Oleoylethanolamide in the homeostatic and non-homeostatic control of eating

A Dissertation in Fulfillment of the Requirements for the

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Index

1. Chapter I:
General Introduction

2. Chapter II:
Role of the area postrema in the hypophagic effects of oleoylethanolamide

3. *Chapter III:*

Evaluation of the brain distribution of oleoylethanolamide and its analogues after acute systemic administration in rats

4. *Chapter IV:*

Satiety factor oleoylethanolamide prevents binge-like palatable food consumption induced by stress in female rats with a history of food restriction

5. *Chapter V:*
Conclusions

Chapter I

General introduction

1.1. Obesity and eating-related disorders

Obesity and overweight, defined as abnormal or excessive fat accumulation, are currently considered as major worldwide public health issues [1]. According to the World Health Organization (WHO), obesity is recognized as a global epidemic that has been spreading all over the world contributing to the higher incidence of major health problems, thus increasing mortality. The high levels of intra-abdominal or visceral fat that are associated with this clinical condition are the most responsible for the development of the main obesity comorbidities such as hypertension, dyslipidemia, type 2 diabetes, osteoarthritis, and changes in the reproductive system [2].

Obesity is a multifactorial disease and among the several conditions that predispose to the weight gain and consequently to obesity, it is well known that genetic, environmental and physiologic factors play a key role [3].

Moreover, some medications can contribute to the development of this complex disease by increasing appetite, reducing the basal metabolic rate and by stimulating adipose cells proliferation as well. Among the pharmacological classes that might be more commonly involved in this process there are antidepressants and corticosteroids [4,5]. More rarely obesity is the consequence of other medical conditions including Prader Willi [6] and Cushing syndrome [7], hypothyroidism or hypothalamic dysfunctions [8].

The current most commonly used method for classifying obesity is the body mass index (BMI), calculated as weight (kg) divided by the square of the height (m^2), which ranges between 25 and 29.9 in overweight individuals whereas it is equal to or more than 30 in obese-classified patients [9,10]. Furthermore, waist circumference, another marker of excess body fat, has becoming discriminating as a measure of overweight/obesity [11].

The WHO estimated that in 2016 more than 1.9 billion adults, over the age of 18 years old, were overweight, of these over 650 million were obese [12].

Since over the past 40 years it has been registered a huge increase in the percentage of overweight and obese individuals worldwide, it has been estimated that by 2030 the 38% of the world's adult population will be overweight and another 20% will be obese [13].

Moreover, changes in body weight are frequently accompanied by psychological and psychosocial problems thus leading to persistent disrupted eating behaviours, which are defined as eating disorders (EDs).

EDs are currently considered, likewise obesity, major health problems worldwide and they are characterized by altered dietary habits and impaired physical health. The 5th edition of the Diagnostic and Statistical Manual of Mental Disorders (DSM-V) recognizes three different EDs: anorexia nervosa (AN), bulimia nervosa (BN), and binge eating disorder (BED) [14]. The latter will be abundantly discussed in the following paragraph since it is one of the main topic of this experimental dissertation.

Although EDs can occur in adults or young individuals indiscriminately, adolescence represents the most vulnerable period for the onset of these diseases [15].

The main criteria for classifying an individual affected by AN are represented by: a persistent caloric restriction followed by a considerable body weight loss with a BMI of 17.5 or even less [16]; a strong fear to gain weight along with a distorted body image perception [14].

AN occurs more commonly among women than men (80-90% of patients with AN are female) and it reaches a peak incidence in adolescents between 14 and 17 years [15]. Moreover, AN is often linked with depressive disorder [17], obsessive compulsive disorder and autism spectrum disorder [18], counting the highest mortality of any psychiatric disease [19].

BN is primarily characterized by recurrent overeating episodes followed by compensatory purging behaviours such as vomiting, use of laxative or excessive physical activity whose purpose is avoiding body weight gain [17]. Likewise the AN, BN is also more common in females than males occurring in 1-2% of women mainly between 20 and 30 years [20]. BN can also take place along with other psychological problems such as affective disorders, impulse control disorders, attention deficit/hyperactivity disorder (ADHD), drug dependence, anxiety and dissociative disorders [17].

1.1.1. The binge eating disorder (BED)

BED is the most frequent eating-related maladaptive disorder, characterized by compulsive and distressing overeating of high palatable food (HPF), a food with high fat or sugar content and consequently rich in kilocalories.

The above-delineated situation is called "binge eating episode", which is defined, following the description of the DSM-V, by two main features: 1) the individual consumes a huge quantity of food in a short period of time, 2) along with a strong sense of loss of control, feeling of shame, guilt, disgust and anxiety [15].

Binge eating episodes occur at least once a week for three months. BED is classified as moderate when the weekly binge eating episodes are 1-3, while it is considered as severe when they become more frequent rising to 14 or even more [14].

It is possible to distinguish three different feeding behaviours, which can be at the bases of binge eating episodes:

- *Restrictive eating:* food consumption is predominantly driven by the appearance, smell and brand of the food; this particular behaviour causes loss of control and overeating episodes more frequently;
- *Emotional eating*: feeding as an escape strategy to fight negative emotions such as anxiety, sadness and stress;
- *External eating:* the feeding behaviour is mainly triggered by external stimuli including the sight and smell of the food [21].

Due to its critical features, BED leads to reduced social and emotional functioning, health impairments and worsens the quality of life [15].

BED differs from the other eating disorders, since it is not characterized by extreme compensatory strategies such as vomiting, use of laxative or increased physical activity [14,22].

It has been observed that the excessive intake of certain foods under specified conditions is able to alter not only eating behaviour but also it induces changes in the brain that resemble an addiction-like state. In particular, growing evidence found that one of the central mechanisms disrupted in BED conditions is represented by the dopamine (DA) rewarding circuitry.

BED is not only linked with the DA system, on the contrary it has been also demonstrated that an impairment in brain serotonin or 5-hydroxytryptamine (5-HT) signalling might play a role in the pathophysiology of BED in humans [23–26].

1.2. Neurobiological mechanisms regulating energy homeostasis

Overweight and obesity derive by an imbalance of the energetic metabolic rate. Our body, in fact, uses the food ingested-derived energy to manage the complete functioning of the main physiological systems at basal or active conditions, process that is called energetic balance. The excess energy quantity is normally stored and it is the primary responsible for fat accumulation leading to obesity development.

Several mechanisms partake in the control of energy balance by sending pro-satiety or pro-appetite signals to the brain in order to regulate energy consumption. Among these, white adipose tissue and liver play a key role in storing energy in the form of fat (energy storing) and in regulating the energy storage and consumption cycle, respectively [27].

The central nervous system (CNS) exerts the most crucial role in controlling feeding behaviour and energy homeostasis by interacting with several other systems. Most of the individuals, in fact, are able to maintain a stable body weight [28] thanks to a homeostatic regulation involving hypothalamic nuclei which integrate orexiant and anorexiant signals coming from other brain regions or from the periphery [29]. The hypothalamus (HYPO) is a structure of the CNS located on the sides of the third ventricle [30] that regulates the energy metabolism by producing several neuropeptides [29]. Moreover, it also partakes in neuronal networks that consist of non-peptidergic neurotransmitters including noradrenaline (NA), DA, 5-HT, histamine and endocannabinoids [29]. The hypothalamic system is also connected with extra-hypothalamic areas primarily located in the brainstem such as the dorsal motor nucleus of the vagus (DMV), the nucleus of solitary tract (NST), the area postrema (AP) and the parabrachial nucleus (PB) [31]. In this regard, it has been demonstrated that hindbrain neurons from the NST and the AP project to the HYPO by sending signals and building integrative networks [29].

Among the several nuclei of the HYPO, the arcuate (ARC), paraventricular (PVN), supraoptic (SON), and tuberomamillary (TMN) nuclei are the most studied nuclei for their pivotal role in integrating appetite-regulating signals.

The ARC is situated in close contact with the median eminence (ME), a circumventricular organ with fenestered capillaries and a weak blood brain barrier (BBB) [32]. Therefore, the ARC, via the ME, can represent a receptive region for circulating signals regulating energy balance such as leptin and insulin [33]. It consists of two distinct neuronal populations which are called "first order" neurons and exert opposite effects in regulating energy homeostasis: 1) the agouti-related protein (AgRP)/neuropeptide Y (NPY) neurons that are responsible for stimulating food intake, and 2) those co-expressing the pro-opiomelanocortin (POMC) and cocaine-and amphetamine regulated transcript (CART) exerting anorexigenic effects [34] (Fig. 1.1). Both the two neuronal populations, POMC/CART and NPY/AgRP neurons, project to a variety of hypothalamic nuclei such as PVN, the lateral hypothalamic area (LHA) and

the perifornical area (PFA), each containing ''second-order'' neurons that integrate the information (Fig. 1.1).
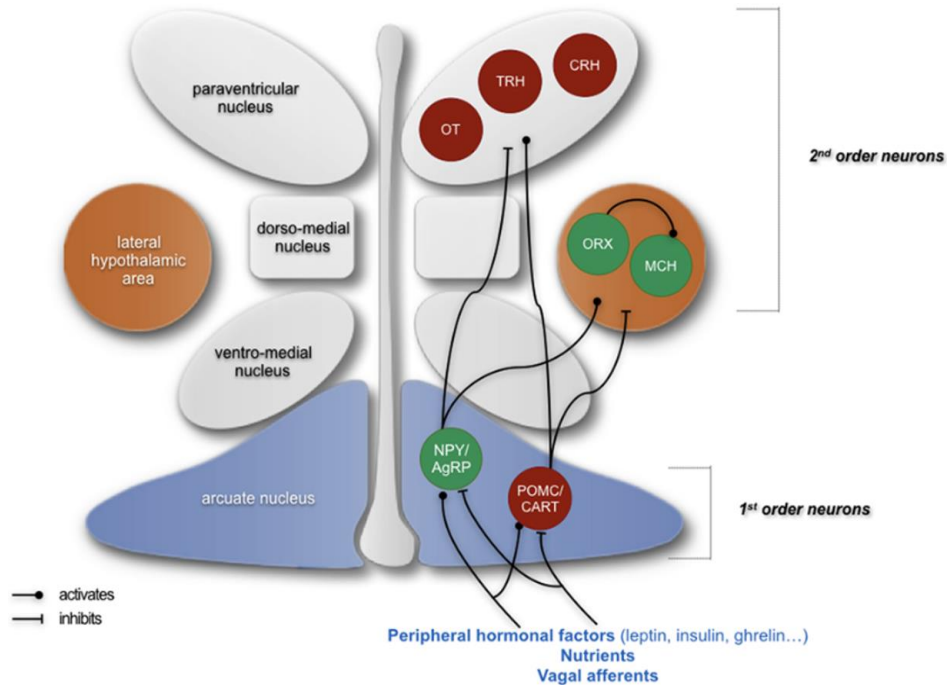
Fig. 1.1: schematic representation of the neuropeptidergic hypothalamic networks involved in the homeostatic control of food intake. Abbreviations: CRH, corticotropin-releasing hormone; MCH, melanin-concentrating hormone; NPY/AgRP, neuropeptide Y/agouti-related protein; ORX, neurons producing orexigenic peptides orexin; OT, oxytocin; POMC/CART, pro-opiomelanocortin/cocaine-and amphetamine regulated transcript; TRH, thyrotropin-releasing hormone [42].

The PVN represents a crucial hypothalamic nucleus involved in the control of feeding behaviour [35–37]. It is composed of magnocellular and parvocellular divisions. The magnocellular neurons are oxytocinergic and vasopressinergic neurons, which release oxytocin (OXY) and vasopressin (AVP) in the neurohypophysis [38]. On the other hand, the medial parvocellular neurons contain corticotropin-releasing hormone (CRH) and thyrotropin-releasing hormone (TRH) which are secreted into the hypophysial portal vessels in the ME [39], whereas the dorsal, ventral and lateral subdivisions of parvocellular neurons mainly project to the periaqueductal grey matter, PB and NST [38–41]. The SON neurons, likewise those located in the PVN, have been demonstrated to produce either OXY or AVP and they all project to the neurohypophysis. Furthermore, these neurons can also co-release a variety of other peptides such as cholecystokinin (CCK) and CRH, among others.

