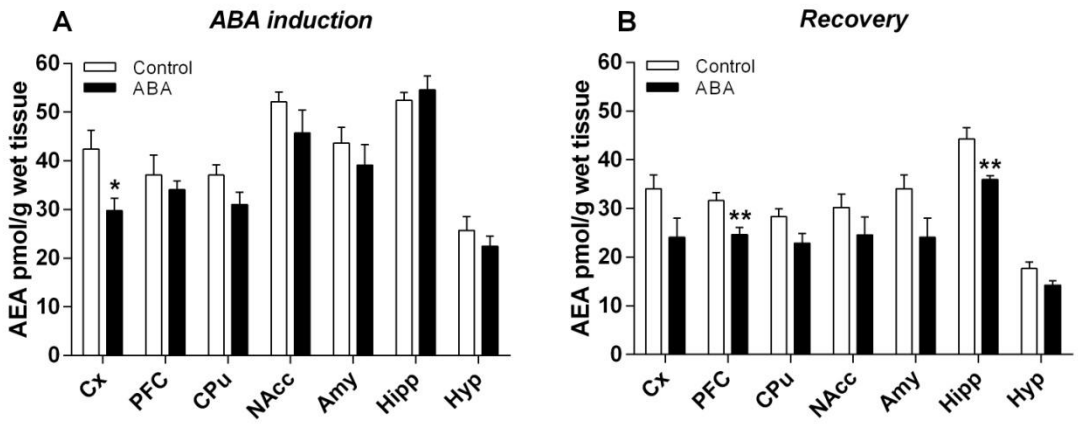


Figure 9. AEA brain region levels in *ABA* and *Control* rats at the end of the *ABA induction* (A) and the *Recovery* (B) phases. Values are expressed as the mean ± SEM (n=8 each group). * $p < 0.05$, ** $p < 0.01$ *vs* corresponding *Control* group.

As shown in figure 9, at the end of the *ABA induction* phase, reduced concentration of AEA was found in the Cx of *ABA* animals compared to *Control* animals (unpaired Student's t test: t(14) = 2.728, p = 0.0163). No significant differences were detected in the other brain regions analyzed in this phase [unpaired Student's t test: PFC t(14) = 0.6852, p = 0.5044; CPu t(14) = 1.820, p = 0.0903; NAcc t(14) = 1.241, p = 0.2348;

Amy t(14) = 0.8315, p = 0.4197; Hipp t(14) = 0.6531, p = 0.5243; Hyp t(14) = 0.9071, p = 0.3808]. When looking at the end of the following *Recovery* phase, significantly lower concentration of AEA was found in the PFC and in the Hipp of *ABA* animals compared to *Control* animals [unpaired Student's t test: PFC t(14) = 3.199, p = 0.0064; Hipp t(14) = 3.323, p = 0.0050]. No significant difference was found in the other regions analyzed at the end of this phase [unpaired Student's t test: Cx t(14) = 2.063, p = 0.0582) CPu t(14) = 2.110, p = 0.0534; NAcc t(14) = 1.333, p = 0.2040; Amy t(14) = 2.063, p = 0.0582; Hyp t(14) = 2.077, p = 0.3808].

3.1.5.2. *2-arachidonoylglycerol levels*

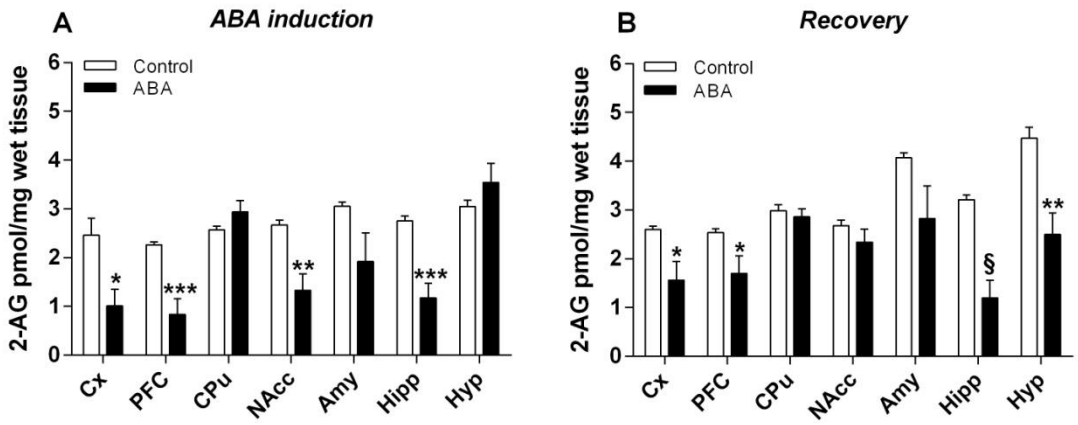


Figure 10. 2-AG brain region levels in *ABA* and *Control* rats at the end of the *ABA induction* (A) and the *Recovery* (B) phases. Values are expressed as the mean ± SEM (n=8 each group). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, § $p < 0.0001$ *vs* corresponding *Control* group.

As shown in figure 10, at the end of the *ABA induction* phase reduced level of 2-AG was revealed in the Cx, PFC, NAcc and Hipp of *ABA* animals compared to *Control* animals [unpaired Student's t test: Cx t(14) = 2.974, p = 0.0100; PFC t(14) = 4.362, p = 0.0007; NAcc t(14) = 3.805, p = 0.0019; Hipp t(14) = 4.930, p = 0.0002]. Moreover, no statistically significant differences were found in the CPu, Amy and Hyp [unpaired

Student's t test: CPu t(14) = 1.468, p = 0.1642; Amy t(14) = 1.907, p = 0.0773; Hyp t(14) = 0.7357, p = 0.4750]. At the end of the following restoration period *ABA* 2-AG levels still remained significantly lower in the Cx, PFC and Hipp when compared to *Control* animals [unpaired Student's t test: t(14) = 2.679, p = 0.0180; t(14) = 2.286, p = 0.0384; t(14) = 5.389, $p < 0.0001$, respectively]. Furthermore, 2-AG levels measured in the NAcc were partially restored after *Recovery* since no significant difference was detected in *ABA* animals compared to *Control* animals [unpaired Student's t test: NAcc t(14) = 1.437, p = 0.1728]. Notably, significantly reduced level of 2-AG was revealed also in the Hyp [unpaired Student's t test: t(14) = 3.971, p = 0.0014], while, no differences were observed in the CPu and Amy [unpaired Student's t test: t(14) = 0.6268, p = 0.5409; t(14) = 1.835, p = 0.0879, respectively].

3.1.5.3. *Palmitoylethanolamide levels*

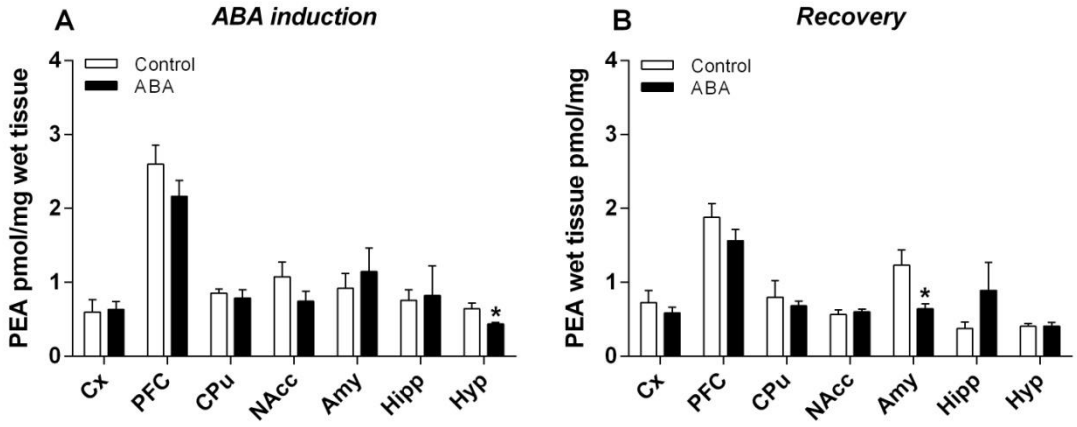


Figure 11. PEA brain region levels in *ABA* and *Control* rats at the end of the *ABA induction* (A) and the *Recovery* (B) phases. Values are expressed as the mean ± SEM (n=8 each group). * $p < 0.05$ *vs* corresponding *Control* group.

As shown in figure 11, at the end of the *ABA induction* phase significantly reduced concentration of PEA was found in the Hyp of *ABA* animals compared to *Control*

animals (unpaired Student's t test: t(14) = 2.867, p = 0.0142). No statistically significant differences were detected in the other brain regions analyzed [unpaired Student's t test: Cx t(14) = 0.1583, p = 0.8765; PFC t(14) = 1.311, p = 0.2109; CPu t(14) = 0.5379, p = 0.5991; NAcc t(14) = 1.369, p = 0.914; Amy t(14) = 0.6043, p = 0.5553; Hipp t(14) = 0.1579, p = 0.8769]. Interestingly, reduced PEA levels in the Hyp were completely reverted at the end of the *Recovery* phase [unpaired Student's t test: t(14) = 0.05876, p = 0.9540]. Moreover, reduced PEA levels were detected in the Amy at the end of this phase, while no significant difference was found in the other regions tested [unpaired Student's t test: Amy t(14) = 2.676, p = 0.0181 Cx t(14) = 0.7580, p = 0.4610; PFC t(14) = 1.303, p = 0.2136; CPu t(14) = 0.5116, p = 0.6169; NAcc t(14) = 0.5266, p = 0.6067; Hipp t(14) = 1.428, p = 0.1770].

3.1.5.4. *Oleoylethanolamide levels*

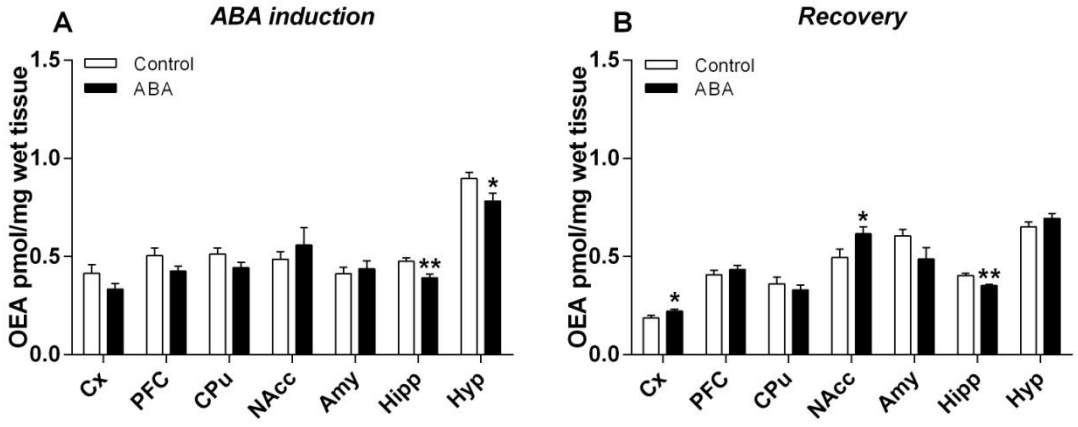


Figure 12. OEA brain region levels in *ABA* and *Control* rats at the end of the *ABA induction* (A) and the *Recovery* (B) phases. Values are expressed as the mean ± SEM (n=8 each group). * $p < 0.05$, ** $p < 0.01$ *vs* corresponding *Control* group.

As shown in figure 12, at the end of the *ABA induction* phase reduced concentration of OEA was found in the Hipp and Hyp of *ABA* animals compared to *Control* animals

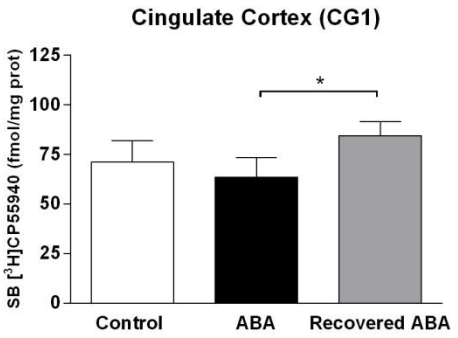
[unpaired Student's t test: Hipp t(14) = 3.312, p = 0.0051; Hyp t(14) = 2.195, p = 0.0455]. No other statistically significant difference was observed in the other brain regions in this phase [unpaired Student's t test: Cx t(14) = 1.560, p = 0.1410; PFC t(14) = 1.694, p = 0.1123; CPu t(14) = 1.699, p = 0.1115; NAcc t(14) = 0.7247, p = 0.4806; Amy t(14) = 0.4781, p = 0.6399]. Interestingly, while OEA levels in the Hipp still remained significantly lower in *ABA* rats at the end of the *Recovery* phase [unpaired Student's t test: t(14) = 3.880, p = 0.0017], levels measured in the Hyp were completely reverted [unpaired Student's t test: t(14) = 1.199, p = 0.2505]. Notably, increased OEA concentration were detected in the Cx and NAcc [unpaired Student's t test: CX t(14) = 2.186, p = 0.0463; NAcc t(14) = 2.171, p = 0.0476]. No significant difference was observed in the other brain regions analyzed [unpaired Student's t test: PFC t(14) = 0.8010, p = 0.4365; CPu t(14) = 0.7665, p = 0.4561; Amy t(14) = 1.788, p = 0.0954].

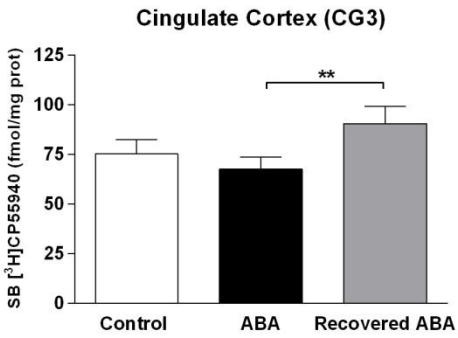
3.1.6. *CB1R density analysis*

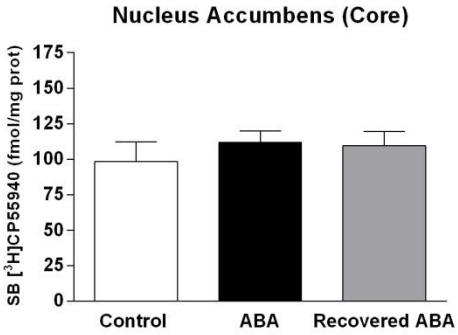
Table X shows the densitometric analysis of [^{3}H]CP-55,940 binding in *ABA* animals at the end of the *ABA induction* phase (*Anorexia* group) and at the end of the following *Recovery* phase (*Recovery* group) compared to *Control* group. One-way ANOVA analysis revealed a significant effect difference among the three experimental groups in the CG1 [$F_{(2, 9)}$ = 5.138, p = 0.0325], CG3 [$F_{(2, 9)}$ = 9.980, p = 0.0052], LH [$F_{(2,9)}$ = 11.04, p = 0.0038] and DG [$F_{(2, 9)}$ = 4.849, p = 0.0372]. Compared to *Control* group, Bonferroni's *post-hoc* test revealed that [^{3}H]CP-55,940 binding levels were significantly reduced in *Anorexia* group compared to *Control* animals in the LH and DG areas (* $p < 0.05$). Animals restored from ABA (*Recovery* group), showed significantly higher CB1R density levels in the LH when compared to *Anorexia* group (** $p < 0.01$). Interestingly, we observed a significant increased receptor density in the CG1 (* $p < 0.05$) and CG3 (** $p< 0.01$) areas in the *Recovery* group. No statistically significant differences were found in the other brain regions analyzed in both phases studied [One-way ANOVA: CPu $F_{(2, 9)}$ = 0.6331, p = 0.5530; NAcc Core $F_{(2, 9)}$ = 1.726, p = 0.2320; NAcc Shell $F_{(2, 9)}$ = 1.872, p = 0.2090; CA1 $F_{(2, 9)}$ = 0.4645, p = 0.6427; CA2 $F_{(2, 9)}$ = 0.9533, p = 0.4205; CA3 $F_{(2, 9)}$ = 0.5291, p = 0.6064; Amy $F_{(2, 9)}$ = 0.09863, p = 0.9070; VMH $F_{(2, 9)}$ = 1.117, p = 0.3688].

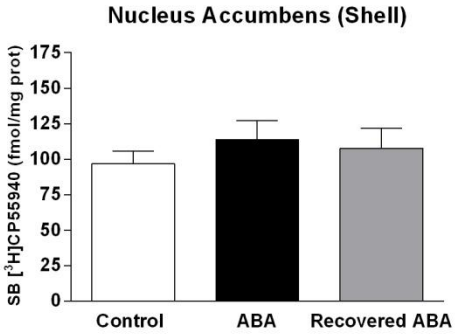
Brain area	**Control** ***(fmol/mg prot)***	**ABA** ***(fmol/mg prot)***	**Recovered ABA** ***(fmol/mg prot)***
CG1	71.1 ± 5.4	63.5 ± 4.8	84.4 ± 3.6 *
CG3	75.3± 3.6	67.7 ± 3.1	90.6 ± 4.3 **
NAcc Core	98.2± 7.0	111.8 ± 4.1	109.5 ± 5.1
NAcc Shell	96.8± 4.5	113.6 ± 6.8	107.7 ± 7.1
CPu	156.0 ± 12.3	174.7 ± 8.5	164.0 ± 13.9
Amy	156.1 ± 5.1	156.0 ± 15.1	161.1 ± 2.6
LH	185.5 ± 7.2	152.8 ± 7.4 *	189.9 ± 2.2 **
VMH	179.3 ± 15.6	146.4 ± 15.3	168.2 ± 16.6
CA1	318.0 ± 15.7	301.7 ± 16.4	297.0 ± 16.3
CA2	318.5 ± 9.9	317.7 ± 14.5	294.3 ± 16.8
CA3	304.5 ± 8.5	294.5 ± 12.6	289.0 ± 11.0
DG	283.0 ± 13.3	220.4 ± 15.5 *	226.2 ± 17.9

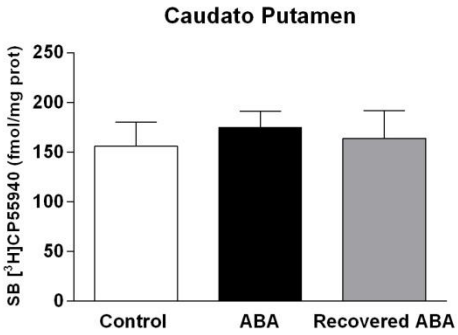
Table 1. CB1R density in selected brain areas. Values represent the mean ± SEM of density reading (six tissue sections for each brain area per animal), expressed as fmol/mg protein.

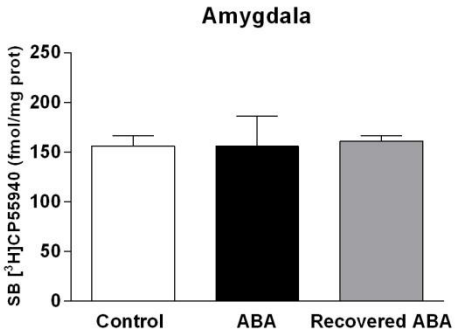

Cingulate Cortex (CG1)
SB [3H]CP55940 (fmol/mg prot)
125
100
75
50
25
0
*
Control
ABA
Recovered ABA

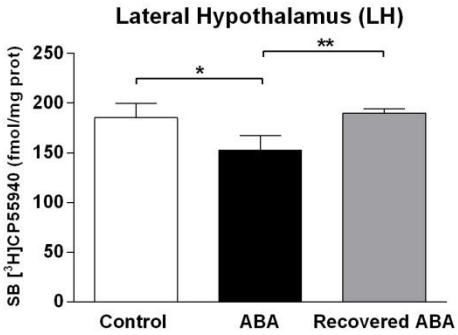

Cingulate Cortex (CG3)
SB [3H]CP55940 (fmol/mg prot)
125
100
75
50
25
0
**
Control
ABA
Recovered ABA

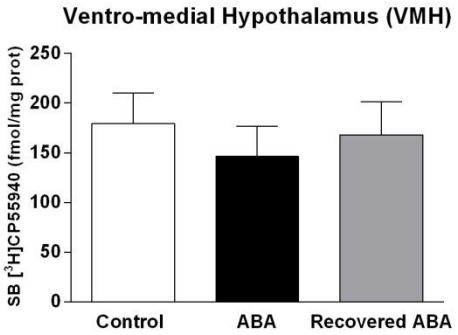

Nucleus Accumbens (Core)
SB [3H]CP55940 (fmol/mg prot)
175
150
125
100
75
50
25
0
Control
ABA
Recovered ABA


Nucleus Accumbens (Shell)
SB [3H]CP55940 (fmol/mg prot)
175
150
125
100
75
50
25
0
Control
ABA
Recovered ABA


Caudato Putamen
SB [3H]CP55940 (fmol/mg prot)
250
200
150
100
50
0
Control
ABA
Recovered ABA


Amygdala
SB [3H]CP55940 (fmol/mg prot)
250
200
150
100
50
0
Control
ABA
Recovered ABA


Lateral Hypothalamus (LH)
SB [3H]CP55940 (fmol/mg prot)
250
200
150
100
50
0
*
**
Control
ABA
Recovered ABA


Ventro-medial Hypothalamus (VMH)
SB [3H]CP55940 (fmol/mg prot)
250
200
150
100
50
0
Control
ABA
Recovered ABA

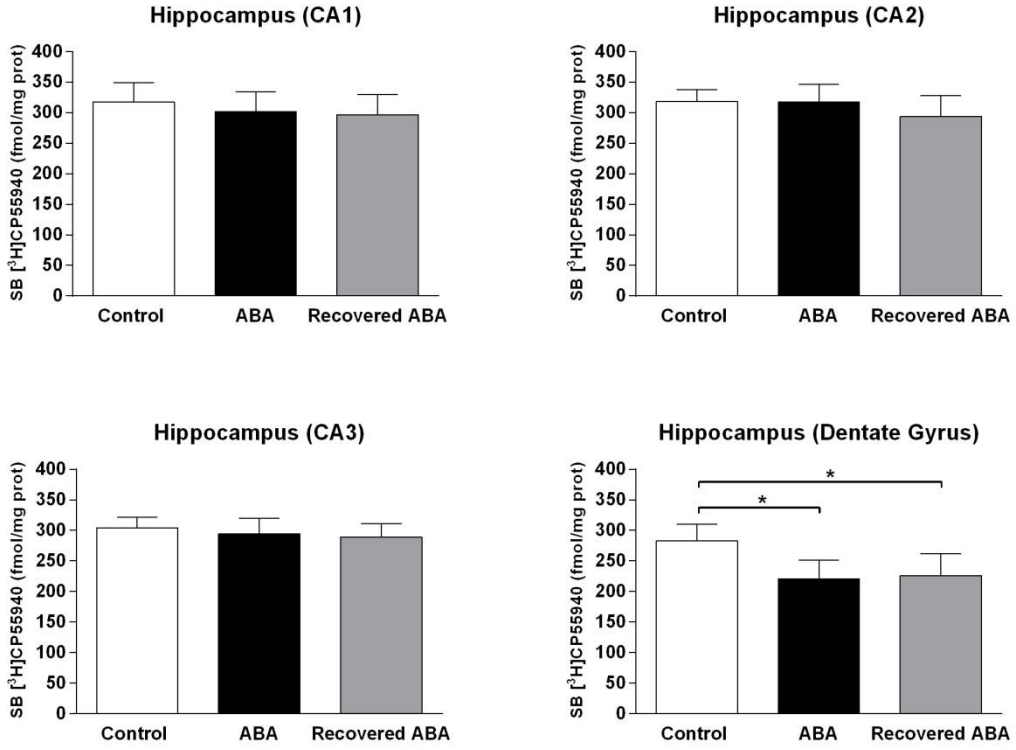


Figure 13. Effect of the *ABA induction* and *Recovery* phases on the CB1R density in different brain areas. Data are expressed as mean ± SEM of density reading (six tissue sections for each brain area per animal) expressed as fmol/mg protein of [^{3}H]CP-55,940 specific bound. (* $p < 0.05$, ** $p < 0.01$, n = 4 each group)

3.2. Experiment 2: pharmacological study

3.2.1. *ABA induction*

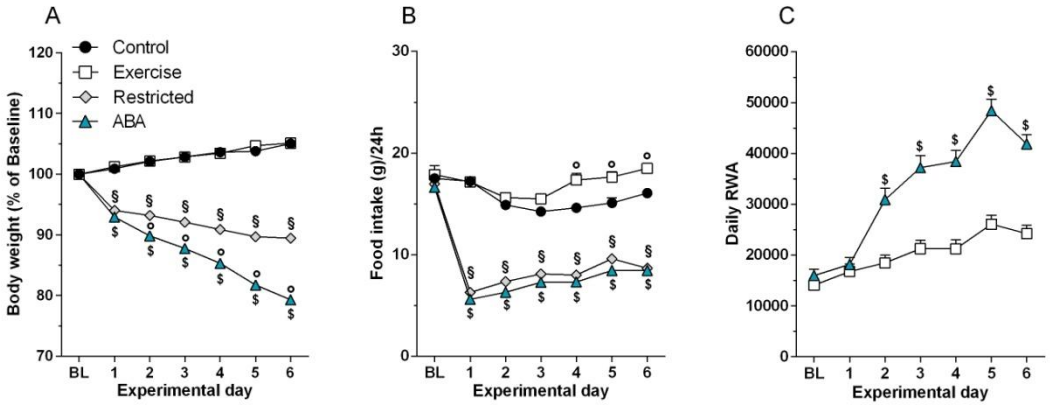


Figure 14. Body weight (A), food intake (B) and daily RWA (C) during the six days of the *ABA induction* phase. All data are presented as mean ± SEM.