Several lines of evidence suggest that the TMN is the main histaminergic cell group in the rat brain which projects to a number of brain regions such as the nucleus accumbens (Acb), the prefrontal cortex (PFC) and other hypothalamic nuclei [43]. Thanks to its

widespread innervation, the TMN exerts a control in many physiological functions by regulating the daily food consumption, the glucose absorption as well as emotional and memory processes [44,45].

The above-described complex neuronal network is responsible for the long-term regulation of energy balance and is primarily involved in the maintaining of a stable body weight.

On the other hand, a short-term regulation modulates the daily consumption of food, the number and duration of meals and it is modulated by the physico-chemical signals that are generated in the gastrointestinal tract upon the meal ingestion [46]. Therefore, the short-term regulation of feeding behaviour occurs at the level of mesencephalic nuclei such as DMV and NST, that project to the PB, that, in turn, sends innervation to the PVN [46] (Fig. 1.2).

The NST is considered as an entry point for a variety of peripheral signals that are responsible for lowering the quantity of food consumed upon a meal [47]. As already mentioned in the text, projections from the NST, in particular noradrenergic neurons deriving from the A2 region, reach the hypothalamic neurons in the PVN [47]. Moreover, growing evidence showed that the ablation of these noradrenergic projections prevents the anorexigenic effects of many pro-satiety endogenous signals, such as the CCK. The NST consisted of distinct neuronal populations that, depending on their specific localization within different NST subnuclei, react in different ways to the central and visceral stimuli [48]. These subnuclei are innervated in a selective and specific manner by afferents fibres arising from specific segments of the gastrointestinal tract [48]. For instance, the gustatory nerves end in the most rostral portion of the NST [49], whereas the vagal afferents mainly project to the caudal part of the nucleus [50]. Furthermore, the neurons of the dorsomedial subnucleus (SolDM) of the NST are particularly sensitive to gastric distention [51], on the contrary the neurons of the commissural part (SolC) mostly respond to the duodenal signals and those located in the medial portion (SolM) are responsive to both gastric and duodenal signals [51].

Moreover, it has been demonstrated that the SolM responds to leptin that acts directly on SolM neurons to reduce food intake [52]. In 1986, Edwards et al., demonstrated that the lesions that extensively damage both medial and commissural subnuclei of NST markedly attenuate CCK-suppression of food intake [53].

An additional brain region crucially involved in food intake regulation is the AP, a circumventricular organ highly vascularized that displays a weak BBB by virtue of its

lack of tight junctions and the presence of fenestrated capillaries [54], features that give to the AP the appearance of a sponge.

The AP is the most caudal of the sensory circumventricular organs [54], located at the apex of the calamus scriptorius in the dorso-medial medulla oblongata, at the base of the fourth ventricle and it appears as a hump of tissue surrounded by the NST [31].

The AP consists of specialized ependymal cells that make up a unique capillary architecture in the ventricles and central canal [55] and it can be subdivided into several zones depending on cell type location and projection patterns [54]. The central and mantle zones predominantly contain neuronal cell bodies and axons which are in close contact with the ependymal cells, while in the ventral zone mainly reside glia [56].

Although the AP has been considered, for many years, for its function as the "chemoreceptor trigger zone" [57,58], it is now well recognized also its role in the regulation of cardiovascular system, metabolism, immune function as well as in the control of cerebrospinal fluid (CSF) balance [54]. In this context, recent studies have suggested that the AP expresses a variety of receptors for many hormones involved in the control of feeding behaviour including amylin, glucagon-like peptide-1 (GLP-1), ghrelin, CCK, NPY, AVP, substance P and insulin [59–68]. Moreover, through its efferent projections, the AP can in turn signal to important autonomic control centres behind the BBB and regulate the autonomic nervous system [54]. Studies conducted in rats in which the AP has been lesioned, recognise the important role of this hindbrain region in the homeostatic control of food intake and body weight as well as in the modulation of peripheral signals [69]. The AP is therefore considered as an entry and integration point in the CNS for a variety of blood-borne substances [54].

Because of its anatomical location, the AP is in close contact with the NST, mostly at -13,80 mm from the bregma where it reaches the maximum extent [31]. Several evidence [70,71] suggests that AP sends major and minor efferents to several nuclei in both the brainstem and the HYPO.

AP primarily projects to the lateral parabrachial nucleus (LPBN) and to the subnuclei of the NST, among which the most innervated is the SolM [70–76] (Fig. 1.2). Furthermore, minor projections innervate the nucleus ambiguus, DMV, dorsal regions of the tegmental nucleus, cerebellar vermis, paratrigeminal nucleus, ventrolateral catecholaminergic column in the medulla, and the spinal trigeminal [70,71] (Fig. 1.2). Moreover, the AP also receives afferents from the NST and LPBN but also from functionally distinct regions of the brain such as the hypothalamic PVN and

dorsomedial nucleus (DMH) [70,71,77]. Additionally to this central input, the AP receives also afferents from the periphery, in particular from the vagus nerve [70,71] (Fig. 1.2).
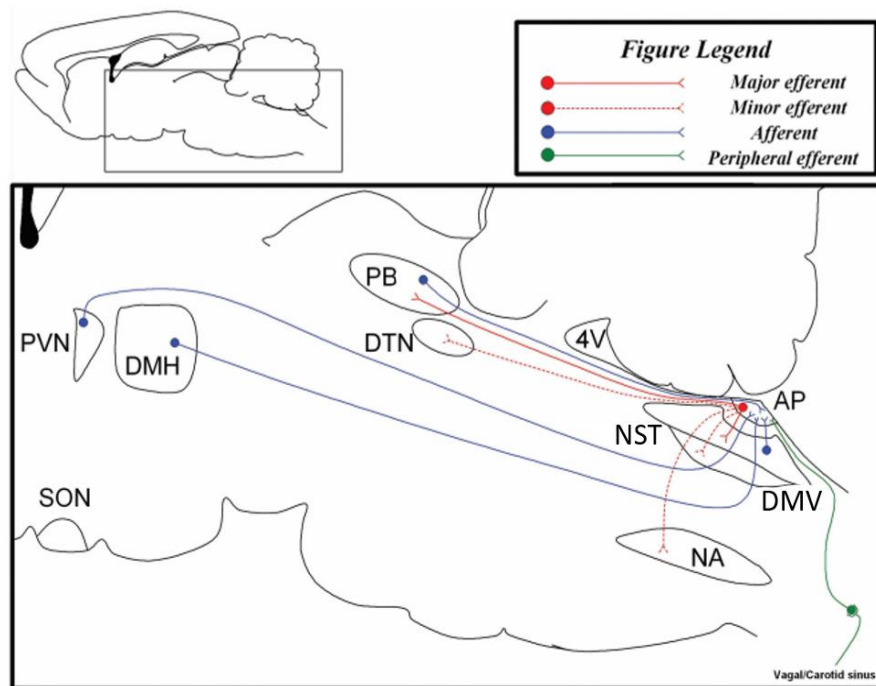
Fig. 1.2: Neuronal projections to and from the area postrema in the central nervous system (afferents (blue) and efferents (red)). 4V = 4th ventricle; AP = area postrema; DMH = dorsomedial hypothalamus; DMV = dorsal motor nucleus of the vagus; DTN = dorsal tegmental nucleus; NA = nucleus ambiguus; NST = nucleus of the solitary tract; PB = parabrachial nucleus; PVN = paraventricular nucleus of the hypothalamus; SON = supraoptic nucleus. Adapted from [54].

In addition to catecholaminergic and serotonergic neurons [78,79], AP has been shown to abundantly express also noradrenergic fibres [76] as well as neurons containing GABA, substance P, encephalin, neurotensin, and CCK [78,80–83]. Moreover, catecholaminergic and CCK neurons, which are located in the central zone of the AP, have been found to project to the NST and to modulate arousal and the emetic reflex [84,85]. While A2 neurons project to oxytocinergic hypothalamic neurons of the SON and have been shown to play a role during experimental fear conditioning [86].

1.2.1. Hormonal signals

A variety of circulating hormones, mainly originating from fat cells, gastrointestinal tract, and endocrine pancreas, partake in the control of energy homeostasis through their actions on the HYPO, brainstem, or afferent autonomic nerves [87] (Fig. 1.3).

Leptin is a 146 amino acids peptidic hormone, produced by adipocytes [88] and involved in the regulation of energy homeostasis, neuroendocrine and immune functions [89]. It has

also been found in several other tissues including placenta, mammary gland, ovary, skeletal muscle, stomach, pituitary gland, and lymphoid tissue [90].

Feeding behavior, e.g., amygdala, cerebral cortex

Lateral hypothalamus
Orexins   MCH

Paraventricular nucleus
Orexigenic   Anorexigenic
Y1R   CCK, MC3R, MC4R

α-MSH

Arcuate nucleus
NPY/AgRP   POMC/CART

Arcuate nucleus

NTS, DMV

Vagus n.

Ghrelin   CCK, GLP-1   Insulin, PP, amylin   PYY   Leptin
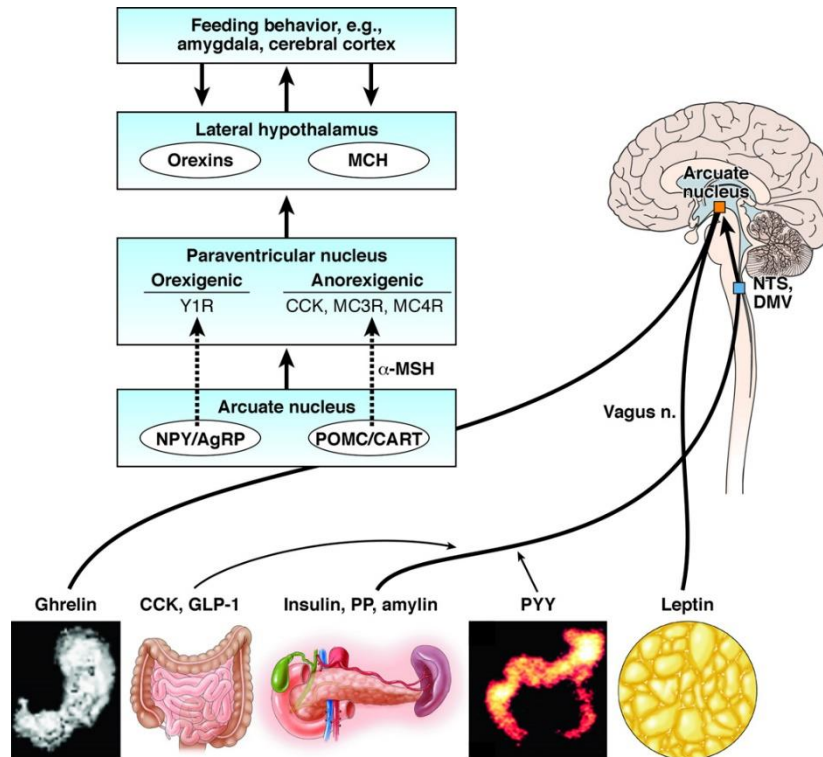
Fig. 1.3: Role of circulating hormones in the central control of energy homeostasis. Gastrointestinal and fat-derived hormones modulate food intake through stimulating specific brain areas such as the arcuate nucleus (ARC) in the hypothalamus and the hindbrain nucleus of solitary tract (NST) and dorsal motor nucleus of the vagus (DMV) [104].

Leptin is transported across the BBB by a saturable transport system [91] and exerts its anorexigenic effect in the ARC by activating POMC/CART [92,93], and inhibiting NPY/AgRP neurons [92,93], thereby reducing food consumption.

Moreover, Leptin is also implicated in stimulating the secretion of neuropeptides that play a role in energy homeostasis such as CRH, TRH and the brain-derived neurotrophic factor (BDNF) [91,94–96]. Beyond interacting with these central neuronal processes it became evident an interplay between leptin and CCK [97]. In particular, leptin appeared to increase the CCK-induced pro-satiety effect [98].

Interestingly, hematic leptin concentration changes in respect to the body fat mass [99].

Leptin is also able to signal rapid negative variations of energetic balance, although they occur independently of body weight fluctuations; during fasting, for example, circulating leptin drastically decreases while body weight does not significantly change [100]. Leptin effects on energy homeostasis regulation are mediated by binding its specific

membrane receptor (Ob-R) that has a single transmembrane domain and is a member of cytokine receptor family [101].