When looking at the body weight during the six days of the *ABA induction* phase (Figure 14), Two-way ANOVA analysis revealed a significant Group x Time interaction effect [$F_{(18, 984)} = 269.91$, $p < 0.0001$]. Bonferroni *post-hoc* analysis showed that *ABA* and *Restricted* animals, significantly loss body weight compared to *Exercise* and *Control* groups ($p < 0.0001$). Moreover, body weight loss was significantly greater in *ABA* compared to *Restricted* animals ($^{\circ}$ $p < 0.0001$). No significant difference between *Exercise* and *Control* animals body weight was observed in this phase. Due to the restricted-feeding schedule, the mean daily amount of food consumed was lower in *ABA* and *Restricted* animals compared to *Control* and *Exercise* rats ($p < 0.0001$). Two-way ANOVA showed a Group x Time significant interaction effect [$F_{(18, 960)} = 32.45$, $p < 0.0001$]. Bonferroni *post-hoc* test revealed no significant differences in the amount of food consumed by *ABA* and *Restricted* animals, but evidenced an increased food intake in *Exercise* animals compared to *Control* animals particularly evident in the last three

days of the *ABA induction* phase ($^{\circ}$ $p < 0.001$). As expected, *ABA* animals progressively developed a physical hyperactivity to the running wheel. Two-way ANOVA analysis revealed a significant Group x Time interaction effect [$F_{(6, 492)} = 23.56$, $p < 0.0001$]. Bonferroni *post-hoc* analysis showed a significant increased mean number of daily running wheel revolutions in *ABA* animals compared to *Exercise* rats from Days 2 to 6 ($^{\$}$ $p < 0.0001$).

3.2.2. *Recovery*

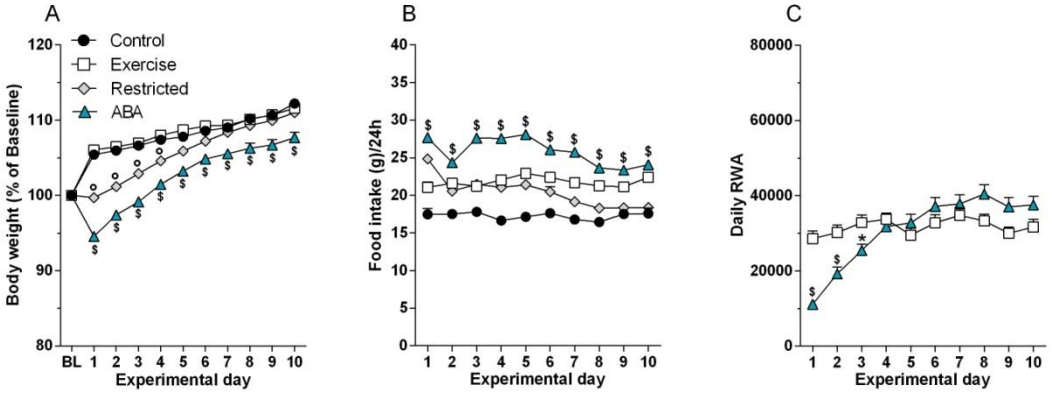


Figure 15. Body weight (A), food intake (B) and daily RWA (C) during the ten days of the *Recovery* phase. All data are presented as mean ± SEM.

As previously described, at the end of the *ABA induction* phase, animals started the *Recovery* phase, during which food was available *ad libitum* for all the experimental groups and running wheels remained unlocked. As regards to body weight, Two-way ANOVA analysis showed a main significant Group x Time interaction effect [$F_{(30, 1610)} = 24.16$, $p < 0.0001$]. Bonferroni *post-hoc* test revealed that even if *ABA* animals came back to the basal body weight by Day 4, they still weighed significantly low than *Control* and *Exercise* for the entire duration of the *Recovery* phase ($^{\$}$ $p < 0.0001$). *Post-hoc* analysis revealed also that *Restricted* animals recovered their 10 % body weight

loss within Day 1 of free feeding and even if their body weight remained significantly lower than *Control* and *Exercise* animals for the first 4 days of *Recovery* ($^{\circ}$ $p < 0.0001$), after that it became to that of no restricted animals. No significant differences were seen in the body weight of *Exercise* and *Control* animals. Notably, chow consumption during this restoration period was different between groups, since Two-way ANOVA revealed a Group x Time significant interaction effect [$F_{(27, 1440)} = 6.89$, $p < 0.0001$]. Bonferroni *post-hoc* analysis showed that *ABA* animals consumed significantly more food than all the other experimental groups for the entire duration of the *Recovery* phase ($^{\$}$ $p < 0.0001$ *vs Control*). Regarding to RWA, Two-way ANOVA analysis evidenced a main significant Group x Time interaction effect [$F_{(9, 720)} = 14.05$, $p < 0.0001$]. Bonferroni *post-hoc* analysis revealed a significant reduction in *ABA* RWA compared to *Exercise* animals on Days 1 and 2 ($^{\$}$ $p < 0.0001$) and on Day 3 ($p < 0.05$).

3.2.3. *Effect of THC*

3.2.3.1. *Effect of THC on body weight*

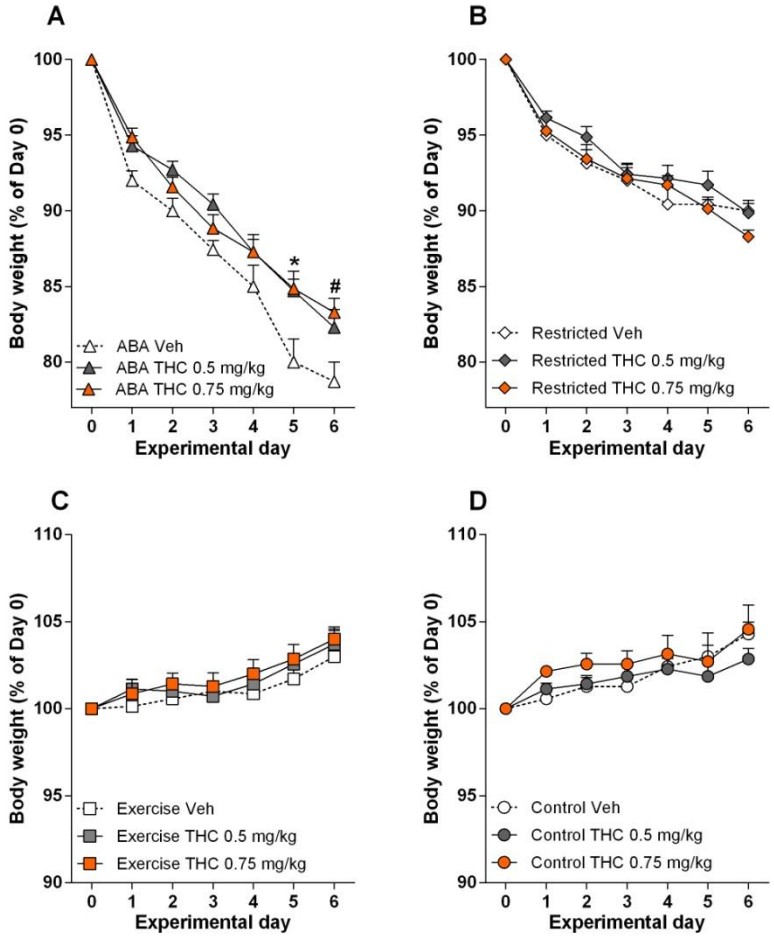


Figure 16. Dose-response curves showing the percentage of body weight in *ABA* (A), *Restricted* (B), *Exercise* (C) and *Control* (D) animals treated with vehicle or Δ^9-THC (0.5, 0.75 mg·kg^{-1}, IP) during the six days of the *ABA relapse* phase. Results are expressed as mean ± SEM.

As shown in figure 16, sub-chronic administration of Δ^9-THC significantly reduced body weight loss in *ABA* animals during the *ABA relapse* phase. Two-way ANOVA analysis revealed a significant interaction effect between the variables Treatment and duration in Days [$F_{(12,\ 108)} = 2.34$, $p = 0.0104$]. Bonferroni *post-hoc* analysis confirmed that both doses of Δ^9-THC tested (0.5 and 0.75 mg·kg^{-1}) significantly reduced *ABA*

body weight loss on Days 5 and 6 of treatment (Δ^9-THC 0.5 mg·kg^{-1}: * p< 0.05 and ** p < 0.01; Δ^9-THC 0.75 mg·kg^{-1}: ** p < 0.01). No significant effect on body weight was observed in the other experimental groups [Two way ANOVA: *Restricted* $F_{(12, 108)}$ = 0.95, p = 0.5010; *Exercise* $F_{(12, 108)}$ = 0.35, p = 0.8995; *Control* $F_{(12, 108)}$ = 0.90, p = 0.5473].

3.2.3.2. *Effect of THC on food intake*

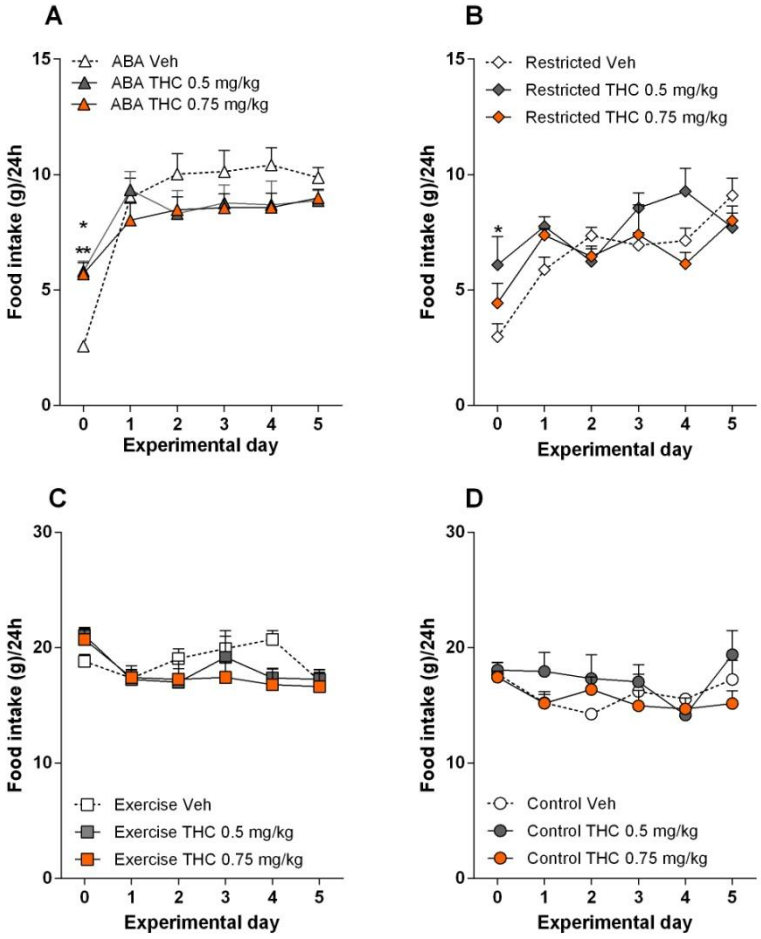


Figure 17. Dose-response curves showing the amount of food consumed by *ABA* (A), *Restricted* (B), *Exercise* (C) and *Control* (D) animals treated with vehicle or Δ^9-THC (0.5, 0.75 mg·kg^{-1}, IP) during the *ABA relapse* phase. Results are expressed as mean ± SEM.

The mean daily amount of food consumed by *ABA* animals during the *ABA relapse* was significantly affected by Δ^9-THC administration (Figure X). Application of Two-way ANOVA showed a significant interaction between variables Δ^9-THC Treatment and duration in Days [$F_{(10, 90)} = 3.89$, $p = 0.0002$]. Bonferroni *post-hoc* test revealed that Δ^9-THC, at both doses tested, significantly increased the intake of food in *ABA* rats as compared to vehicle-treated rats on Day 0 (* $p < 0.05$ and ** $p < 0.01$, respectively). In the same way, on Day 0, Δ^9-THC at the dose of 0.5 mg/kg, significantly increased food consumption also in *Restricted* rats [Two-way ANOVA: $F_{(10, 90)} = 2.80$, $p = 0.0046$; *post-hoc test:* * $p < 0.05$].

3.2.3.3. *Effect of THC on RWA*

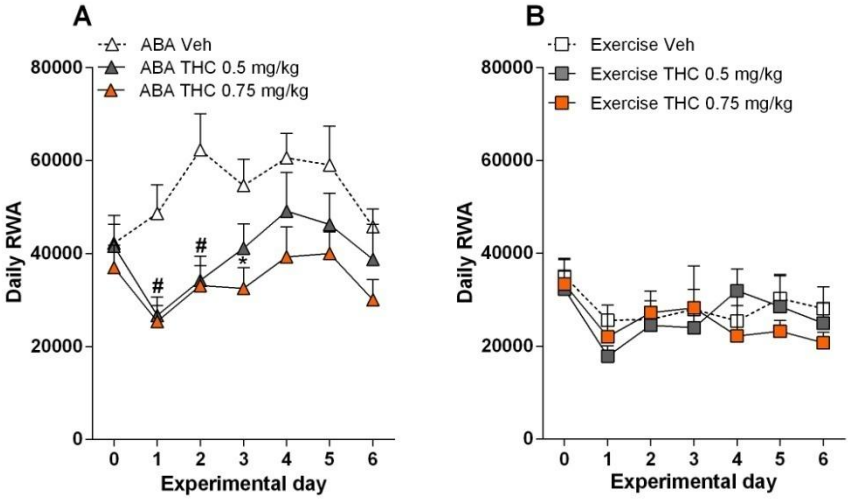


Figure 18. Dose-response curves showing the daily RWA of *ABA* (A) and *Exercise* (B) animals treated with vehicle or Δ^9-THC (0.5, 0.75 mg·kg^{-1}, IP) during the six days of the *ABA relapse* phase. Results are expressed as mean ± SEM.

As illustrated in figure 18, the daily injection of Δ^9-THC affected RWA in *ABA* animals. Two-way ANOVA analysis evidenced a significant effect of Treatment [$F_{(2, 108)} = 6.20$, $p = 0.0089$] and Day [$F_{(6, 108)} = 4.87$, $p = 0.0002$], but not a significant effect of the

interaction Treatment x Day [$F_{(12, 108)}$ = 1.41, p = 0.1729]. The following individual One-way ANOVA showed that, compared with vehicle-treated rats, both doses of Δ^9-THC were effective in reducing RWA on Days 1, 2 and 3 [Day 1 $F_{(2, 18)}$ = 8.16, p = 0.0030; Day 2 $F_{(2, 18)}$ = 6.668, p = 0.0068; Day 3 $F_{(2, 18)}$ = 5.023, p = 0.0185]. Furthermore, Δ^9-THC administration did not modify RWA in *Exercise* rats [Two-way ANOVA: $F_{(12, 108)}$ = 0.71, p = 0.7428].

3.2.4. *Effect of CP-55,940*

3.2.4.1. *Effect of CP-55,940 on body weight*

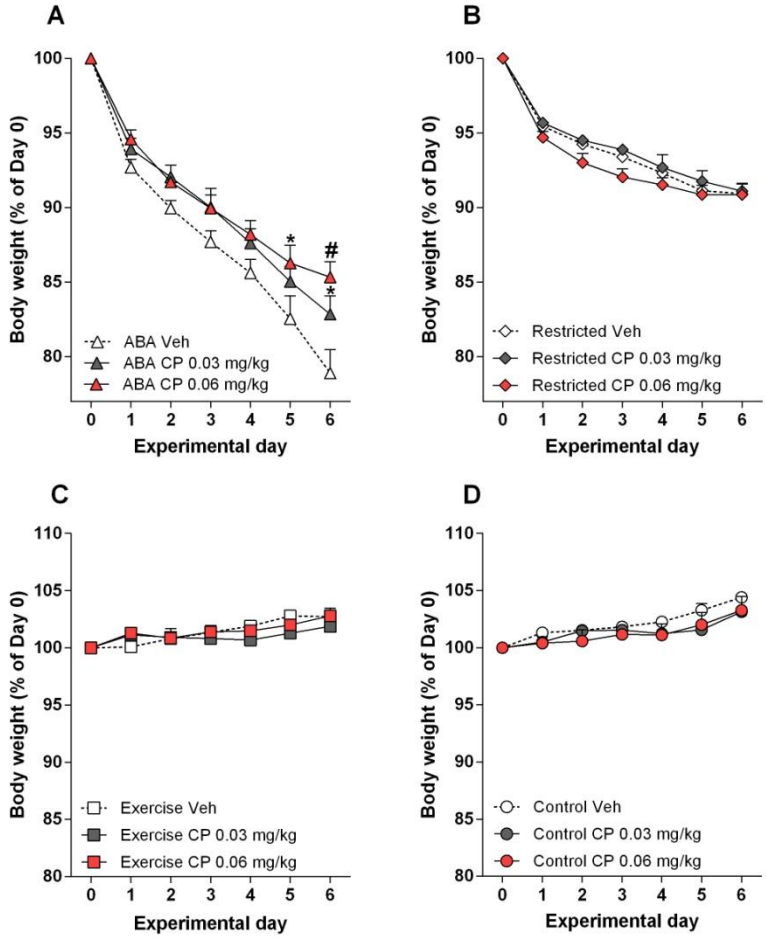


Figure 19. Dose-response curves showing the percentage of body weight in *ABA* (A), *Restricted* (B), *Exercise* (C) and *Control* (D) animals treated with vehicle or CP-55,940 (0.03, 0.06 mg·kg^{-1}, IP) during the six days of the *ABA relapse* phase. Results are expressed as mean ± SEM.

As shown in figure 19, during the *ABA relapse*, CP-55,940 affected *ABA* body weight. Two-way ANOVA analysis revealed a significant interaction effect between variables CP-55,940 Treatment and duration in Days [$F_{(12,\ 108)} = 2.15$, $p = 0.0195$]. Bonferroni *post-hoc* analysis showed that sub-chronic treatment with CP-55,940, at both doses tested (0.03, 0.06 mg·kg^{-1}), was able to reduce body weight loss in *ABA* rats compared

to vehicle-treated animals. In particular, the higher dose tested (0.06 mg·kg^{-1}) significantly attenuated weight loss on Days 5 and 6 (* $p < 0.05$, *** $p < 0.001$, respectively), while, the lower dose (0.03 mg·kg^{-1}) was effective on Day 6 (* $p < 0.05$). No significant differences of body weight between vehicle- and CP-treated rats were revealed in the other experimental groups [Two-way ANOVA: *Restricted* $F_{(12, 108)} = 1.01$, $p = 0.4495$; *Exercise* $F_{(12, 108)} = 1.63$, $p = 0.0939$; *Control* $F_{(12, 108)} = 0.78$, $p = 0.6672$].

3.2.4.2. *Effect of CP-55,940 on food intake*

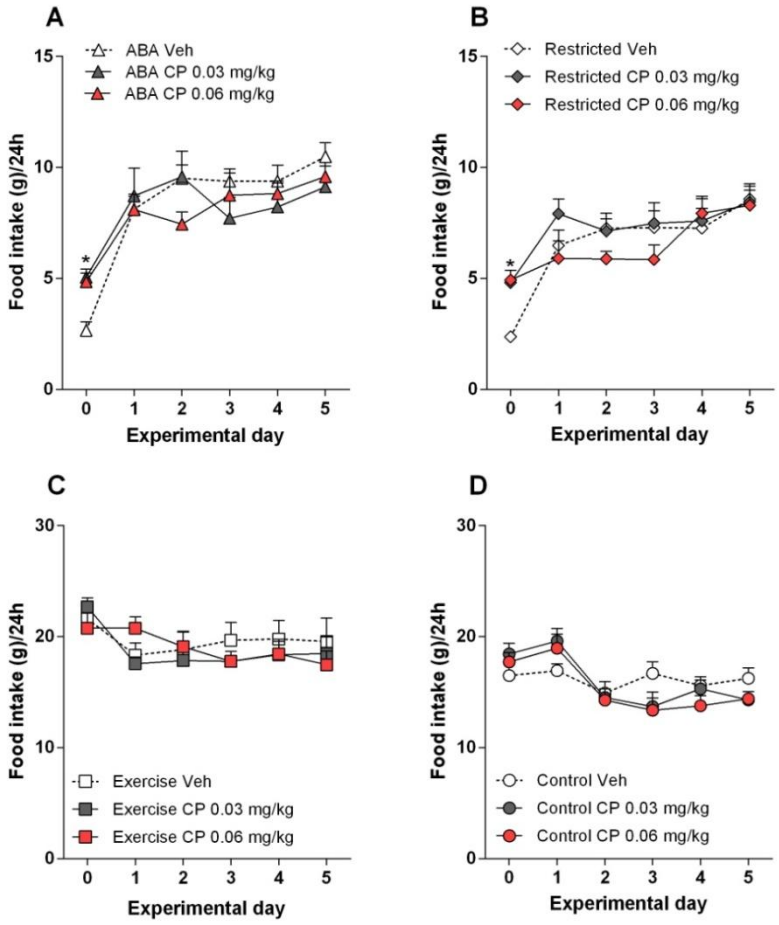


Figure 20. Dose-response curves showing the amount of food consumed by *ABA* (A), *Restricted* (B), *Exercise* (C) and *Control* (D) animals treated with vehicle or CP-55,940 (0.03, 0.06 mg·kg^{-1}, IP) during the *ABA relapse* phase. Results are expressed as mean ± SEM.

As shown in figure 20, CP-55,940 administration affected the mean daily amount of food consumed during the *ABA relapse*. Application of Two-way ANOVA analysis revealed a significant effect of the interaction between CP-55,940 Treatment and duration in Days [$F_{(10,\ 90)} = 2.82$, $p = 0.0043$]. Bonferroni *post-hoc* test revealed significant increased food intake in CP-55,940 treated *ABA* rats compared to vehicle-treated animals at both doses tested (0.03, 0.06 mg·kg^{-1}) on Day 0 (** $p < 0.01$ and * $p < 0.05$, respectively). On Day 0 both doses significantly increased food intake also in *Restricted* animals [Two-way ANOVA: $F_{(10,\ 90)} = 1.96$, $p = 0.0477$; *post-hoc* * $p < 0.05$]. CP-55,940 administration did not affect food intake in *Exercise* and *Control* groups [Two-way ANOVA: $F_{(10,\ 90)} = 1.28$, $p = 0.2557$; $F_{(10,\ 90)} = 1.72$, $p = 0.0878$, respectively].

3.2.4.3. *Effect of CP-55,940 on RWA*

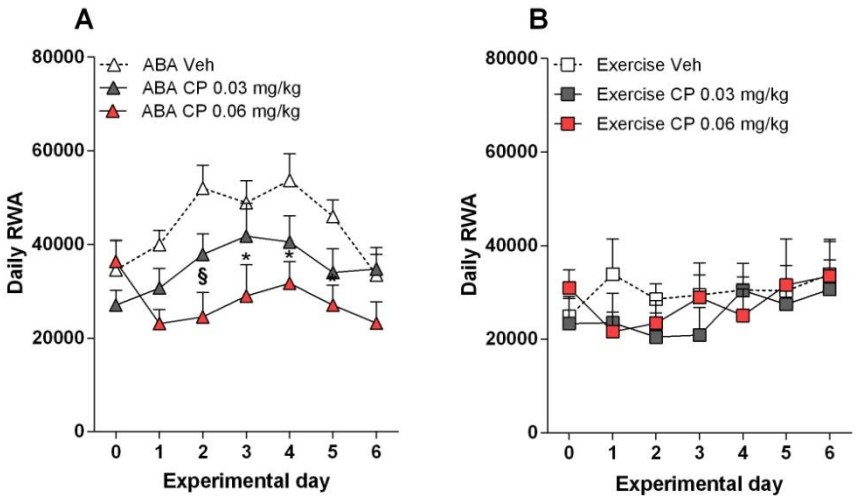


Figure 21. Dose-response curves showing the RWA of *ABA* (A) and *Eercise* (B) animals treated with vehicle or CP-55,940 (0.03, 0.06 mg·kg^{-1}, IP) during the six days of the *ABA relapse* phase. Results are expressed as mean ± SEM.

The daily administration of CP-55,940 affected RWA in *ABA* animals. Two-way ANOVA analysis revealed a significant effect of the interaction between CP-55,940 Treatment and duration in Days [$F_{(12, 108)} = 2.45$, $p = 0.0073$]. Bonferroni *post-hoc* test revealed a significant reduction in the RWA of *ABA* rats treated with the higher dose of CP-55,940 (0.06 mg·kg^{-1}) on Day 2 ($^{\$}$ $p < 0.01$) and from Day 3 to Day 5 (* $p < 0.05$). No effect was found in *Exercise* rats [Two-way ANOVA: $F_{(12, 108)} = 0.76$, $p = 0.6931$].