Finally, it has been demonstrated that the direct leptin administration in the ARC inhibits feeding [102]; moreover, ARC lesions dampen the hypophagic effect of peripherally administered leptin [103], thus suggesting the ARC crucial role in mediating leptin effects on energy homeostasis. The gastrointestinal tract also produces other hormones that are involved in the short-term regulation of appetite by predominantly interacting with the HYPO.

In this context, although ghrelin has been originally identified as the main responsible for growth hormone secretion [105], it is now recognised as one of the peripheral orexigenic factor implicated in sending to the CNS short-term modifications of energy balance.

Ghrelin is a peptide hormone of 28 amino acids secreted by stomach and duodenum [105], which exerts, compared to leptin, opposite effects at hypothalamic level. In fact, it activates the anabolic pathway increasing dietary intake and reducing energy expenditure. Moreover, it has been shown that circulating ghrelin levels increase immediately before eating thus promoting food intake [99,100]. By contrast, in a post-prandial phase, hematic ghrelin levels significantly decrease [99,100].

In the HYPO ghrelin-containing neurons are mainly located in the ARC, which is also a leptin target [106]. From here, ghrelin neurons project to NPY/AgRP expressing fibres in order to prevent leptin-induced inhibition of food intake and stimulate orexigenic peptides release; whereas by contacting POMC neurons, ghrelin fibres suppress anorexigenic peptides production [107].

Among other intestinal hormones that play a role in feeding behaviour regulation, the most studied are the CCK and peptide tyrosine-tyrosine (PYY) [108]. CCK is considered as a physiological pro-satiety factor that is produced at both peripheral (intestine) and central level upon food consumption [109]. It is normally released in response to long-chain fatty acids, amino acids and small peptides deriving by proteins ingestion [110,111].

CCK binds two different receptors: CCK receptor type 1 (CCK1) and 2 (CCK2). The former has been identified in peripheral tissues including pancreas, gall bladder, vagal afferents innervating intestine [112] as well as in CNS areas involved in food intake regulation such as NST, AP, and DMH [63]. While the latter has a different distribution, being mostly located in the cortex, HYPO, vagal afferents and gastric mucosa.

Intravenous injection of physiological CCK doses in humans has been demonstrated to increase appetite sensation and reduce food consumption [113].

PYY is a 36 amino acids peptide mostly containing tyrosine, produced by enteroendocrine L-cells of the distal gastrointestinal tract, whose plasmatic levels increase within 30 minutes after a meal consumption [114]. PYY consists of 2 different isoforms: a 36 amino acids isoform (PYY_{1-36}) and another who misses the first two amino acids (PYY_{3-36}). A line of evidence showed that circulating PYY_{3-36} levels are particularly high after meal consumption [114] thus suggesting the anorexic effect of this hormone peptide. The hypophagic effects of PYY_{3-36} appeared to be mediated by the ARC. Moreover, both the two isoforms exert their action by binding NPY receptors family [115]; in particular, PYY_{1-36} shows the same affinity for all the receptor subtypes, while PYY_{3-36} displays a higher selective affinity for Y2 receptor [116].

Finally, other two peripheral hormones regulating energy homeostasis are represented by insulin and amylin.

Insulin is a metabolic hormone produced by the β-cells of the pancreatic islets of Langerhans and consists of two polypeptide chains which are linked together by two disulfide bonds: an A chain of 21 amino acids and a B chain of 30 amino acids.

Insulin is known to act on hypothalamic neurons located in the ARC, where insulin receptors are abundantly expressed, thus exerting a pivotal role in regulating glucose homeostasis and reducing energy intake [117]. It has been observed that insulin intracerebroventricular (i.c.v.) infusion or systemic injection induce a dose-dependent reduction of food intake [118] through NPY inhibition and POMC stimulation [119].

Both insulin and leptin are able to activate POMC neurons, however they appeared to differently regulate AgRP, since leptin is responsible for its inhibition while insulin is important for its stimulation [120].

Amylin is a polypeptide of 37 amino acids which is co-secreted, along with insulin, from pancreatic mammalian β-cells in response to food consumption, in a ratio of 1:100 amylin:insulin [121]. Likewise insulin, amylin displays a role in glucose metabolism regulation; moreover, it also appears to lessen digestion through reducing digestive secretions (bile, enteric and pancreatic fluids) and to inhibit plasmatic glucagon production [121].

Amylin represents a pro-satiety physiological signal [122]; in fact, it has been demonstrated to exert an anorexic effect even during fasting conditions [27], through both central and peripheral mechanisms. Amylin inhibitory effect on food intake seems to be

mediated by the activation of hindbrain regions including the AP [123], as well as the NST and LPB [124].

A large body of evidence demonstrated amylin ability to exert its hypophagic effect also through serotonergic, histaminergic, and dopaminergic systems [124]. Moreover, amylin appeared to communicate, at the level of brainstem, with other signals involved in short-term control of food intake such as CCK, GLP-1 and PYY [121].

1.2.2. Neuropeptidergic system

A major player in energy homeostasis regulation in the CNS is represented by the hypothalamic neuropeptidergic system that consists of a variety of orexigenic and anorexigenic peptides, which are released at both synaptic and soma-dendritic level and modulate the neuronal networks by binding specific receptor targets [125]. As already mentioned in the text, the neurons populating the HYPO are classified in "first-order" and "second-order" neurons. The former are represented by the neurons co-expressing POMC/CART and those co-expressing NPY/AgRP both located in the ARC, while the latter consist of the PVN neurons releasing TRH, CRH and OXY (Fig. 1.1).

Among the neuropeptides stimulating appetite NPY, AgRP, orexins and the melanin-concentrating hormone (MCH) are the most studied.

Although the NPY displays different actions in the control of feeding behaviour, its most evident effect is the stimulation of food intake after its central administration [126]. Furthermore, it has been observed that acute food deprivation [127] or chronic food restriction [128,129] induced the increase of hypothalamic NPY mRNA levels, which returned to normal levels after refeeding [130].

NPY synthesis in the ARC and its release at the level of PVN are regulated by inhibitory afferent signals including leptin and insulin, and by stimulatory signals such as ghrelin [126].

5 different G protein-coupled receptors (GPCRs) for NPY have been identified: Y1, Y2, Y4, Y5 and Y6 [131,132] and they are widely distributed throughout the brain, including cortex, hippocampus (Hipp), amygdala (AMY), and HYPO.

Likewise NPY, AgRP is another orexigenic peptide of 132 amino acids which induces a sensation of appetite when i.c.v. injected in the PVN or in the DMH [133].

In vitro and *in vivo* studies have demonstrated that both POMC and AgRP neurons express leptin and insulin receptors and are targeted by the respective hormones to increase POMC mRNA expression and decrease NPY and AgRP mRNA levels [134,135].

Orexin-A and orexin-B are appetite stimulant neuropeptides which are produced in the PFA, DMH and LHA [136,137] and bind two distinct GPCRs: orexin receptor 1 (OxR1), mainly expressed in the ventromedial region of the HYPO (VMH), and orexin receptor 2 (OxR2) mostly present in the PVN [138]. Evidence showed that after being i.c.v. administered, orexin-A and orexin-B caused increased food intake in rats, while fasting led to upregulation of prepro-orexin mRNA [137]. Orexin secreting neurons project their axons broadly throughout the brain and, particularly, to the NPY neurons of the ARC, which express OxR1 [139].

The MCH is an orexigenic neuropeptide of 19 amino acids abundantly expressed in the LHA [140] and targeting two central receptors: MCHR1 and 2 [141,142]. The crucial role of MCH in controlling feeding is demonstrated by the observation that its injection into the rat lateral ventricle induces increased food intake [143].

Besides the orexigenic signals, growing interest is given to a number of anorexigenic peptides.

CART is a pro-satiety neuropeptide located in several regions of the HYPO including the DMH, PFA, PVN, and the ARC [94] as well as in the periphery. It has been observed that CART mRNA co-localizes with AVP and CRH containing neurons [94] in the PVN and with POMC in the ARC [144].

Several evidence supported the anorexic effect of CART, for instance its i.c.v. administration showed to inhibit food intake in rats [145], result that has been further sustained by the injection of an antibody against CART peptide 82–103 which has been demonstrated to stimulate feeding [145].

Melanocortins are bioactive lipids deriving from the tissue-specific post-translational cleavage of the precursor molecule POMC [95]. The POMC coding gene is physiologically expressed in several tissues including pituitary gland, skin, immune system, and hypothalamic neurons [96].

The long-term regulation of energy homeostasis mediated by POMC is a consequent of α-melanocyte-stimulating hormone (α-MSH) release, which occurs after POMC neurons stimulation [34]. α-MSH, in turn, induces satiety by binding to downstream melanocortin-3 and melanocortin-4 receptors (MC3 and MC4) in the PVN [34]. Consistently, it has been found that the ability of POMC neurons to suppress feeding is consecutive to melanocortin receptors activity [146].

On the contrary, it has been found that AgRP is a MC3/4-R competitive antagonist of α – MSH and lowers food intake by reducing α –MSH signaling [147].

CRH is the main physiological regulator of the adrenocorticotropic hormone (ACTH) secretion by pituitary gland, and growing evidence suggests its involvement in energy balance regulation [148]. It is a 41-amino acid neuropeptide largely expressed in PVN neurons and, in mammalian brain, it is also abundantly present in extrahypothalamic regions, including the olfactory bulb, bed nucleus of the stria terminalis (BNST), medial preoptic area, PVN, LHA, and central amygdala (CeA).

Moreover, when centrally injected, CRH inhibits food consumption and reduces body weight in rats through stimulating POMC-related peptides synthesis and secretion in the hypophysis [149]. It has been observed that, when peripherally administered in human, CRH significantly increases energetic expenditure and fat oxidation [118]. Furthermore, leptin infusion appeared to stimulate CRH expression, whereas the pre-treatment with a CRH antagonist dampens leptin-induced food intake and body weight reduction [118].

1.2.2.1. Oxytocinergic system

Among the satiety stimulating neuropeptides, a great deal of attention has been attracted by OXY, a 9-amino acid peptide crucially involved in feeding behaviour and energy homeostasis regulation [119–121]. OXY is synthesized as a part of a precursor protein, preprohormone, which is cleaved with the consequent release of a nonapeptide, the neurophysin [150]. Neurophysin appeared to be non-biologically active, however it seems to protect OXY against enzymatic damage and to mediate OXY inclusion into neurosecretory vesicles [151,152].

The major OXY neurosecretory system is represented by the hypothalamic-neurohypophysial system whose neurons, having the cell body in the PVN and SON, project their axons to the posterior lobe of the pituitary gland (neurohypophysis) as well as to the ARC, ME, lateral septum (LS), and medial amygdala nucleus (MeA) [153].

Different observations suggest that OXY is primarily synthesized into the magnocellular neuronal population of the PVN and SON [154] that, once activated, are responsible of OXY release in the neurohypophysis. OXY is then secreted from the posterior lobe of the pituitary gland into the blood circulation where it can exert its effects by binding OXY receptors (OXTR), which are located throughout the body [155]. Furthermore, it has been observed that after being secreted into the blood stream, or after its peripheral stimulation (during milk suckling or vaginal dilatation), cerebral OXY levels do not change, thus suggesting that OXY does not readily permeate the BBB [156].

Several evidence suggests that in the PVN and SON OXY can be released at both axonal and dendritic level [157].

The observation that hypothalamic oxytocinergic neurons project, among other destinations, also to the anterior part of the pituitary gland [158], suggested OXY ability to act as a hypothalamic regulation factor able to physiologically modulate the adenohypophysial hormones including prolactin adrenocorticotropic hormone [159], and gonadotropins [160].

OXTR, is a GPCR coupled to a Gq/11α protein that has been identified not only in the CNS but also in the periphery [156]. Interestingly, its distribution changes according to the species. For instance in the rat brain, in addition to PVN and SON, OXTR is also widely expressed in the olfactory system, cortex, thalamus, basal ganglia, VMH, BNST, CeA, ventral subiculum (vSUB), Hipp, Acb, brainstem, and spinal cord [155]; while in human brain it is abundantly distributed in pars compacta of substantia nigra (SN), globus pallidus, anterior cingulate and medial insula [155].