3.2.5. *Effect of rimonabant*

3.2.5.1. *Effect of rimonabant on body weight*

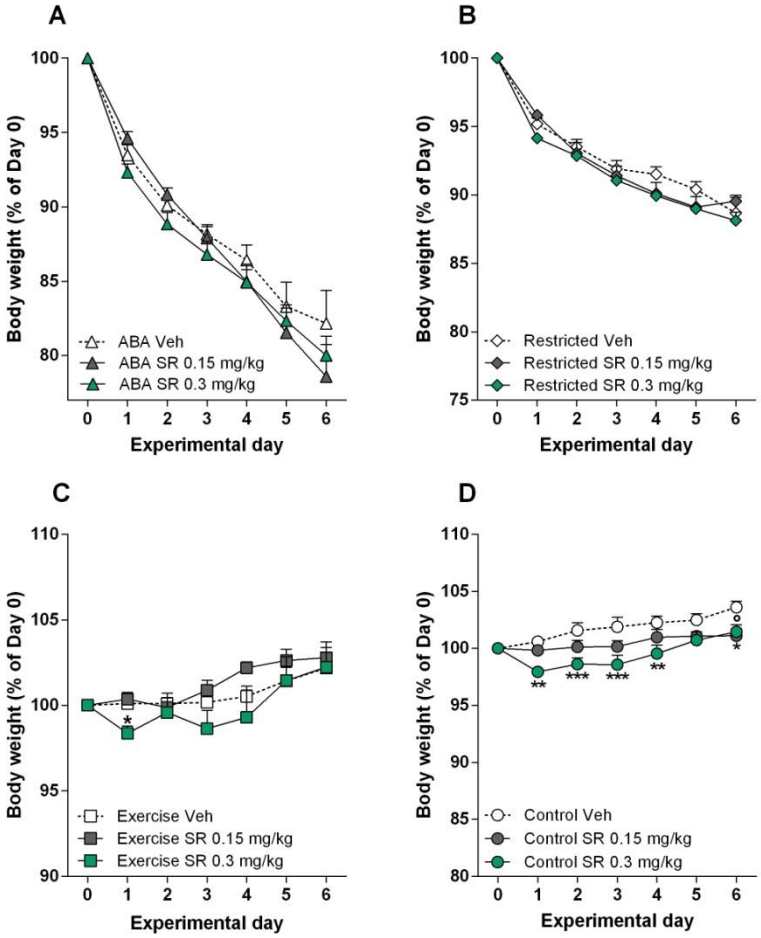


Figure 22. Dose-response curves showing the percentage of body weight in *ABA* (A), *Restricted* (B), *Exercise* (C) and *Control* (D) animals treated with vehicle or Rimonabant (0.15, 0.3 mg·kg^{-1}, IP) during the six days of the *ABA relapse* phase. Results are expressed as mean ± SEM.

As shown in figure 22, during the *ABA relapse*, rimonabant did not affect body weight loss in *ABA* and *Restricted* animals. Two-way ANOVA analysis revealed a significant effect of the interaction between variables Treatment and duration in Days only in *Control* treated animals [$F_{(12, 126)} = 2.485$, $p = 0.1609$], but not in the other experimental groups [*ABA*: $F_{(12, 126)} = 1.429$, $p = 0.0059$; *Restricted*: $F_{(12, 126)} = 1.752$, $p = 0.0635$;

Exercise: $F_{(12, 126)} = 1.334$, $p = 0.2077$]. Bonferroni *post-hoc* analysis evidenced the significant effect of the higher dose of rimonabant (0.3 mg·kg^{-1}) in reducing body weight in *Control* animals (Days 1 and 4: ** $p < 0.01$; Days 2 and 3: *** $p < 0.001$; Day 6: * $p < 0.05$). The higher dose tested (0.3 mg·kg^{-1}) reduced body weight in *Exercise* animals on the Day 1 [One-way ANOVA: $F_{(2, 21)} = 5.577$, $p = 0.0144$; * $p < 0.05$].

3.2.5.2. *Effect of rimonabant on food intake*

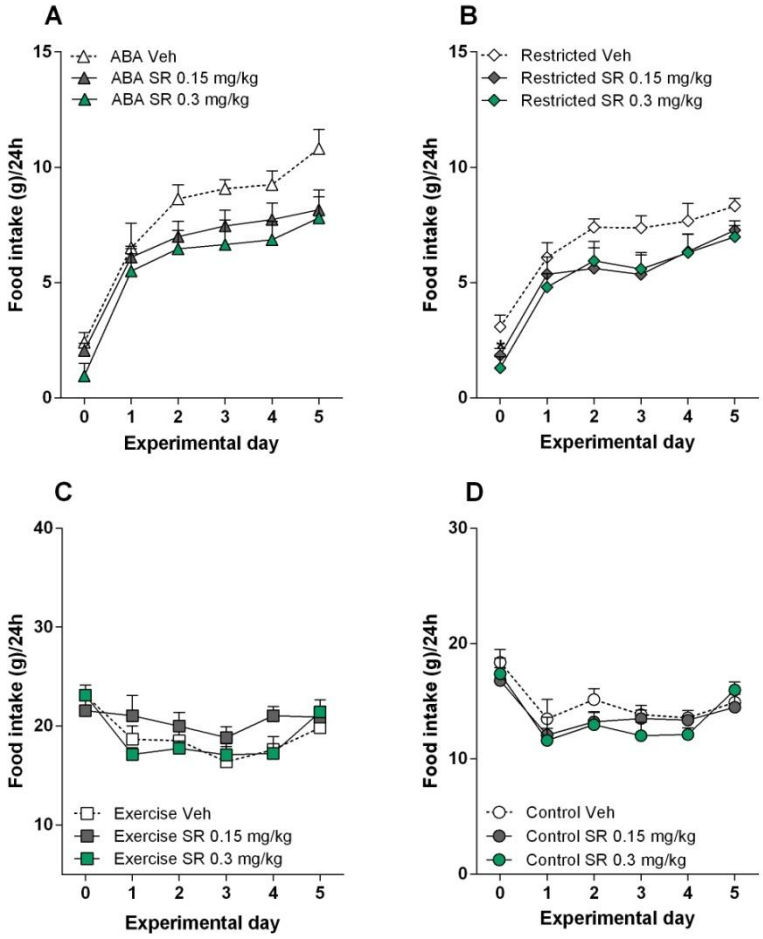


Figure 23. Dose-response curves showing the food intake of *ABA* (A), *Restricted* (B), *Exercise* (C) and *Control* (D) animals treated with vehicle or Rimonabant (0.15, 0.3 mg·kg^{-1}, IP) during the *ABA relapse* phase. Results are expressed as mean ± SEM.

Rimonabant administration did not modify the daily mean amount of food consumed during the *ABA relapse* in all the experimental groups analyzed [Two-way ANOVA: *ABA* $F_{(10, 105)} = 0.5887$, $p = 0.8200$; *Restricted* $F_{(10, 105)} = 0.2247$, $p = 0.9933$; *Exercise* $F_{(10, 105)} = 1.778$, $p = 0.0736$; *Control* $F_{(10, 100)} = 0.4704$, $p = 0.9055$]. The following One-way ANOVA analysis, revealed a significant effect of the higher dose tested (0.3 $mg \cdot kg^{-1}$) in reducing the intake of food in *Restricted* animals on Day 0 [$F_{(2, 21)} = 4.569$, $p = 0.0225$].

3.2.5.3. *Effect of rimonabant on RWA*

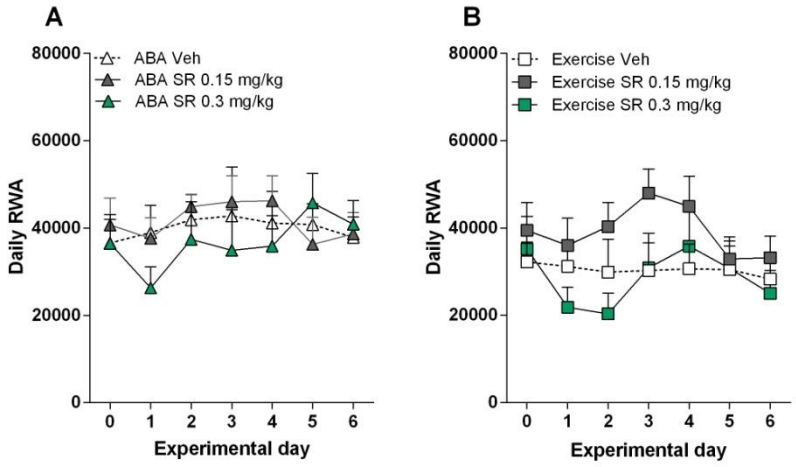


Figure 24. Dose-response curves showing the RWA of *ABA* (A) and *Exercise* (B) animals treated with vehicle or Rimonabant (0.15, 0.3 $mg \cdot kg^{-1}$, IP) during the six days of the *ABA relapse* phase. Results are expressed as mean ± SEM.

As reported in figure 24, the daily administration of rimonabant did not affect RWA in *ABA* and *Exercise* animals. Two-way ANOVA analysis revealed no significant interaction effect between variables Treatment and Days [*ABA*: $F_{(12, 126)} = 1.236$, $p = 0.2658$; *Exercise*: $F_{(12, 126)} = 0.9309$, $p = 0.5186$].

3.2.6. *Plasma measurements*

3.2.6.1. *Leptin, ghrelin and corticosterone basal levels*

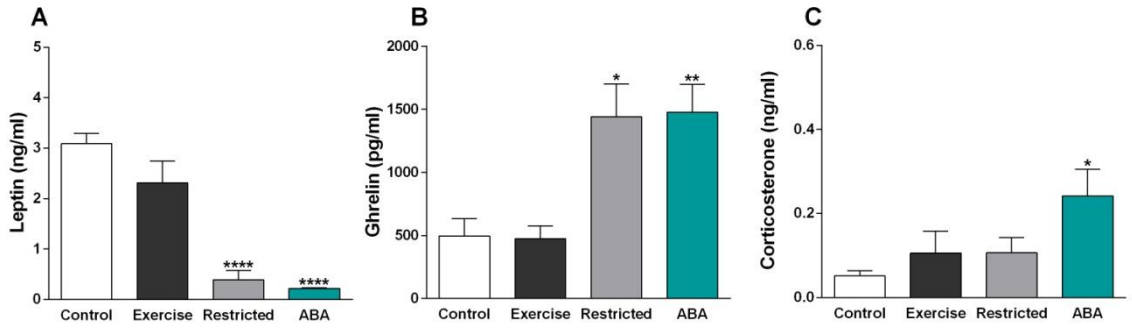


Figure 25. Basal plasma levels of leptin (A), ghrelin (B) and corticosterone (C), measured at the end of the *ABA relapse* phase. Data are expressed as the mean ± SEM. * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$ *vs* corresponding *Control*.

As shown in figure 25, at the end of the *ABA relapse*, significantly altered levels of leptin, ghrelin and corticosterone were observed. One-way ANOVA analysis of basal leptin levels yielded a significant result [$F_{(3, 17)} = 25.39$, $p < 0.0001$]. Bonferroni's *Post hoc* test evidenced significantly reduced levels in *ABA* and *Restricted* animals compared to *Control* animals ($p < 0.0001$). Ghrelin basal levels were significantly modified in this phase [One-way ANOVA: $F_{(3, 13)} = 10.78$, $p = 0.0008$]. Bonferroni's *post-hoc* test evidenced an increased concentration of ghrelin in *ABA* ($p < 0.01$) and *Restricted* ($p < 0.05$) animals compared to *Control* rats. Notably, corticosterone levels in *ABA* animals were significantly increased compared to *Control* animals [One-way ANOVA: $F_{(3, 16)} = 3.199$, $p = 0.0517$; Bonferroni's *post-hoc* test: $p < 0.05$].

3.2.6.2. *Effect of Δ^9-THC on leptin levels*

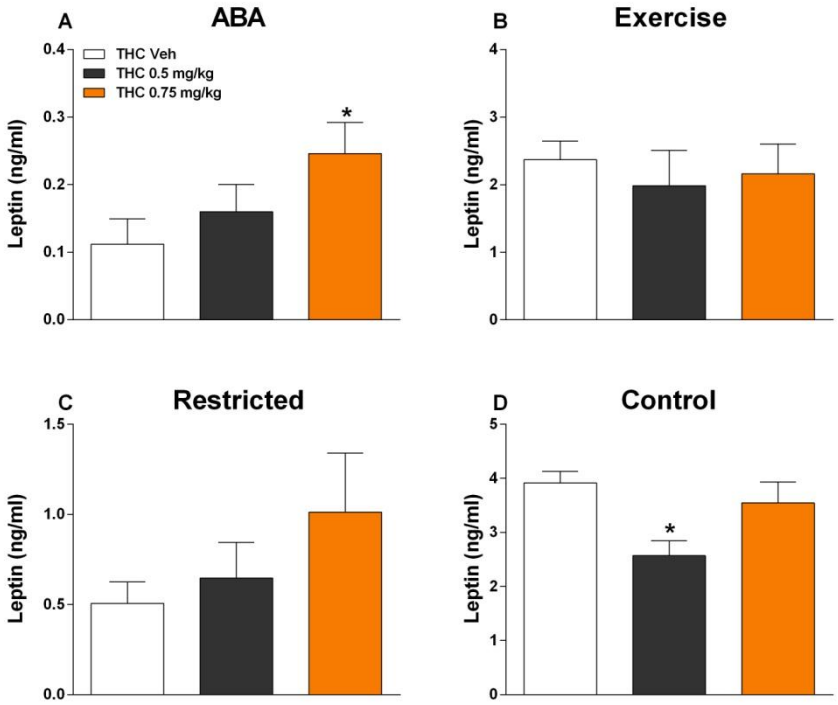


Figure 26. Effect of Δ^9-THC administration (0.5, 0.75 mg·kg^{-1}, IP) on leptin plasma levels in *ABA* (A), *Exercise* (B), *Restricted* (C) and *Control* (D) groups. Data are expressed as the mean ± SEM. * $p < 0.05$.

As shown in figure 26, Δ^9-THC treatment was able to affect leptin plasma levels at the end of the *ABA relapse*. In particular, the higher dose tested (0,75 mg·kg^{-1}) significantly increased leptin levels in *ABA* animals [One-way ANOVA: $F_{(2, 13)} = 2.779$, $p = 0.0989$; Bonferroni's *post-hoc* test: $p < 0.05$]. Moreover, the lower dose tested (0.5 mg·kg^{-1}) significantly reduced leptin levels in *Control* animals [One-way ANOVA: $F_{(2,14)} = 5.929$, $p = 0.0136$; Bonferroni's *post-hoc* test: $p < 0.05$]. No other significant effect of treatment was observed in *Exercise* [$F_{(2, 13)} = 0.2245$, $p = 0.8019$] and *Restricted* [$F_{(2, 15)} = 1.286$, $p = 0.3052$] animals.

3.2.6.3. *Effect of Δ^9-THC on ghrelin levels*

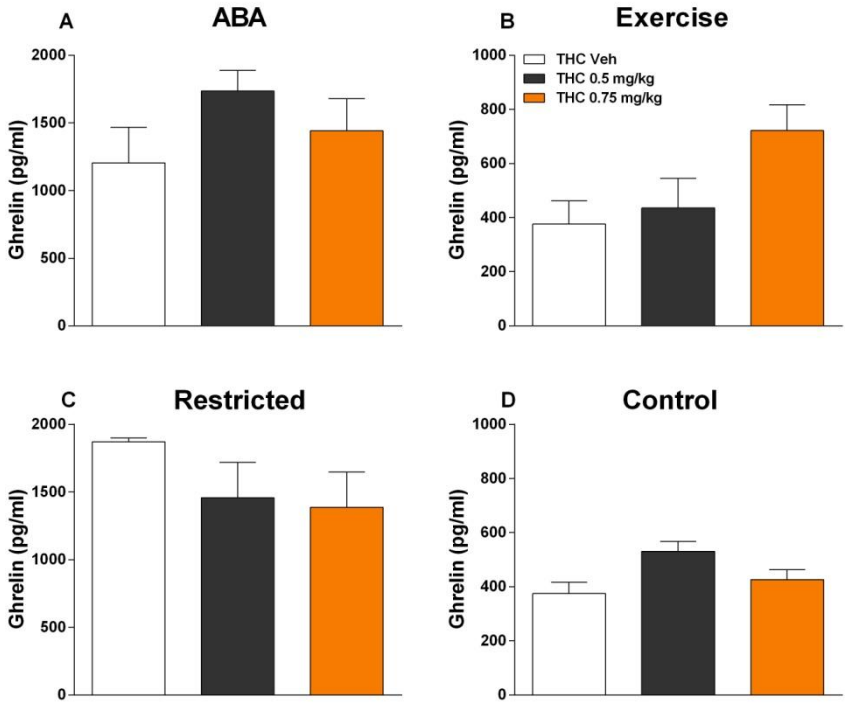


Figure 27. Effect of Δ^9-THC administration (0.5, 0.75 mg·kg^{-1}, IP) on ghrelin plasma levels in *ABA* (A), *Exercise* (B), *Restricted* (C) and *Control* (D) groups. Data are expressed as the mean ± SEM.

As shown in figure 27, Δ^9-THC administration, at both doses tested (0.5, 0.75 mg·kg^{-1}), did not modify ghrelin plasma levels at the end of the *ABA relapse* in all the experimental groups analyzed [One-way ANOVA: *ABA* $F_{(2, 6)} = 1.426$, $p = 0.3114$; *Exercise* $F_{(2, 6)} = 3.585$, $p = 0.0946$; *Restricted* $F_{(2, 5)} = 0.9490$, $p = 0.4473$; *Control* $F_{(2, 6)} = 4.391$, $p = 0.0669$)].

3.2.6.4. *Effect of Δ^9-THC on corticosterone levels*

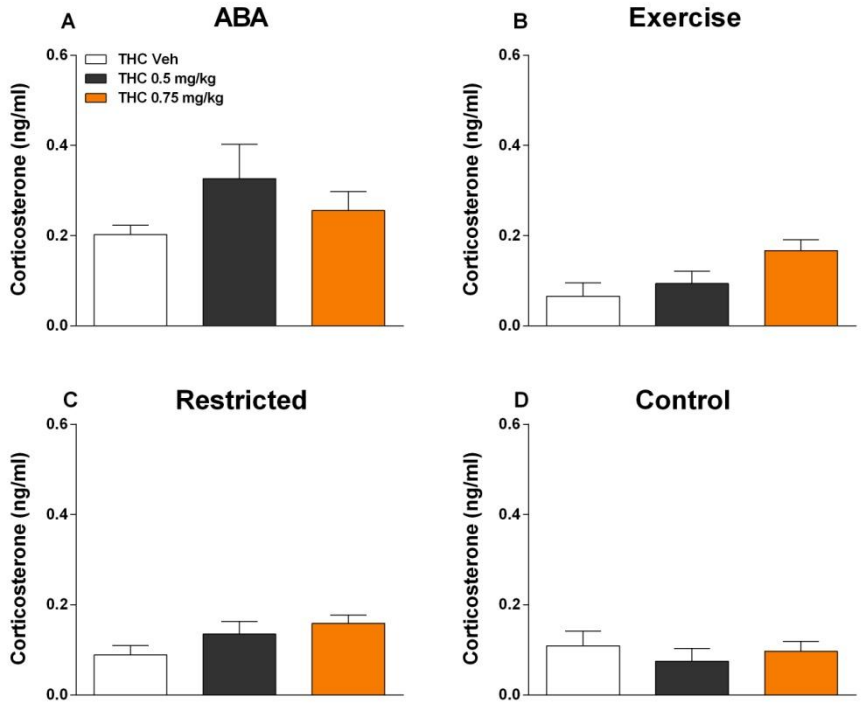


Figure 28. Effect of Δ^9-THC administration (0.5, 0.75 mg·kg^{-1}, IP) on corticosterone plasma levels in *ABA* (A), *Exercise* (B), *Restricted* (C) and *Control* (D) groups. Data are expressed as the mean ± SEM.

As shown in figure 28, Δ^9-THC treatment, at both doses tested (0.5, 0.75 mg·kg^{-1}), did not change corticosterone plasma levels in all the experimental groups analyzed [One-way ANOVA: *ABA* $F_{(2, 9)} = 2.421$, $p = 0.1442$; *Exercise* $F_{(2, 12)} = 3.702$, $p = 0.0559$; *Restricted* $F_{(2, 15)} = 2.463$, $p = 0.1189$; *Control* $F_{(2, 14)} = 0.9384$, $p = 0.6788$].

3.2.6.5. *Effect of CP-55,940 on leptin levels*

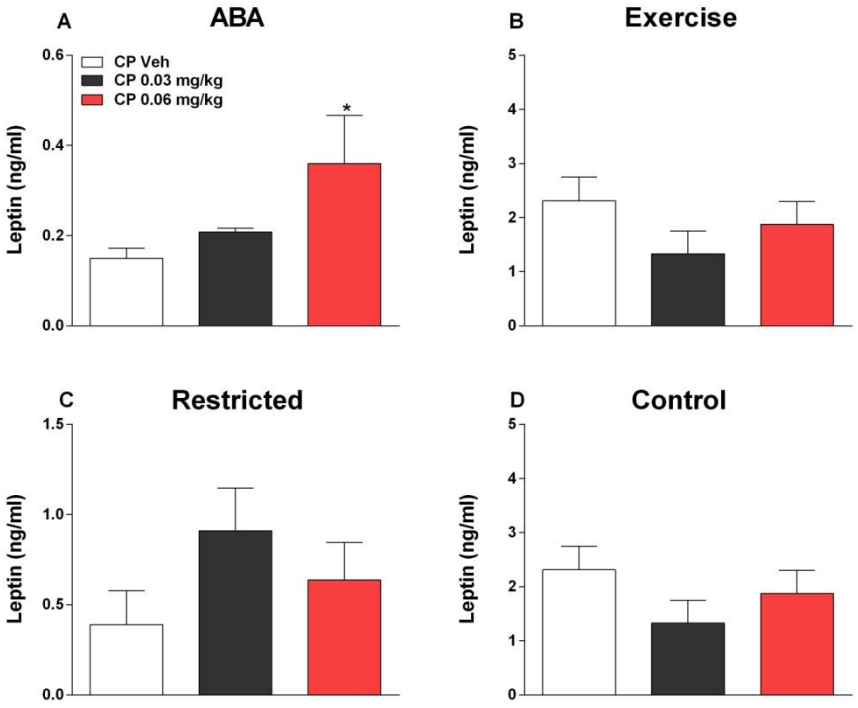


Figure 29. Effect of CP-55,940 administration (0.03, 0.06 mg·kg^{-1}, IP) on leptin plasma levels in *ABA* (A), *Exercise* (B), *Restricted* (C) and *Control* (D) groups. Data are expressed as the mean ± SEM. * $p < 0.05$.

As shown in figure 29, CP-55,940 administration, at the higher dose tested (0,06 mg·kg^{-1}) significantly increased leptin plasma levels in *ABA* animals [One-way ANOVA: $F_{(2, 15)} = 2.953$, $p = 0.0429$; Bonferroni's *post-hoc* test: $p < 0.05$]. No other significant effect of the treatment was revealed in *Exercise* [$F_{(2, 15)} = 1.319$, $p = 0.2967$], *Restricted* [$F_{(2, 11)} = 1.373$, $p = 0.2963$] and *Control* [$F_{(2, 12)} = 2.018$, $p = 0.1756$] groups.

3.2.6.6. *Effect of CP-55,940 on ghrelin levels*

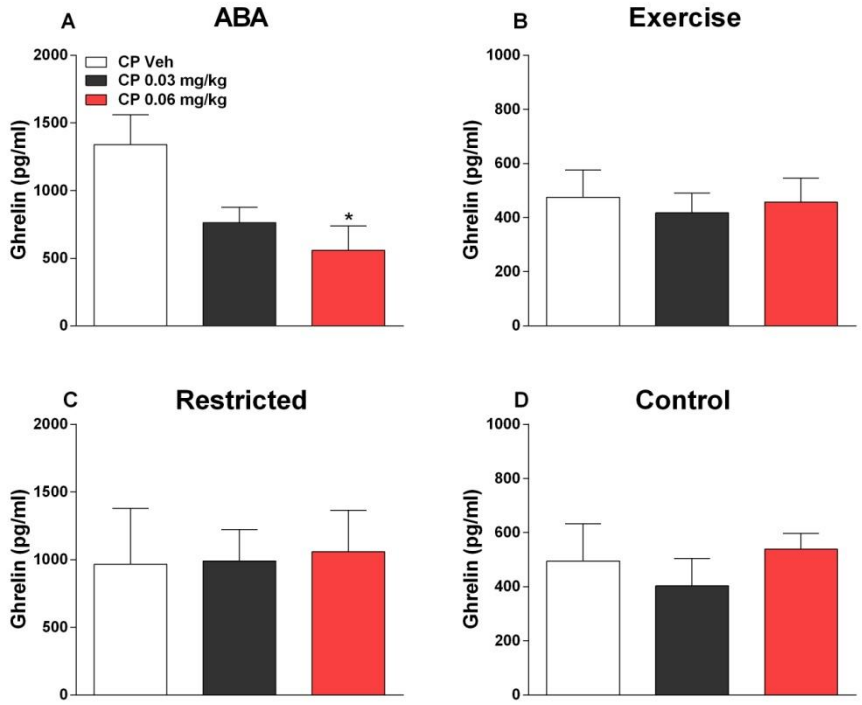


Figure 30. Effect of CP-55,940 administration (0.03, 0.06 mg·kg^{-1}, IP) on ghrelin plasma levels in *ABA* (A), *Exercise* (B), *Restricted* (C) and *Control* (D) groups. Data are expressed as the mean ± SEM. * $p < 0.05$.

As shown in figure 30, CP-55,940 administration, at the higher dose tested (0,06 mg·kg^{-1}) significantly reduced ghrelin plasma levels in *ABA* animals [One-way ANOVA: $F_{(2, 13)} = 5.415$, $p = 0.0195$; Bonferroni's *post-hoc* test: $p < 0.05$]. No other effect of treatment was seen in *Exercise* [$F_{(2, 13)} = 0.09985$, $p = 0.9057$], *Restricted* [$F_{(2, 12)} = 0.02402$, $p = 0.9763$] and *Control* [$F_{(2, 9)} = 0.2979$, $p = 0.7494$] animals.