Interestingly in the periphery OXTR is present in a variety of organs including uterus, mammary gland, ovary, kidney, heart, bone, and endothelial cells [155].

Thanks to its wide distribution in the body, the oxytocinergic system plays a crucial role in many physiological functions. For instance, it has an uterotonic action and therefore it is involved in the induction of labor [161,162]. Growing evidence suggests an involvement of OXY in the regulation of social behaviour [163,164] that is dependent on the context: when the social cues are considered as "safe" OXY is able to improve sociality; whereas if they are interpreted as "unsafe" OXY induces defensive behaviour [165,166].

A large body of evidence indicates that OXY is also released during stressful conditions including conditioned fear, pain, electric footshock, exposure to novel environments, and restraint stress [156]. Moreover, concerning OXY capability to dampen the stress-induced hypothalamic-pituitary-adrenal (HPA) axis activation [167], it has been shown an interplay between the hypothalamic oxytocinergic system and the corticotrophin-releasing factor (CRF) system [168].

Furthermore, OXY has been shown to participate in the control of autonomic and somatic effects, such as sexual behaviour in both rodents [163] and humans [163,169], the cardiovascular system [170] and analgesia [171,172].

Interestingly, due to its effects in the control of social behaviour, OXY appeared to improve social cognition, functioning and repetitive behaviour in autism spectrum disorders [156].

A large body of evidence suggests the pivotal role of OXY in the regulation of feeding behaviour and energy homeostasis, being OXY particularly involved in stimulating appetite suppression [119–121]. Intravenous infusion of both OXY and OXTR receptor agonists has been demonstrated to induce a dose-dependent reduction of food intake [121,173,174]. Subsequent studies revealed that OXY acts as "homeostatic" inhibitor of feeding behaviour. In fact, it plays a role in gastric motility and in the response of stomach distension, which occurs upon food consumption [175]. Olszewski and collaborators found increased oxytocinergic neurons activity in concomitance with the meal termination [175], thus suggesting their function in mediating satiety.

Moreover, studies conducted in rodents revealed that OXY plays a role in the conditioned taste aversion (CTA) mechanisms [156].

Several studies have been conducted in order to investigate a possible interaction between OXY and other feeding related peptides. In this context, Olszewski demonstrated that α-MSH injection in the PVN, reduces food intake because of a concomitant increase of the percentage of neurons co-expressing c-Fos and OXY [176]. Furthermore, CART [177] and CCK [178] injection determined increased oxytocinergic neuronal activity in PVN and SON and/or increased OXY release, while a cerebral injection of OXTR antagonists has been demonstrated to prevent CRH-induced hypophagic effect [179] and to attenuate the pro-satiety leptin action [180].

OXY is not only involved in the control of energy homeostasis but it also regulates body weight and fat [181]. Finally, McCann and Rogers found that oxytocinergic neurons modulate gastric motility by increasing the excitability of sensorial NST fibres and DMV motoneurons [182].

1.2.3. Neurotransmitter systems

It is now well recognised the existence of an interplay between the monoaminergic neurotransmitters (such as DA, 5-HT and NA) along with neuropeptidergic and hormonal signals in regulating eating behaviour. Also the neurotransmitter histamine participates in the regulation of feeding consumption and it is considered a neural satiety signal; in fact it has been demonstrated that pharmacological treatments that increase the brain histamine availability are able to suppress eating, whereas administration of histamine antagonists increases food consumption and body weight [183].

- *Dopaminergic system*

Knowledge accumulated during the last years investigated DA role in the control of food intake, particularly in the rewarding aspects of food [184–186].

DA is the predominant catecholamine neurotransmitter, which is synthetized within the ventral tegmental area (VTA) and the SN of the midbrain, from here DA neuronal cell bodies project to various brain structures.

We distinguish three different circuitries: 1) a nigrostriatal pathway originating in the SN and projecting to the dorsal part of the striatum such as the caudate putamen (CPu), involved in the regulation of movements; 2) a mesocorticolimbic pathway where dopaminergic cell bodies, residing in the VTA, project to both limbic (such as Acb and AMY) and cortical (such as PFC and medial prefrontal cortex (mPFC)) regions, thus controlling reward, and reinforcement behaviours as well as emotional and motivational responses; and 3) a tuberoinfundibular circuit which connects the ARC, where reside the cell bodies, and the ME [187] (Fig. 1.4).
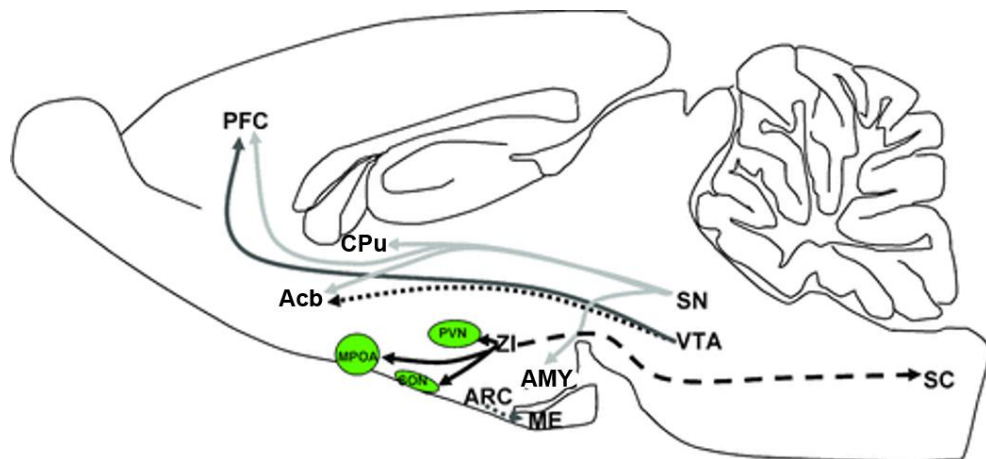
Fig. 1.4: Dopaminergic pathways in the rat brain. The mesocorticolimbic circuit connects the VTA and both limbic (Acb, black-dotted line) and cortical (PFC, dark gray line) areas; the nigrostriatal circuit connects the SN to the CPu and others brain regions including the PFC, Acb and AMY (light gray line). The tuberoinfundibular pathway connects the ARC and the ME (dark gray dotted line).
Acb, nucleus accumbens; AMY, amygdala; ARC, arcuate nucleus; CPu, caudato putamen; ME, median eminence; MPOA, medial preoptic nucleus; PFC, prefrontal cortex; PVN, paraventricular nucleus; SC, spinal cord; SON, supraoptic nucleus; VTA, ventral tegmental area; ZI, zona incerta. Adapted from [187].

It has been demonstrated that DA effects in the regulation of feeding vary depending on the brain sites of action and on the variety of receptor subtypes [35]. For instance, mesolimbic dopaminergic circuitry is responsible of the rewarding aspects of consuming HPF [188], whereas DA release in the HYPO suppresses food intake by reducing the duration of meal consumption [93,189].

Growing evidence showed that DA release is linked with both short-term and long-term control of feeding [189,190]. Moreover, several findings suggested that a different expression in the HYPO of dopaminergic receptors in obese and lean phenotypes is directly involved in the regulation of meal size and meal number [189].

Among the different neurotransmitters implicated in the rewarding effects of food, DA represents the best characterized, in fact several evidence revealed that the ingestion of HPF induces DA release in the striatum of both humans and rodents [191,192]. In particular, the first exposure to HPF stimulates an increase in the firing of DA neurons, whose cell bodies are located in the VTA, with the consequent DA release in the Acb [193]. On the other hand, DA response habituates following situations of repeated HPF exposures [194].

The involvement of dopaminergic circuitry in regulating food reward is strictly correlated to the motivational aspects driving the desire for the food, referred to as "wanting" [195]. The dopaminergic brain regions involved in this rewarding aspect of food are represented by the striatal nuclei such as CPu and Acb [196].

This concept is opposed to the mechanism involved in the hedonic properties of food, referred as "liking" which are predominantly regulated by opioid and cannabinoid systems [195]. Conversely, in the hedonic aspects of food are implicated the LHA, ventral pallidum (VP), orbitofrontal cortex (OFC), Acb [197–199] and insula [200].

Brain-imaging studies revealed that the food cue-induced increase of DA release in humans is associated with the motivational salience of food (wanting) [201], while opioid or cannabinoid receptors activation enhances the liking of the food, thereby stimulating appetite.

- *Serotonergic system*

5-HT is a monoamine neurotransmitter derived from the amino acid tryptophan and it is synthesized both peripherally (in the gastrointestinal tract) and centrally [202–204].

Among the variety of functions that 5-HT exerts including reproduction, mood, sleep, and cognition, a very important physiological role appeared to be the control of feeding behaviour [189].

In this context, it has been demonstrated that peripheral administration of 5-HT inhibits food intake by reducing meal size in rats [205]. This effect seems to be associated to the indirect activation of the CNS, which occurs through the recruitment of ascending vagal afferent fibres [206–208]. 5-HT, in fact, does not cross the BBB.

Central 5-HT is released by the 5-HT-synthesizing neurons, whose cell bodies are located in the brainstem, particularly in the caudal and rostral raphe nuclei, and densely innervate many regions of the brain [189].

More specifically, the 5-HT neurons of the caudal raphe project to the cerebellum, midbrain, pons, medulla, and most segments of the spinal cord, while 5-HT neurons whose cell bodies reside in the rostral raphe project in regions including cortex, Hipp, thalamus, HYPO, striatum, and AMY [209,210].

In particular, it has been observed that 5-HT fibres synaptically contact POMC and AgRP ARC neurons thereby affecting melanocortin neuronal activity [211,212].

The role of central 5-HT in eliciting satiety has been demonstrated by the observations that the administration of different compounds acting as direct or indirect agonists at central 5-HT synapses cause a significant reduction of food intake [189].

Moreover, it has been found that during feeding, 5-HT release increased in the HYPO [213].

A large body of evidence shows the existence of an interplay between 5-HT and DA, in particular it has been observed that DA release is partially regulated by 5-HT release [214–216].

To this regard, it is important to mention that meal size and meal number are independently regulated by distinct brain regions. In particular, several evidence demonstrated that the meal size is influenced by the intra-LHA DA and 5-HT, whereas the meal number is modulated by DA and 5-HT interaction within the VMH [217–219].

Thanks to 5-HT properties in inducing satiety, a number of drugs enhancing 5-HT transmission have been used for obesity treatment. In this context, several evidence supported an important role for 5-HT_{2C} receptors in the inhibitory control of eating.

For example, mice lacking these receptors have been found to be hyperphagic thus developing obesity [220–222]. Moreover, they showed a lower responsiveness to dexfenfluramine [223], GLP-1, and CCK [224]. This aspect will be further discussed in the next sessions of the text.

- *Noradrenergic system*

NA is a catecholamine neurotransmitter synthesized in the dorsal vagal complex and in the locus coeruleus (LC) of the brainstem. In particular, Dalhström and Fuxe have recognised in mammalian brainstem several distinct groups of NA neurons from A1 to A7 [225].

These regions contain the caudal ventrolateral medulla (A1), the NST (A2) the reticular regions around inferior olivary nuclei (A3) and the ependyma of the fourth ventricle (A4), the ventrolateral pons (A5), the LC (A6) and the rostral caudal pons (A7) [225,226].

From these hindbrain regions, noradrenergic fibres have been found to project both caudally to the spinal cord and rostrally to the HYPO, thalamus and cortex [93]. In particular, it has been shown that the NA neurons innervating the HYPO are those originating from A1, A2, and A6 regions [40,227].

Several observations demonstrated the crucial involvement of the noradrenergic neurotransmission in controlling food intake [93]. For instance, particularly in the noradrenergic neurons projecting to the PVN, NA has been found to co-localize with NPY [93]. Moreover, likewise NPY, intra-PVN injection of NA in rats appeared to strongly increase feeding and body weight [228] through the activation of the α_2-receptor [229]. Conversely, NA by binding to α_1- and β_2-receptors exert opposite effects on food intake [229].

The observation that elevated NA signaling in the PVN or other hypothalamic areas produced an increase of leptin deficiency-induced hyperphagia, suggests that NA acts as an anabolic effector in the control of energy homeostasis of CNS [93].

Further evidence confirming the role of noradrenergic tone in feeding behaviour regulation is represented by the observation that A2 neurons are activated when food intake is inhibited [230].

Moreover, in order to investigate the role of noradrenergic neurons located in the NST in mediating anorexic responses to gastric stimulation and in conveying gastric sensory signals to the HYPO and AMY, these hindbrain noradrenergic fibres have been lesioned through the microinjection of saporin, a toxin conjugated to an antibody against the enzyme responsible for NA synthesis, dopamine-β-hydroxylase (DBH) [231].

These experiments demonstrated the involvement of noradrenergic neurons of the NST in mediating CCK-anorexia and the hyphotalamic responses to gastric sensory stimulation [231].

- *Histaminergic system*

Histamine is an organic nitrogenous compound that is produced from the amino acid L-histidine by the enzyme histidine decarboxylase (HDC) and it has been isolated for the first time by Sir Henry Dale and colleagues from ergot in 1910 [232]. The first evidence of histamine presence in the brain dates back to 1919 [233], however its role as a

neurotransmitter became evident only several decades later with the morphologic characterization of histamine- synthesizing neurons [234].

The central location of histaminergic neurons is represented by the above-mentioned TMN of the HYPO, from here these neurons project to the whole CNS through three major pathways: two ascending fibre groups reaching the forebrain structures and one ascending way that innervates the spinal cord [235–237] (Fig. 1.5).
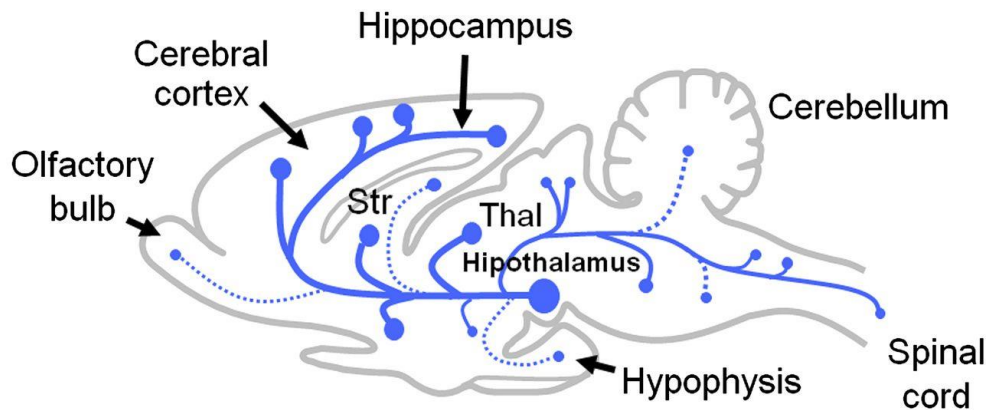
Fig. 1.5: The histaminergic pathways in the rat brain [234].

Histamine is known to be involved in local immune responses and, as a neurotransmitter, it regulates many physiological functions [234] including arousal [238], sleep-wake cycle [239], seizure activity [239], release of hypothalamic hormones (AVP, OXY, prolactin, ACTH and β-endorphin) [240–243], thermoregulation [244], glucose and lipid metabolism [245], blood pressure [239] and food intake suppression [44,246].

Although histamine is able to activate both G protein-coupled receptors [247] and ligand-gated ion channels [239], its physiological functions are mainly mediated by the binding of the former referred to as H_1R [248], H_2R [249], H_3R [250], H_4R [251].

Among histamine biological functions, a crucial role is represented by the regulation of eating behaviour. Histamine, in fact induces satiety being released during the appetitive phase of food intake [252]. Moreover, the observation of increased hypothalamic histamine levels during the feeding state of rats that have been fasted for 24 hours demonstrated a role for histamine also in regulating the consummatory phase of eating behaviour [253,254]. Preclinical data collected over the years concerning the administration of histamine within the brain of laboratory rats [255,256] or cats [257] revealed histamine ability to induce a long-term inhibition of food intake.

Moreover, additional evidence of the pro-satiety action of histamine comes from the observation that both the activation of H_1R within the PVN and VMH and the blockade of brain H_3R autoreceptors (that increases histamine release) are able to suppress food consumption [258]. These receptors have been recognized to regulate energy intake and expenditure and their activation has been demonstrated to modulate feeding circadian rhythms [44,259].

Furthermore, there is consistent evidence indicating that a variety of regulatory peptides involved in the homeostatic control of food intake, including orexin, leptin, GLP-1, TRH, amylin and the emerging gut-derived pro-satiety compound oleoylethanolamide (OEA), require an intact histaminergic system to exert their orexigenic or anorexigenic effect [45,260].

1.3. Neurobiological mechanisms regulating stress

The way by which our body reacts to the exposure of stressful agents (physical, chemical or emotional stress) consists of an adaptation response whose objective is the homeostasis maintenance.

The mechanisms accounting for this process consider both the catecholamines release from adrenal medulla and the activation of the HPA axis that is responsible for higher cortisol levels [261]. As previously described in the text, medial parvocellular neurons of PVN containing CRF, project to the ME where they release this hormone into the hypophysial portal vessels [39]. From here, CRF reaches corticotropic cells of anterior pituitary gland [261] where it binds to its receptor, thus leading to ACTH secretion within the systemic circulation (Fig. 1.6). ACTH, in turn, binds to specific receptors expressed by the adrenal cortex in order to stimulate synthesis and release of corticosterone (CORT) in the general circulation, a hormone crucially involved in the body adaptation mechanisms to the stress response [262].

Among the stress factors characterizing the stressful conditions, both systemic and psychological factors result in HPA activation.

In particular, systemic stress factors are also associated with an activation of brainstem and circumventricular organs that directly project to the PVN neurons. Moreover, it is also possible that HPA axis hyperactivation, occurring during stressful conditions, is dependent on catecholaminergic ascendant input [261].

On the other hand, psychological stress factors evoke excitement conditions leading to the so-called "fight-or-flight" homeostatic stress response, which is linked to

glucocorticoids and neuro-hormones secretion and to the activation of the sympathetic part of the autonomic nervous system.

Both systemic and emotional stress factors are elaborated in a variety of limbic structures, including AMY, Hipp, and PFC regions of the brain receiving projections from sub-cortical and cortical areas and involved in regulating emotional or memory processes and conditioned responses [263].

The above-mentioned limbic regions also modulate HPA axis activation and collaborate in the autonomic stress responses [264].

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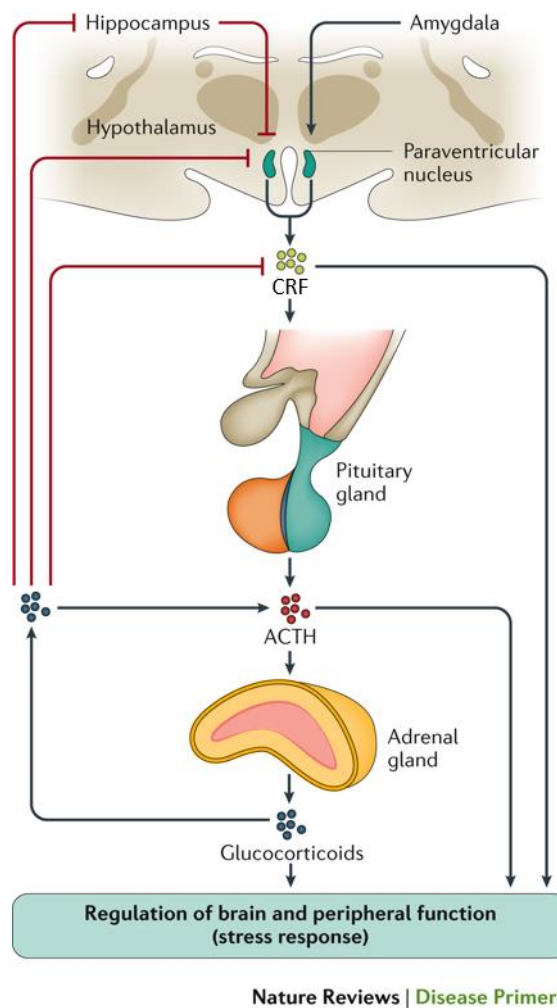
Fig. 1.6: Regulation of the hypothalamic–pituitary–adrenal axis (HPA) [265].

Limbic modulation of the stress responses is primary mediated by oligosynaptic inputs to the PVN and other preautonomic brain regions. In this context, the PVN is considered as the principal integrator of stress signals [264].

In particular, the vSUB provides glutamatergic input to primarily inhibitory PVN relays, thus limiting psychogenic stress responses [264].

On the other hand, AMY, that is a structurally complex brain region, differently regulates systemic and psychogenic stressor responses. For instance, GABAergic projections from the CeA are involved in the regulation of responses to systemic stressors, while those from the MeA are responsible for the modulation of psychogenic stressor responses. Finally, the basolateral amygdala (BLA) via glutamatergic projections, within and outside the AMY, regulates both the acute and the chronic psychogenic stress response [264].

As far as the cortex, the prelimbic cortex (PL) via glutamatergic projections directed to inhibitory PVN relays suppresses the responses to emotional stress, whereas the infralimbic cortex (IL) appeared to activate autonomic responses to psychogenic stress, via direct (NST) or indirect (CeA) projections.

CRF effects on physiological and behavioural stress responses are mediated by binding to its receptors: CRF receptor type 1 (CRF1) and 2 (CRF2). Between the two receptors, the CRF1 represents the most abundant and it is predominantly expressed within HYPO, VTA, AMY, and cortical structures; while CRF2 distribution is restricted to the HYPO, dorsal raphe (DR), and LS [266–268].

It has been reported that, CRF1 antagonists reduce stress-induce *food-seeking* in rats [269]. Moreover, an interplay between CRF and the dopaminergic and oxytocinergic systems has been predominantly studied in the last years [270,271].

For instance, the HPA stimulation leads to the activation of the mesolimbic dopaminergic pathway, which is associated to the reward circuitry. In fact, it has been demonstrated that the high CRF levels followed stressful events exert a role on DA neurons of the VTA, which projects to both limbic and prefrontal regions [272].

Moreover, experiments conducted in rodents reported that a variety of stressful stimuli including pain, fear conditioning and the exposure to novel environments induce an increase of plasmatic OXY concentration [161], which leads to the decrease of the stress-induced HPA axis activation [167]. OXY represents, in fact, an important regulator of the anxiety disturbances related to the physiological stress response [164].

1.4. Neurobiological mechanisms regulating food addiction

Growing evidence considers obesity not only as a metabolic disease but also as the consequence of persistent disrupted eating behaviours thus affecting not only the physical but also the psychosocial health [273].

In this context, it is current knowledge that overeating is linked to neurobiological and psychological aspects, such as mood disturbances, altered reward perception and motivation, and addictive behaviour [274].

From this point of view, it is now prevailing the tendency to consume certain foods, particularly those high in sugar or in fat and therefore highly palatable, for pleasure and not to maintain energy homeostasis. Due to its intrinsic features, the HPF has an important impact on mood regulation so that it is becoming frequent the activity to overconsume these nutrients to self-medicate from negative emotional conditions such as anxiety, depression, mental fatigue [275].

Based on this background, it is now clear that there are many similarities between HPF consumption and reinforce behaviours related to the use of abuse drugs [276–279]. In fact, HPF has been demonstrated to induce CNS modifications similarly to those evoked by drugs of abuse [276–279]. In this context, it has been defined the concept of food addiction, suggested for the first time by Randolph in 1956 [280].

The first indirect evidence showing affinities between excessive food consumption and drugs of abuse comes from the pharmacological data, since common therapies that are known to influence the intake of abuse drugs have been also shown to attenuate compulsive overeating occurring, for example, during binge eating episodes. Among these drugs, we recognize naloxone [281], naltrexone, baclofen [282,283], topiramate [284], Rhodiola rosea and hypericum perforatum extracts [285,286], adenosine A_{2A} receptor agonists [287], orexin 1 [288] and CRF1 receptor antagonists [289].

Moreover, additional molecular and neurochemical evidence suggests that uncontrollable HPF consumption stimulates reward dopaminergic and opioid systems [273], which appear deeply readapted in people suffering from BED and obesity, thus leading to a reward hyposensitivity state [290–293]. Moreover, this readaptation seems to be the consequence of disrupted striatal D2 receptors signalling [294–297].