3.2.6.7. *Effect of CP-55,940 corticosterone levels*

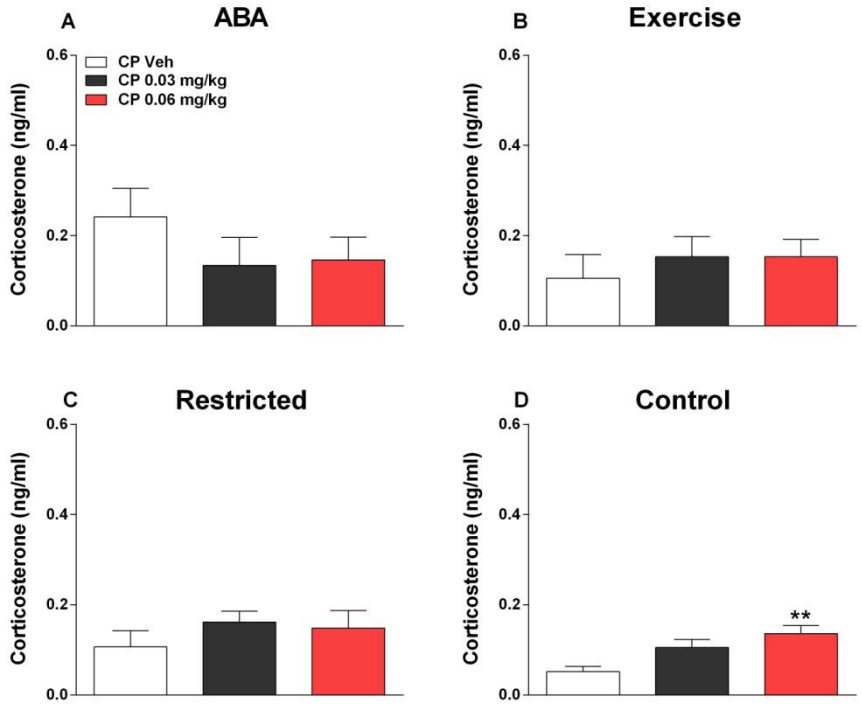


Figure 31. Effect of CP-55,940 administration (0.03, 0.06 mg·kg^{-1}, IP) on corticosterone plasma levels in *ABA* (A), *Exercise* (B), *Restricted* (C) and *Control* (D) groups. Data are expressed as the mean ± SEM. ** $p < 0.01$.

As shown in figure 31, CP-55,940 administration, at the higher dose tested (0,06 mg·kg^{-1}) significantly increased corticosterone plasma levels in *Control* animals [One-way ANOVA: $F_{(2, 11)} = 7.759$, $p = 0.0079$; Bonferroni's *post-hoc* test: $p < 0.01$]. No other significant effect of treatment was revealed in *ABA* [$F_{(2, 12)} = 1.011$, $p = 0.3928$], *Exercise* [$F_{(2, 14)} = 0.3499$, $p = 0.7107$] and *Restricted* [$F_{(2, 11)} = 7.759$, $p = 0.0079$] rats.

Chapter 4. Discussion

The present study was designed to deeply investigate the involvement of the endocannabinoid system in the neurobiology of anorexia nervosa. The possible neuro-anatomical and functional alterations of the endocannabinoid system as well as its potential role as a new pharmacological target in the treatment of this dramatic eating disorder, has been studied in young adolescence female rats subjected to the ABA model of anorexia nervosa showing behavioral and endocrine anorexic-like dysregulations typical of the human condition (Routtemberg and Kuznesof, 1967). Findings confirmed previous descriptions of a robust ABA phenotype displayed by rats undergoing a restricted regime of feeding associated with free physical activity. As a consequence of the limited access to food (1.5 h/day) and the concomitant availability of a running wheel, *ABA* rats significantly increased their RWA and dramatically reduced their body weight (Hall and Hanford 1954; Routtenberg and Kuznesof, 1967; Routtenberg, 1968). Interestingly, *Restricted* rats, that were subjected to the same food restrictive protocol, but had no access to the running wheel, lost significantly less body weight compared to the *ABA* group of animals, while *ABA* rats run significantly and progressively more than *Exercise* rats during the six days of the *ABA induction* phase. These findings indicate that the combination of the two variables in the ABA paradigm (*i.e.*, food restriction and physical activity) was effective in producing the food restriction-induced hyperactivity typical of *ABA* animals well described in the literature (Broocks *et al.*, 1990; Duclos *et al.*, 2005). In fact, when looking at the human condition, physical hyperactivity is present in 30-80 % of anorexic patients (Davis *et al.*, 1994; Klein *et al.*, 2007) and is generally considered a sort of strategy to lose weight

that may deteriorate into uncontrolled and obsessive-compulsive behavior (Davis *et al.*,1995; Holtkamp *et al.*, 2003).

Following the *ABA induction* phase, animals started the *Recovery* phase, designed to evaluate the effect of diet restoration on food consumption, body weight and RWA. During this period, food was available *ad libitum* and *ABA* and *Exercise* groups still had free access to the running wheel (Ratnovsky and Neuman, 2011; Achamrah *et al.*, 2016). For the entire duration of the *Recovery* phase, *ABA* animals consumed significantly more food compared to all the other groups evaluated. This enhanced food intake resembles the human condition in which anorexic patients need to eat an even larger amount of food compared to healthy controls to recover to a normal body weight (Kaye *et al.*, 1986, 1988). Such a hyperphagic reaction was associated with a significant physical hypoactivity particularly evident during the first days of restoration. This result is consistent with a previous study showing a reduction in the physical activity of ABA rats during *Recovery*, explained by authors as an energy balance reaction that clarifies the induced compensatory increment in food intake (Dixon *et al.*, 2003). Interestingly, under this condition, *Restricted* animals gained weight more rapidly than *ABA* animals that at the end of this phase still weight significantly less than all the other groups. This effect may indicate the necessity of a longer period of restoration to get back to a normal body weight. In agreement, it has been shown that after recovery, women with anorexia nervosa tend to maintain lower body mass indices compared to healthy people (Dellava *et al.*, 2011).

Together with the behavioral alterations reproduced during the *ABA induction* and *Recovery* phases of this study, *ABA* animals also displayed a typical endocrine dysregulation revealed by plasma measurements of the principal appetite regulating

hormones, leptin and ghrelin, and of the stress hormone corticosterone. In particular, with respect to *Control* group, at the end of the *ABA induction* phase *ABA* rats presented decreased plasma concentrations of leptin in agreement with previous findings reporting reduced peripheral serum and plasma leptin levels in anorexic patients (Grinspoon *et al.,* 1996; Hebebrand *et al.,* 1997) and in animals exposed to the ABA model of anorexia nervosa (Pardo *et al.,* 2010). Leptin is an anorexigenic messenger mainly synthesized in adipose tissues, and its concentration is related to the percentage of body fat since it increases with high adiposity and decreases after body fat loss (Maffei *et al.,* 1995). It functionally acts as a signalling hormone which, by means of specific receptors, affects energy homeostasis by increasing metabolism and limiting food consumption (Calandra *et al.,* 2003). Moreover, our results confirmed previous reports of increased ghrelin concentration in the plasma of both anorexic patients and ABA animals (Otto *et al.,* 2001; Krsek*et al.,* 2003; Pardo *et al.,* 2010). It has been demonstrated that central and peripheral administration of ghrelin are able to increase feeding in humans and rodents (Wren *et al.,* 2000; Druce *et al.,* 2005; Gilg and Lutz, 2006). The major proposed pathway of ghrelin-mediated increased appetite consists of its production at the gastrointestinal level and direct activation of the NPY/AgRP neurons in the hypothalamus (Chen *et al.,* 2004; Denis *et al.,* 2015). Due to their ability to modulate feeding, leptin (decreased) and ghrelin (increased) plasmatic concentration in *ABA* animals could be interpreted as a response to a situation of unbalanced caloric intake and energy expenditure.

We also found increased corticosterone plasma levels in *ABA* rats compared to *Control* rats at the end of the *ABA induction* phase. The activity of the hypothalamic-pituitary-adrenal axis (HPA) in the ABA model of anorexia nervosa has been already shown to

be increased (Burden *et al.*,1993). Duclos *et al.* (2009) found that the interaction between the increased activity of the HPA axis and the physical hyperactivity associated with caloric restriction is dependent upon corticosterone levels. They showed that diet-induced physical hyperactivity was absent in adrenalectomized rats and dose-dependently increased with corticosterone administration (Duclos *et al.*, 2009). On these basis, increased corticosterone in *ABA* rats may drive physical hyperactivity. Interestingly, we found that all these hormonal alterations were completely recovered at the end of the *Recovery* phase since leptin, ghrelin and corticosterone came back to basal *Control* group levels. This result is in agreement with previous findings of recovered leptin and ghrelin levels in anorexic patients undergoing a regime of food restoration (Stroe-Kunold *et al.*, 2016; Otto *et al.*, 2001). Thus suggesting that all these hormonal alterations may be a consequence rather than a cause of the anorexia nervosa condition and therefore secondary to the restricted consumption of food.

Biochemical study

Our data evidenced a significant reduction of arachidonic acid in the caudato putamen, amygdala and hippocampus, and a significant increase in the hypothalamus of *ABA* animals compared to *Control* rats at the end of the *ABA induction* phase. These altered levels were not completely reverted by the following *Recovery* phase. Arachidonic acid is known to be one of the most abundant polyunsaturated omega-6 fatty acid (PUFA) in the brain and its involved in different physiological functions (Bazinet *et al.*, 2014; Khan and He, 2015). It is an integral component of phospholipids membranes in mammals which acts as a precursor of a numerous biologically active lipid mediators generally indicated as eicosanoids (*e.g.*, prostaglandins, leukotrienes) and endogenous

cannabinoids (Fan *et al.,* 2012; Alexander *et al.,* 2007). Furthermore, arachidonic acid is also a common product of the MAGL and FAAH catalyzed hydrolysis reactions of 2-AG and AEA (Blankman *et al.,* 2007). On these basis, the altered arachidonic acid levels in our *ABA* animals on one hand may influence the subsequent altered endocannabinoids production, on the other hand could be secondary to an altered endocannabinoids metabolism (Cascio and Marini, 2015). It is interesting to note that arachidonic acid was significantly increased only in the hypothalamus, a key center in the regulation of feeding behavior. This alteration may be in some way related to the anorexic-like properties of arachidonic acid. In fact, Doggett and Jawaharlal (1977) demonstrated that both intraperitoneal and intragastric arachidonic acid administration were able to inhibit food intake in food-deprived rats. The authors also showed that this effect was similar to that induced by administration of the prostaglandine $F_{2\alpha}$, and that prior administration of the prostaglandin synthetase inhibitor indomethacin completely reverted this anorectic activity, thus suggesting that arachidonic acid may have to be converted to prostaglandins to exert its action on food consumption (Doggett and Jawaharlal, 1977).

When looking at measurements of endocannabinoids levels, our study evidenced altered levels of AEA and 2-AG in different regions of the brain in *ABA* animals. In particular, AEA levels were found to be significantly reduced in the cortex of *ABA* rats compared to *Control* rats at the end of the *ABA induction* phase, while 2-AG levels were reduced in the cortex, prefrontal cortex, nucleus accumbens and hippocampus, which are brain regions well known to be involved in the regulation of feeding behavior as well as mood and cognition (Morton *et al.,* 2006). These results are in agreement with previous findings reporting a dual effect of short or long-term food deprivation on brain

endocannabinoids levels in rodents. In particular, Kirkham and collaborators (2002) reported significantly increased concentrations of AEA and 2-AG in the limbic forebrain and of 2-AG in the hypothalamus of rats after 24h food deprivation. One year later, Hanus and colleagues (2003) found decreased 2-AG levels in the whole brain (including the hippocampus and the hypothalamus) of mice exposed to 12 days of food restriction. Interestingly, authors showed that reduction of 2-AG in the hypothalamus was dependent on the severity of food restriction, and confirmed previous findings of increased 2-AG levels in the mouse brain after 24h full starvation (Hanus *et al.*, 2003). These results seem to underline different strategies to cope with short or long-lasting starvation. The authors tried to explain the elevated 2-AG levels in the short term as a natural trigger of searching for food and eating behavior, while as regards prolonged starvation, survival may be aided by downregulating this orexigenic signal and reducing appetite and motivation to eat.

As regards to the fatty acid ethanolamides OEA and PEA, we found that both were significantly decreased in the hypothamalus of *ABA* rats at the end of the *ABA induction* phase. OEA and PEA are ligands for the peroxisome proliferator-activated receptor-α (PPARα) and the transient receptor potential vanilloid type-1 (TRPV1) channel, and have been recently known to act as mediators in eating modulation (Gaetani *et al.*, 2003; Lo Verme *et al.*, 2005; Movahed *et al.*, 2005). Primarily produced in the small intestine, upon the ingestion of dietary fat, OEA acts as a satiety signal in the CNS (Fu *et al.*, 2003; Romano *et al.*, 2014, 2015). Lower intestinal levels of OEA, that increase upon refeeding, have been reported in food-deprived animals, thus suggesting a stimulatory effect of feeding on OEA synthesis (Rodríguez de Fonseca *et al.*, 2001). Supporting this hypothesis, administration of OEA was found to produce a dose- and

time-dependent inhibition of food intake in mice and rats (Oveisi *et al.,* 2004; Nielsen *et al.,* 2004). Recent findings showed the anorexic and fat-losing effects of PEA administration in a rat model of obesity (Mattace Raso *et al.,* 2014) and demonstrated that histamine signalling is involved in the acute effect of OEA on food consumption (Provensi *et al.,* 2014). On the basis of these evidence, reduced OEA and PEA levels in the hypothalamus of *ABA* rats could be explained as a secondary effect due to the restricted regime of feeding imposed during the *ABA induction* phase. Notably, these reduced concentrations were in part reverted in the hypothalamus at the end of the *Recovery* phase, during which *ABA* animals had free access to food.