Interestingly, another evidence supporting the link between obesity and drug addiction comes from the observation that the disruption of the reward dopaminergic circuitry occurring during food overconsumption is similar to that observed after intravenous cocaine or heroin self-administration [298–300].

Human brain imaging data showed an increase in DA levels in the brain of both addicted individuals and obese people with BED [301,302]. This effect appeared to be parallel to a reduced expression of DA receptors observed during obesity and drug dependence conditions [290].

In particular, in BED patients the higher DA release, which occurs upon exposure to food cues, has been described as an index to classify the severity of the disorder [302].

Moreover, the symptoms described during food withdrawal including headaches, sweats, irritability, and panic [303], are also similar to those found in drugs addicted individuals such as symptoms resembling tolerance, withdrawal, and craving [291].

The aversive emotional state that triggers the negative reinforcement of addiction such as stress and negative moods (depression, anxiety), is also predominant in food addiction and can cause uncontrollable food consumption in humans through the involvement of a three-step cycle characterized by binge/intoxication, withdrawal/negative affect and preoccupation/anticipation [304–306].

It is important to mention that obesity and BED are not synonyms of food addiction, which is neither necessary nor sufficient for obesity [277,307].

To conclude, the inclusion of food addiction in the DSM-V suggests the similarities between people suffering from EDs and drug addicted individuals [308].

This observation indicates that the neuronal circuitry engaged in the two pathological conditions are the same [273].

1.5. Pharmacological treatment for obesity and BED

- *Obesity*

The growing prevalence of obesity highlights the need to deal with this public health issue by using specific anti-obesity treatments as an adjunct to a complete weight-loss program including diet and physical activity. Moreover, for patients with a BMI of 40 kg/m^2 or greater, who failed an adequate exercise and diet program (with or without therapy), the most effective anti-obesity approach is represented by a bariatric surgery, which reduces the size of the stomach [309].

The European Medicines Agency (EMA) recommends that, in order to be considered efficient against obesity, a drug needs to induce a response that is more than 10% weight loss at the end of a 12-month period [310].

The anorexiant drugs that have been used over the last years are numerous, however most of them has been withdrawn from the market due to their several side effects. Some examples are represented by d-fenfluramine, a 5-HT reuptake inhibitor, that was withdrawn from the U.S. market in 1997 after reports of heart valve disease and pulmonary hypertension [311]; rimonabant, a cannabinoid receptor type 1 (CB1) antagonist which is not more available since 2008 because of its effects in increasing

the risk of psychiatric disorders such as depression and suicidal tendency [312]; sibutramine, a NA and 5-HT reuptake inhibitor that displayed severe cardiovascular side effects when administered in patients who already showed cardiac disorders [313] and therefore withdrawn from the market in 2010.

Over the last 20 years, the pharmacological research identified novel compounds for long-term obesity treatment and, in particular, three drugs have been approved both by EMA and Food and drug administration (FDA) and are currently on the market (Orlistat, Liraglutide and naltrexone/ bupropion combination therapy).

Orlistat is a synthetic hydrogenated lipostatin derivative acting as a specific gastrointestinal lipase inhibitor, which reduces dietary fat absorption by the 30%, thereby consequently lowering body weight [314].

Orlistat treatment is also associated with an important reduction of the cardiovascular risk factor by lowering total cholesterol, low-density lipoproteins, triglycerides and arterial blood pressure [315]. Moreover, through decreasing body weight it also improves glycemic control and it has been shown to reduce the type 2 diabetes incidence to 6.3% after 4 years of treatment [315]. However, Orlistat is associated with several adverse effects such as liquid and soft stools, abdominal discomfort, flatulence, faecal urgency, and/or incontinence [314]. At least one of these effects, which are related to Orlistat pharmacologic mechanism of action, occurred in up to 95% of orlistat-treated patients during clinical trials [316]. Nevertheless, they occurred during the first week of treatment and did not persist for more than 4 weeks [314].

Liraglutide, an acylated form of the human GLP-1, represents another pharmacological target for obesity management. Thanks to its structural change, Liraglutide is able to spontaneously aggregate in the injection site and to bind circulating albumin therefore having a greatly prolonged half-life compared to native GLP-1 [15]. Although originally developed for type 2 diabetes mellitus treatment, Liraglutide was demonstrated to lower body weight and then approved by both the FDA (2014) and EMA (2015) for obesity therapy [317].

The most commonly reported adverse events are gastrointestinal complaints such as nausea, diarrhoea and vomiting [317].

In order to manage both homeostatic and hedonic aspects of eating, a combination therapy containing naltrexone and bupropion has been approved in 2014 in the US and in 2015 in Europe [318,319].

In particular, Naltrexone is a high-affinity μ-opioid receptor antagonist while bupropion is a NA and DA transporter inhibitor [320]. It has been demonstrated that the reduced food intake followed by naltrexone/bupropion treatment is strictly related to an increase in POMC neuronal activity [318], an effect that is primarily stimulated by bupropion action and subsequently amplified by naltrexone-induced inhibitory effects on β-endorphin [321]. Moreover, thanks to the effects of naltrexone in reducing dopamine levels in the Acb, this combination therapy decreases the reward value of food and the preference for the so-called HPF [322–324] thus addressing one of the goals of obesity management pharmacotherapy.

Among the most common adverse effects of naltrexone/bupropion (nausea, vomiting, migraine, and dizziness), increased blood pressure and cardiac frequency appeared to be related to its mechanism of action, being bupropion a sympathomimetic substance.

Other two pharmacological approaches, Lorcaserin and Phentermine/Topiramate combination, have been approved by FDA in 2012 and are currently available on the US market. Lorcaserin is a selective $5-HT_{2C}$ receptor agonist, which inhibits appetite and reduces body weight, showing classical side effects such as constipation, fatigue, dry mouth, headache and dizziness [325].

Phentermine/Topiramate is an extended-release formula designed for long-term obesity therapy and indicated for lowering body weight and increasing resting energy expenditure [320]. Phentermine is a sympathomimetic compound, which inhibits appetite by increasing NA in the HYPO and consequently stimulating β2-adrenergic receptors [326]. On the other hand, Topiramate is a non-specific drug originally indicated as anticonvulsant and proposed to act at several cellular targets including voltage-gated sodium channels, high-voltage-activated calcium channels, gamma-aminobutyric acid (GABA-A) receptors, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)/kainate (AMPA/KA) receptors, and carbonic anhydrase isoenzymes [327]. The specific mechanism by which Topiramate reduces body weight is still debated, however it has been proposed that its effects in reducing compulsive or addictive food cravings are mediated by topiramate's antagonism of AMPA/KA receptors [328]. Furthermore, topiramate reduction of food intake seems to be mediated, at least in part, by the activation of GABA-A receptors [329].

It is important to mention that initially topiramate was approved as a monotherapy for obesity but it was withdrawn because of its neuropsychiatric and cognitive side effects

[330]. As a combination formula, Phentermine/Topiramate therapy shows common adverse effects including constipation, dry mouth, paraesthesia, and insomnia [320].

Moreover, an additional anti-obesity approach considers the supplementation of diet with naturally occurring nutritional ingredients such as the N-oleoyl-phosphatidyl-ethanolamine (NOPE) conjugated with epigallocatechin gallate (EGCG) [331].

NOPE is a phospholipid present in animal and vegetable foods and hydrolysed by the membrane phospholipase D into N-oleyl-ethanolamide (NOE) which is known to exert an anorexic effect through peroxisome proliferator activated receptor-alpha (PPAR-α) activation, when systemically administered in rat [331]. Whereas EGCG is a standardized green tea extract and has been shown to promote lipolysis and, consequently, weight and fat loss [332] by inhibiting catechol-O-methyltransferase (COMT)-induced degradation of catecholamines [333]. Several evidence showed that, when NOPE is orally administered, NOE is rapidly hydrolysed in the stomach in oleic acid and ethanolamine and therefore it is not able to reach the intestine and to exert its anorexic action. Moreover, the conjugation with EGCG prevents the rapid breakdown of NOPE improving its availability [331].

- *BED*

To date there are no efficacious therapies for the treatment of BED, especially because of the complexity of the pathology, but also because of their high relapse rates and a wide range of side effects [334,335]. The main outcome criteria for BED treatment should represent a reduction of binge eating episodes and the improvement of the disrupted feeding behaviour, the control of the psychopathological aspects of the disease, and the reduction of the possible excessive body weight [21].

BED seems to be sensitive to three classes of drugs: antidepressant drugs, central appetite suppressors, and antiepileptic agents.

The possibility to use antidepressant therapies for the treatment of BED is related to the high correlation existing between BED and major depression [336]. Moreover, the overeating occurring during binge eating episodes is similar to that observed in people suffering from BN, which is treated with antidepressant drugs.

Furthermore, several evidence demonstrated that the impairment in brain 5-HT signalling is one of the mechanism underlying the pathophysiology of BED [23–26].

In keeping with this observation, it has been recently reported that fluoxetine and d-fenfluramine, selective 5-HT reuptake inhibitors that increase 5-HT content, are able to suppress BED [337], by reducing the number of binge episodes in obese patients [338–340].

However, d-fenfluramine, as already mentioned in the text, it was withdrawn from the market for its cardiopulmonary adverse effects [311].

Interestingly, an important interplay of the serotonergic and dopaminergic pathways has been recently found in the regulation of BED, as suggested by Xu and collaborators, who showed that lorcaserin attenuates BED in mice fed with a high fat diet, by stimulating central dopaminergic neuronal activity [341].

Anti-obesity agents may also be used for treating BED since there is a significant overlap between both diagnoses [342–347]. In this context, naltrexone, which is used in combination with bupropion for obesity management, has been reported to regulate both the homeostatic and the hedonic aspect of food consumption.

For example, naltrexone has been demonstrated to decrease DA levels in the Acb, leading to a decrease in food seeking and binge-like eating [322,348,349], thus reducing the preference for highly palatable, high-fat, and high-sugar foods [323,324,350].

Among its several mechanisms of action, it has been observed that topiramate's antagonism of AMPA/KA receptors seems to improve BED [351] and reduce other addictive behaviours [352] through reducing compulsive or addictive food cravings [328].

The only FDA-approved (in 2015) medication for the treatment of moderate to severe BED in adults is the Lisdexamfetamine (LDX), a pro-drug of d-amphetamine [353], which primarily acts by enhancing dopaminergic and noradrenergic neurotransmission in the brain [353]. Beyond influencing the hedonic system, it has been demonstrated that LDX also up-regulates CART expression in animal models [354,355].

However, it has several limitations since it causes serious adverse effects in patients with cardiac damages (cardiomyopathy, heart arrhythmia, coronary artery disease), which, unfortunately are quite frequent comorbidities in the population affected by eating disorders [356,357].

Less important side effects of LDX are insomnia, weight loss, and headache [358]. Furthermore, being a psychostimulant there is a considerable risk of abuse. LDX is only approved in the US, its use in other countries is still off-label.

1.6. Oleoylethanolamide and N-acylethanolamides as lipid mediators

Nowadays, the limited and poorly efficacious anti-obesity therapies highlight the urgency to find novel pharmacological targets for controlling aberrant feeding behaviour. In this context, a great deal of attention has been dedicated to N-acylethanolamides (NAEs), a family of phospholipid-derived signalling molecules, which have been shown to participate in many physiological and pathological conditions [273,359–362].

Even though NAEs have been isolated from animal and vegetal tissues more than 50 years ago [363,364], they did not attract any attention until anandamide (AEA) discovery, a polyunsaturated NAE binding to cannabinoid receptors [365]. Hence, the identification of AEA as an endogenous cannabinoid ligand [366] has raised the interest for the other members of NAEs family thus recognizing their role in many biological functions including the regulation of food intake [359]. The first NAE to be discovered was palmitoylthanolamide (PEA), followed by OEA.

Among NAEs, PEA, OEA, stearoylethanolamide (SEA) and linoleoylethanolamide (LEA) are the best characterized and, in addition to feeding, they have been shown to exert roles in conditions like inflammation, pain and memory consolidation [367,368]. Furthermore, due to their high expression in the CNS, growing evidence established their protective effects in neurodegenerative and neuropsychiatric disorders [369–372].

Among NAEs, the most important compound that has been studied for its pivotal role in the regulation of feeding behaviour is represented by the endogenous lipid signal OEA. Although OEA shares with its monounsaturated analogue, AEA, biosynthesis and degradation pathways [362,373], it exerts opposite effects in the control of feeding behaviour and lipid metabolism. In fact, OEA does not induce cannabimimetic effects and it does not show any affinity for the cannabinoid receptors [374].

OEA is generated by enterocytes of the proximal small intestine from oleic acid, which is released upon the absorption of dietary fat [366]. Growing evidence demonstrated the pro-satiety effect of OEA since it is able to significantly reduce food intake and body weight in both lean and obese rodents [36,37,359,375–384] as well as in humans [385].

1.6.1. Synthesis and metabolism

OEA and NAEs are synthesized on-demand from a phospholipid precursor within the membrane lipid bilayer. OEA biosynthesis is under the control of the sympathetic nervous system [386,387] and is mediated by two reactions (Fig. 1.7A). The first step is represented by the transfer of an acyl group, oleic acid, from the stereospecific numbering-1 (sn-1) position of a membrane phospholipid, such as phosphatidylcholine, to the amine group of phosphatidylethanolamine (PE) [366] (Fig. 1.7A). The enzyme responsible for catalyzing this reaction, named as N-acyltransferase (NAT), is a calcium-dependent enzyme involved in the production of a chemically heterogeneous family of N-acylphosphatidylethanolamines (NAPEs) [362,388]. The second step is the release of OEA from NAPE, which contains oleic acid at the amine position (Fig. 1.7A). This reaction is catalyzed by a NAPE-selective phospholipase D (NAPE-PLD) that hydrolyzes the distal phosphodiester bond of NAPE [362,388].

Other NAEs may be produced by a similar biosynthetic pathway that involves the synthesis of the respective NAPEs as precursor [389].

NAPE-PLD has been purified and molecularly cloned by Dr. Ueda and his collaborators [373] and it is able to hydrolyze, with similar efficiency, a variety of analogs of the NAPE family [388]. Moreover, experiments conducted in NAPE-PLD knock out (KO) mice revealed the existence of a redundant OEA biosynthetic pathway since in these mice OEA endogenous synthesis was not completely prevented [390,391].

In this regard, a calcium-independent mechanism, through which NAPEs can be generated, has been identified and it involves the phospholipase A/acyltransferase (PLA/AT) family proteins [392].

The main mechanism by which biological OEA effects are terminated is represented by the enzymatic hydrolysis of OEA to oleic acid and ethanolamine [362] (Fig. 1.7B). The intracellular enzymes involved in this process are amidases named as: fatty acid amide hydrolase (FAAH) [393] and N-acylethanolamine acid amidase (NAAA) [394]. FAAH belongs to the serine-hydrolase family [393] and, although it is widely distributed in all mammalian tissues, it is predominantly expressed in the CNS, liver and small intestine [396]. By contrast, NAAA, which belongs to the family of fatty acid cysteine hydrolases, is mainly expressed in the periphery rather than in the brain. In particular, NAAA has been found in high concentrations in lung, spleen, thymus, intestine and in alveolar macrophages of rats [397] and has no homology with FAAH [394].

NAAA also differs from FAAH, since it mostly prefers PEA as a substrate for hydrolysis [394,398,399].

Moreover, it has been demonstrated that NAAA might play a role in regulating NAEs levels during inflammation processes [398,400]. However, its role in OEA metabolism remains poorly understood.

Interestingly, a line of evidence showed that CD36, the fatty acid transporter presents on the enterocytes apical surface in the duodenum and jejunum, might contribute to OEA mobilization by facilitating, directly or indirectly, oleic acid absorption [362].
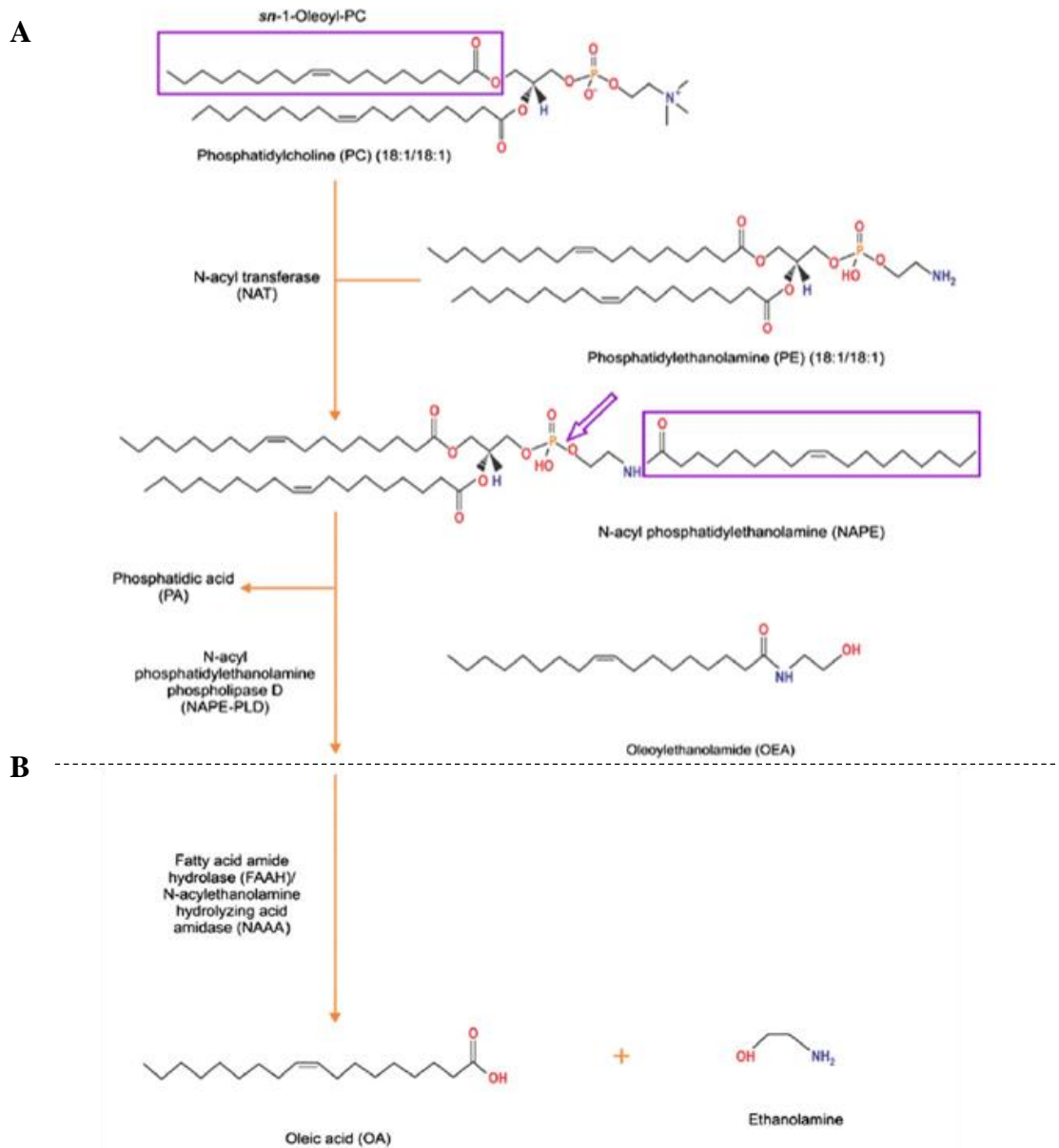
Fig. 1.7: Biosynthesis (panel A) and degradation (panel B) pathways of OEA [395]

1.6.2. Receptors

There is accumulating evidence showing that the pro-satiety effect of OEA is mediated by the activation of intestinal PPAR-α [401], which belongs to the family of peroxisome proliferator activated receptors (PPARs). PPARs are a family of transcription factors constituted by three different isoforms (α, β/δ, and γ), widely expressed in tissues with a higher oxidative capacity such as the cardiovascular system and, in particular, cardiomyocytes, endothelial cells, and vascular smooth muscle cells [402], but also in several brain areas and in peripheral tissues such as kidney, liver and intestine [368,403]. After being activated by a ligand, PPARs stimulate gene expression by creating heterodimers with the retinoid X receptor (RXR), thereby binding to specific peroxisome proliferator response elements (PPREs) in the promotor region of target genes [404]. They are involved in different biological processes, such as energy homeostasis, lipid and lipoprotein metabolism, cell proliferation and inflammation, blood pressure control and hypertensive-related complications, such as stroke and renal damage [405,406].

Furthermore, among the different members of the PPARs family, PPAR-α and PPAR- δ have been demonstrated to exert distinct roles in the regulation of lipid metabolism [407]. In this regard, once activated, PPAR- δ induces the expression of genes involved in the control of fatty acid oxidation and energy dissipation, thus improving lipid profiles and reducing adiposity [408]. PPAR-α plays an important role in the regulation of lipid metabolism and energy homeostasis. As a matter of fact, it has been demonstrated that OEA pro-satiety effects are completely prevented in PPAR-α KO mice that are sensitive to other anorexic compounds, thus revealing the pivotal role of PPAR-α in mediating the satiety-inducing effects of OEA [401].

Moreover, OEA appeared to stimulate the transcription of several target genes which encode for PPAR-α, such as PPAR-α itself, as well as fatty acid translocase (FAT/CD36), fatty acid transport protein 1 (FATP1), and inducible nitric oxide synthase (iNOS) [401].

However, beyond binding PPAR-α there is increasing pharmacological evidence for additional receptor targets in mediating OEA biological action [361,362], such as the transient receptor potential vanilloid 1 (TRPV1) and the orphan GPCR 119 (GPR119) [409,410]. TRPV1 is a member of the vanilloid transient receptor potential cation channel subfamily, abundantly expressed in the cardiovascular system, peripheral nervous

system, CNS and in epithelial cells of the bladder and the gastrointestinal tract. It is known to act by activating protein kinase A (PKA) and the endothelial nitric oxide synthase (eNOS), thus stimulating the production of •NO and the release of calcitonin gene-related peptide and substance P [411,412], which, in turn, lead to the altered ion permeability [413]. TRPV1 has been demonstrated to be involved in mediating inflammation mechanisms and pain [409]. In accordance with this latter information, intraperitoneal (i.p.) or intradermic OEA injections cause short-lasting nociceptive responses in wild-type mice, but not in mice lacking TRPV1 [409].

Overton and colleagues have shown that OEA also acts as a medium-potency agonist for GPR119 (median effective concentration (EC50) ~ 3 μM) [410].

The Gαs coupled-GPR119, primarily expressed in human and rodent pancreas, foetal liver, gastrointestinal tract and in rodent brain, stimulates adenylyl cyclase (AC) leading to increased intracellular adenosine 3′,5′-cyclic monophosphate (cAMP) levels, thus regulating incretin and insulin hormone secretion [414]. In the gastrointestinal tract, GPR119 is expressed in enteroendocrine L-cells, which release GLP-1 in response to food ingestion.

It has been demonstrated that, after an intraluminal infusion, OEA enhances GLP-1 secretion from intestinal L-cells through a GPR119-dependent mechanism [415].

However, OEA hypophagic action was still evident in TRPV1 and GPR119 KO mice thus excluding a direct involvement of these receptors in OEA-induced satiety [400,416].

1.6.3. OEA and the control of food intake

Growing evidence accumulated over the last 20 years demonstrated that OEA acts as a satiety signal since it is able to evoke consistent and sustained food intake suppression in rats and mice, following both i.p. and oral administration [359,377,380,382,401,417–419].

Rodent feeding activity that occurs at dark onset, is organized into episodes called "meals" corresponding to a variable amount of food eaten (meal size) [388]. The time interval between the dark onset and the particular moment in which the animal starts eating is defined as "latency", while the gap occurring between a meal and the subsequent is called "inter-meal interval" and has a variable duration [380,381,417].

Feeding-regulating compounds including neuropeptides, hormones or lipid mediators participate in different aspects of this patterned behaviour.

For instance, the serotonergic anorexiant agent d-fenfluramine affects both latency and meal size [420], whereas CCK exerts a pro-satiety effect by only reducing meal size [421].