A further step of this study was to investigate possible alterations in CB1R density in specific regions of the brain of *ABA* animals. We found a significant reduction of the CB1R density in the lateral hypothalamus of *ABA* animals at the end of the *ABA induction* phase. Interestingly, our data show increased 2-AG concentration in the hypothalamus of *ABA* animals compared to *Control* animals at the end of the same phase. Even if this increase did not reach a significant value, it is plausible that such an altered concentration may have an effect on CB1R density in this particular nucleus of the hypothalamus. The lateral hypothalamus, identified as the "feeding center", is involved in the regulation of eating behavior since its lesion causes hypophagia in animals (Anand and Brobeck, 1951; Teitelbaum and Epstein, 1962; Boyle and Keesey, 1975; Oltmans and Harvey, 1976; Schallert *et al.,* 1977), while its electrical stimulation determines an increase in food and water consumption (Delgado and Anand, 1953; Mogenson and Stevenson, 1967; Coons and Cruce, 1968). Moreover, it has been shown that activation of the endocannabinoid system in the lateral hypothalamus positively modulates food intake (Pérez-Morales *et al.,* 2012; Sánchez-Fuentes *et al.,* 2016). On

the basis of these evidence, increased 2-AG levels in the hypothalamus of *ABA* rats could be interpreted as an attempt to restore an altered eating regulation as a consequence of the restricted schedule imposed, which could have influenced the receptor expression specifically in the lateral hypothalamus. Supporting this hypothesis, several studies demonstrated the physiological effects of chronic administration of CB1R agonists which results in a downregulation of this receptor in different regions of the brain (Breivogel *et al.*, 1999; Sim-Selley and Martin, 2002).

Our data also revealed reduced CB1R levels in the dentate gyrus of the hippocampus of *ABA* animals compared to *Control* rats. Notably, this reduction was associated with a significant decreased concentration of 2-AG in the hippocampus at the end of the *ABA induction* phase. In addition to the typical physiological effects of a receptor downregulation as a result of an increased agonist concentration, it has been shown that a decreased endocannabinoid content could also drive a subsequent decrease in the CB1R density that may be due to the absence of a trophic factor necessary for the receptor expression, in this case, its endogenous agonist (Romero *et al.*, 1995). Alternatively, changes in CB1R density and endocannabinoids content could occur independently, and may be driven by other physiological alterations. For example, Hill and collaborators (2005) showed decreased 2-AG levels and CB1R expression in the hippocampus of rats as a result of a chronic stress exposure (Hill *et al.*, 2005). Moreover, authors reported increased corticosterone plasma levels in the same animals. Interestingly, it has been suggested that glucocorticoids exert a negative regulating action over CB1R transcription (Mailleux and Vanderhaeghen, 1993). Notably, the ABA phenotype, which results from the activity-induced reduction of feeding, has been reported to be emphasized by the stressful effect of social isolation (Boakes *et al.*,

1997). In agreement, we found an increased corticosterone concentration in the plasma of *ABA* animals that, in light of these evidence, could be related to the altered CB1Rs expression reported. Importantly, these reductions were in part reverted to *Control* animals at the end of the *Recovery* phase.

Pharmacological study

ABA rats appeared to respond well to the stimulation of the endocannabinoid system, since some of the typical pathological traits reproduced were positively influenced by pharmacological treatments. In particular, during the *ABA relapse* phase, both natural and synthetic cannabinoid receptor agonists, THC and CP-55,940, were able to significantly attenuate body weight loss in *ABA* animals compared to vehicle-treated rats. On the contrary, both agonists tested did not affect body weight in all the other experimental groups under the same regime of treatment. Moreover, endocannabinoid system stimulation was able to increase food consumption in *ABA* and *Restricted* animals given limited access to food, particularly during the first day of treatment. The lack of effect on food intake observed during the remaining days of treatment supports previous demonstration of a transient hyperphagic effect following THC administration (Rahminiwati *et al.*, 1999). Regarding physical activity, both THC and CP-55,940 significantly attenuated RWA in *ABA* animals without affecting physical activity in *Exercise* animals, which suggests that this effect was specific for *ABA* animals in which the combination of food restriction and free access to a running wheel per sè leads to the development of uncontrolled physical activity. Lewis and Brett (2010) attempted to reverse some of the typical behavioral manifestations of the ABA paradigm by administering THC and obtained a positive effect on food intake but no significant

effects on body weight or RWA. Beside restoration of food consumption, THC-treated ABA mice also displayed high mortality rates, possibly as a consequence of the hypothermic effect of THC and/or of a compromised thermoregulatory system in food-restricted animals (Lewis and Brett, 2010). On the contrary, Verty *et al.* (2011) showed the effectiveness of subchronic THC administration in attenuating weight loss in ABA female rats. More specifically, they found that THC transiently stimulates chow intake and significantly reduces weight loss with a moderate effect on RWA (Verty *et al.*, 2011). The discrepancies among the above mentioned studies could reflect important species-specific differences as administration of cannabinoids produced contrasting results in rats and mice also in other experimental animal models (Berrendero and Maldonado, 2002; Valjent *et al.*, 2002; Patel and Hillard, 2006). Notably, administration of the antagonist rimonabant had a significant effect in reducing body weight only in *Control* animals, without influencing body weight in all the other experimental groups (Karlsson *et al.*, 2015). Moreover, at both doses tested it did not significantly affect food intake and RWA in all experimental groups.

Plasma measurements performed at the end of the *ABA relapse* revealed significant alterations of the hormones leptin, ghrelin and corticosterone similar to those observed at the end of the *ABA induction* phase. In particular, basal leptin plasma levels were found to be significantly decreased in *ABA* animals compared to the *Control* group. Both agonists tested, THC and CP-55,940, were able to revert and increase leptin concentration in *ABA* rats compared to vehicle treated animals. Recent evidence proposed a possible involvement of leptin signal in the hyperactivity displayed by the majority of anorexic patients (Ehrlich *et al.*, 2009). Supporting this idea, leptin administration was demonstrated to be effective in reducing hyperactivity displayed by

ABA animals (Hillebrand *et al.*, 2005, 2008; Exner *et al.*, 2000). As mentioned previously, we found that THC and CP-55,940 reduced RWA in *ABA* animals, an effect that could be associated to the capacity of both cannabinoids to increase leptin signalling in *ABA* animals. In agreement with this hypothesis, it has been recently shown the ability of leptin to reduce locomotor activity in the ABA model by acting in the VTA, which takes part to the mesolimbic reward system where CB1Rs are abundantly expressed (Herkenham *et al.*, 1990; Verhagen *et al.*, 2011). When looking at the other experimental groups, it is interesting to note that also *Restricted* and *Exercise* groups presented reduced leptin plasma levels at the end of this phase. This indicates that even if the combination of this two factors, food restriction and exercise, was more effective in reducing leptin concentration in *ABA* animals, the single variables per sè were also able to influence leptin levels. This result is in agreement with previous demonstration of decreased leptin following diet restriction and exercise in both humans and rats (Ahima *et al.*, 1996; Iwasa *et al.*, 2016; Soch *et al.*, 2016). Notably, these levels were not influenced by the administration of THC or CP-55,940.

Ghrelin plasma levels were found to be increased in *ABA* animals compared to *Control* animals at the end of the *ABA relapse* phase. Pharmacological treatments revealed that administration of the synthetic CB1R agonist CP-55,940 was able to significantly revert the increased plasma levels of ghrelin in *ABA* animals compared to vehicle treated rats. It has been recently shown that ghrelin acts not only as an orexigenic mediator but also influences the regulation of reward and motivated behaviors. For example, a functional brain imaging study conducted on healthy subjects showed the effect of intravenous administration of ghrelin in increasing the neural responses to food pictures in different brain areas involved in reward processes and appetitive behavior (Malik *et al.*, 2008).

Also, recent evidence demonstrated that injection of ghrelin into the third ventricle of mice significantly increased locomotor activity and extracellular dopamine levels in the nucleus accumbens (Jerlhag *et al.,* 2006, 2007). Moreover, interaction between ghrelin and the endocannabinoid system has been previously demonstrated since the inhibitory effect of central and peripheral administration of rimonabant on the orexigenic effect has been shown (Tucci *et al.,* 2004; Rigamonti *et al.,* 2006). On these basis, it can be proposed that an increased secretion of ghrelin in *ABA* animals could be involved in the altered activation of the central reward circuit which overrides the physiological necessities of food ingestion. As such, eating is reduced and physical activity is increased despite the need for calorie ingestion, due to the rewarding related feelings of these extreme condition. Interestingly, ghrelin has been recently tested as a possible candidate for the pharmacological therapy of anorexia nervosa in the ABA model with positive results (Legrand *et al.,* 2016).

At the end of the *ABA relapse* phase, *ABA* rats also showed elevated plasma concentration of corticosterone compared to the *Control* group. A link between higher HPA axis activation and increased running associated with food restriction have been proposed (Duclos *et al.,* 2005). Moreover, both caloric restriction and excessive physical activity by itself are supposed to increase HPA axis activation in anorexia nervosa (MacDonald *et al.,* 2014; Klein *et al.,* 2007). Interestingly, we found that CP-55,940 administration significantly decreased corticosterone plasma levels in *ABA* rats. It has been shown that the endocannabinoid system signalling acts to suppress the HPA-axis activity within the prefrontal cortex, amygdala and hypothalamus (Hill *et al.,* 2012). Moreover, Andries *et al.* (2015) suggested that dronabinol therapy is able to alleviate the increased HPA-axis activity in women with severe and chronic anorexia

nervosa. These results may indicate the possible influence of the endocannabinoid system stimulation in reducing the elevated physical activity through inhibition of the HPA axis hyperactivation already seen in *ABA* animals.

Conclusions

Despite the increasing knowledge on the neurobiology of anorexia nervosa, at present, no pharmacological therapies have been approved for the treatment of this dramatic eating disorder. Our findings show that the endocannabinoid system appear to be dysregulated in animals undergoing the ABA paradigm for anorexia nervosa. Moreover, our results evidenced the positive effect of cannabinoid agonists which positively affect all the typical pathological traits reproduced in anorexic-like animals, particularly reducing the physical hyperactivity and consequently the body weight loss, but also increasing leptin signalling, reducing ghrelin and stabilizing the HPA axis activity during the *ABA relapse* phase. The pharmacological manipulation of the endocannabinoid system shows promising effects for the treatment of anorexia nervosa and for relapse prevention, specifically in subjects where physical hyperactivity plays a central role in the pathogenesis and progression of this disorder.

List of abbreviations

OMDM-2	(S)-N-oleoyl-(10-hydroxybenzyl)-20-ethanolamine
AA	arachidonic acid
ACTH	corticotropin
AEA	N-arachidonoyl-ethanolamine
2-AG	2-arachidonoyl-glycerol
AgRP	agouti-related protein
AMPK	AMP-activated protein kinase
Amy	amygdala
AN	anorexia nervosa
ARC	arcuate nucleus
CART	cocaine- and amphetamine-regulated transcript
CB1R	cannabinoid type 1 receptor
CB2R	cannabinoid type 2 receptor
CNS	central nervous system
CSF	cerebrospinalfluid
CRH	corticotropin-releasing hormone
DA	dopamine
DAG	diacylglycerol
DAGL	diacylglycerol lipase
DSM-V	Diagnostic and Statistical Manual of Mental Disorders
DMN	dorsomedial nucleus
FAAH	fatty acid amide hydrolase
GH-R	ghrelin receptor
GPCRs	G-protein-coupled receptors
Hipp	hippocampus
HVA	homovanillic acid

ICV	intracerebroventricularl
KO	knockout
LH	lateral nucleus
MCH	melanin concentrating hormone
MAGL	monoacylglycerol lipase
NAcc	nucleus accumbens
NAPE	N-acyl phosphatidylethanolamine
NAPE-PLD	N-acyl phosphatidylethanolamine-specific phospholipase D
NAEs	N-acylethanolamines
NAT	N-acyltransferase
NE	norepinephrine
NPY	neuropeptide Y
OB-R	leptin
OEA	N-oleoylethanolamine
PEA	N-palmitoylethanolamine
PET	positron emission tomography
PFC	prefrontal cortex
PVN	paraventricular nucleus
PLC	phospholipase C
PLD	phospholipase D
POMC	pro-opiomelanocortin
5-HT	serotonin
SRIs	serotonin reuptake inhibitors
TRH	thyrotropin releasing hormone
TrpV1	transient receptor potential cation channel vanilloid type 1
VMN	ventromedial nucleus
VTA	ventral tegmental area
α-MSH	α-melanocyte stimulating hormone

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