Conversely, OEA hypophagic effect depends on the feeding state of the animals [380,417] and, in particular, in free feeding rats OEA (i.p, 1–20 mg kg^{-1}) dose-dependently increases the latency to eating onset, prolonging the inter-meal interval, without affecting the meal size, while in food-deprived rats it acts at both levels by delaying feeding onset and reducing meal size [380,417].

It is important to mention that the anorexic effects of OEA are not accompanied by stress, malaise, and nausea. In fact, OEA does not change plasma corticosterone levels and does not induce CTA in rats [359,422].

Moreover, it has been clearly demonstrated that OEA has no effects on drinking behaviour or sodium appetite thus suggesting the specificity of OEA pro-satiety action [359,422].

It has been also demonstrated that, among the structural analogs of OEA such as the endocannabinoid AEA, oleic acid and some other NAEs (including LEA and PEA), OEA is the most active in reducing energy intake. In fact, when administered i.p. to laboratory rats, PEA is less active than OEA in reducing food intake; LEA is similar in potency to OEA, however AEA and oleic acid have no effect [359,423,424], thus suggesting that OEA anorexic effect is structurally selective.

Additional evidence supporting the idea that endogenous OEA participates in satiety induction comes from the observation that OEA physiological levels are strictly dependent on the nutritional status of the animals [361,362].

Moreover, it has been found that a dietary fat intake influences OEA endogenous intestinal levels suggesting a possible role for OEA as intestinal fat sensor [424]. In particular, in rat chronically fed with a high fat-diet (HFD) OEA intestinal levels resulted considerable decreased.

Additionally, several results reported that beyond reducing appetite, OEA might also regulate body weight through modulating peripheral lipid metabolism and enhancing fatty acid uptake in enterocytes [425].

1.6.4. OEA effects in the central nervous system

A large body of evidence revealed that the pro-satiety effect evoked by OEA requires the involvement of selected brain regions, which actively participate in the control of energy homeostasis and feeding behaviour. In this context, it has been observed that after being systemically administered, OEA (10mg kg^{-1} i.p.) significantly increases the transcription of the early gene c-fos (investigated as a marker of neuronal activation) in

the NST and AP of brainstem [37,359,382,383], and in the PVN and SON hypothalamic nuclei [37,359,382,383]. Moreover, in both hypothalamic areas, OEA has been found to simultaneously increase c-fos mRNA and protein in OXY expressing neurons [37,377,426], thus suggesting that the activation of these hypothalamic neuronal populations is accompanied by increased oxytocinergic tone. Interestingly, this effect appeared to be associated with increased levels of OXY mRNA and peptide neurosecretion, along with enhanced circulating OXY levels [37,427].

The OEA-induced neurochemical effects evoked by its systemic administration are highly selective, as demonstrated by recent findings revealing that the levels of additional hypothalamic peptides implicated in energy balance (AVP and TRH in PVN and SON, and POMC expressed in the ARC) are not affected by OEA i.p. injection [37].

Evidence accumulated over the years points to the involvement of different neuronal circuits in mediating OEA action as appetite suppressor, and they include oxytocinergic, noradrenergic and histaminergic neurons [388] (Fig. 1.8). As a matter of fact, the i.c.v. infusion of the OXY antagonist, L-368,889, which selective blocks the cerebral OXTR, has been demonstrated to prevent OEA anorexic effects thus confirming the pivotal role of oxytocinergic transmission in OEA-induced feeding suppression [37].

Moreover, the requirement of hindbrain noradrenergic fibres (that from NST project to the PVN) in mediating OEA's hypophagic effects has been demonstrated in rats subjected to the intra-PVN administration of the toxin saporin (DSAP), a substance able to selectively destroy hindbrain NST-PVN noradrenergic neurons since it is conjugated to an antibody against DBH [383].

Very recently, Provensi and collaborators demonstrated that OEA also requires the integrity of the brain histaminergic system to fully exert its pro-satiety effect [426]. The authors, in fact, showed that mice lacking the enzyme involved in histamine synthesis, such as HDC, or mice pharmacologically deprived of releasable brain histamine did not respond to the hypophagic effect of exogenously administered OEA as normal mice did [426].

Even though OEA has been demonstrated to induce satiety by activating key brain hypothalamic and hindbrain areas, the mechanism by which OEA reaches the brain from the periphery remained poorly understood.

After being systemically or orally administered, OEA quickly reaches the bloodstream, however it cannot readily permeate the brain because of the high expression of its main degrading enzyme, FAAH, in the BBB [428].

For many years, vagal fibres were hypothesized to convey OEA's effects from the periphery to the brain, as suggested by the observation that after a complete subdiaphragmatic vagotomy, the pro-satiety action of OEA is abolished [401]. However, recent findings obtained in rats subjected to a subdiaphragmatic vagal deafferentiation (SDA), demonstrated that the hypophagic action of peripherally administered OEA does not require intact vagal afferents [380].

Given these premises, circumventricular organs, such as the AP in the brainstem, are attracting a great deal of attention for their possible role in allowing the direct access to the brain for circulating peptides and other peripheral signals.

In this background, previous results demonstrated that the systemic administration of OEA at a dose and at a time-point that causes a significant inhibition of eating (10 mg kg^{-1} i.p.), strongly activates neurons of the AP and significantly stimulates c-fos transcription in the SolC, a nucleus of the NST in close contact with the AP [382].

This observation, together with recent findings demonstrating the high expression of PPAR-α in the AP of drug-naïve rats [377], suggests a possible involvement of this circumventricular organ in mediating OEA pro-satiety effect (Fig. 1.8). This hypothesis was then confirmed by novel findings obtained in rats in which the AP was surgically removed. These results revealed the crucial role of this brain area in OEA mechanism of action, since after the ablation of the AP both behavioural and neurochemical effects of OEA were completely abolished [377]. The above-described observations led us to hypothesize that circumventricular organs, thanks to their absence of a functional BBB and being particularly enrich in PPAR-α receptors, could represent a receptive region for circulating OEA, which may exert its central effects through the direct activation of this brain circumventricular organ.

The working model on the central mechanisms mediating OEA's pro-satiety action is depicted in Fig. 1.8A, B.

Furthermore, in the brain OEA is also able to affect monoaminergic transmission. In particular, Yu and collaborators found that after repeated oral administration, OEA increases NA and 5-HT levels in the total brain thus exerting antidepressant-like effects [429]. This result was further confirmed by very recent findings showing that OEA, both acutely and chronically administered, decreases the immobility time of mice exposed to the tail suspension test, a test used to evaluate the depressive-like state in rodents [430].
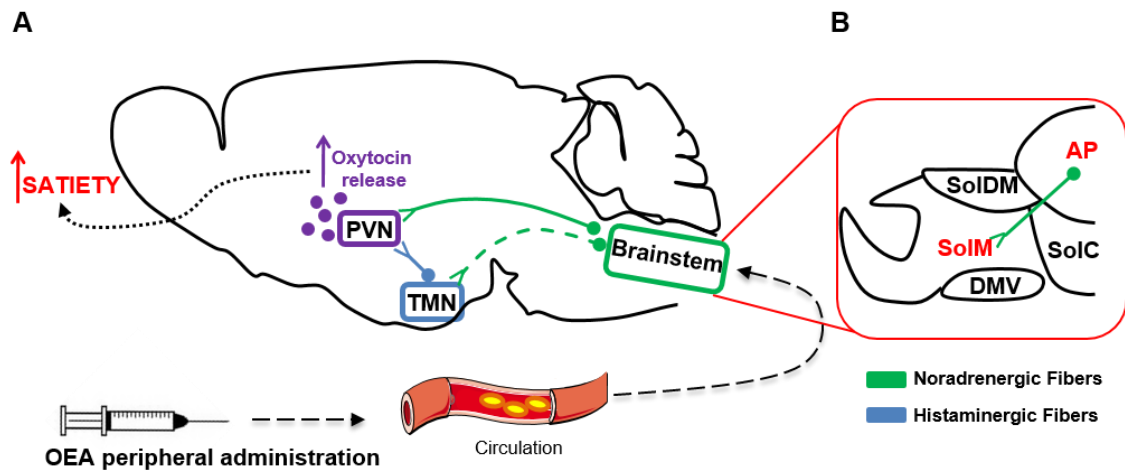
Fig. 1.8: schematic representation showing the central mechanism mediating OEA's hypophagic action. Panel A: Peripherally administered OEA activates the AP, a brainstem region with fenestrated capillaries that lacks a functional blood-brain barrier (BBB). At this level, OEA may cause the activation of noradrenergic neurons projecting to the nucleus of the solitary tract (NST) and especially to the medial part (SolM) (**panel B**). Noradrenergic neurons within the NST could, in turn, activate oxytocinergic neurons of the paraventricular nucleus (PVN), stimulating oxytocin expression and release, and, in addition, could presumably activate the tuberomamillary nucleus (TMN), evoking histamine release from neurons projecting to the PVN. Finally, oxytocin released from neurons of the PVN can centrally act to inhibit eating (adapted by [377]).

This effect was parallel to the OEA-induced increase of 5-HT release (following acute K^+ challenge) in the ventral hippocampus (vHipp) of mice lacking HDC, which appeared to be 'supersensitive' to K^+ administration than wild type animals [430].

Moreover, OEA has been found to influence the stress response by reducing the HPA axis activation, ACTH and CORT levels in an experimental paradigm inducing stress [431,432].

Interestingly, as reported by Tellez and co-authors [433], OEA is also able to modulate feeding behaviour acting within the hedonic non-homeostatic circuits.

1.7. Aim of the thesis

The goal of the present study is to evaluate the role of OEA as a potential novel pharmacological target for the treatment of obesity and eating disorders, two major health problems worldwide [1].

OEA's ability in inducing consistent and sustained food intake suppression in rats and mice, that is mediated by PPAR-α activation [401], has been well characterized over the last two decades from my laboratory and from other research groups [359,377,378,382,417]. In

fact, it is now well recognized that the pro-satiety effect of OEA is strictly dependent on the involvement of key brain hypothalamic and hindbrain areas [37,377,382,383,426].

However, a crucial aspect remained to be fully elucidated, such as the way by which systemically administered OEA can reach the CNS from the periphery and whether it is able to permeate the brain parenchyma.

In this background, circumventricular organs, such as the AP in the brainstem, are attracting a great deal of attention for their possible role in allowing the direct access to the brain for circulating peptides and other peripheral signals. Moreover, previous data showed that the i.p. administration of OEA, strongly activates neurons of the AP and significantly stimulates c-fos transcription in the subpostremal part of the NST, which is the closest sub nucleus to the AP [382].

Based on these premises, in order to better delineate the mechanism underlying the eating-inhibitory effects of OEA, the aim of **chapter II** was to investigate the involvement of the AP in mediating OEA hypophagic action.

To this purpose, in collaboration with Prof. Thomas Lutz at University of Zurich, we subjected rats to a surgical ablation of the AP and evaluated the effects of i.p. OEA administration (10 mg kg^{-1}) on food intake, on Fos expression, on OXY immunoreactivity at both PVN and neurohypophysial level and on the expression of DBH within the brainstem and PVN. Further, we aimed to assess the phenotype of neuronal populations activated by OEA in the brainstem of controls and lesioned rats; to this aim, we assessed, also, whether OEA induced Fos expression co-localized with DBH as marker for noradrenergic neurons. Finally, as last step of our study, we investigated PPAR-alpha expression within the AP.

Furthermore, since there are no observations demonstrating the ability of OEA to permeate the brain parenchyma, in the **chapter III** I aimed to investigate whether systemically administered OEA might directly reach and permeate the CNS through circumventricular organs devoid of a functional BBB, such as the AP and the ME.

To this purpose, in collaboration with Prof. Lutz and Prof Giulio Muccioli at Université Catholique de Louvain, male Wistar rats were sacrificed at different time points (2.5, 5, 15, 30, 60 minutes) after acute administration of OEA (10 mg kg^{-1}, i.p.). Plasma and different brain areas were collected for UPLC-MS/MS quantification of the main NAEs (including OEA, AEA, PEA, SEA, and LEA), and 2-arachidonoyl-glycerol (2-AG). In particular, in order to selectively investigate OEA concentrations within a variety of PPAR-α-expressing cerebral regions, selected brain areas of interest (AP, ME, NST,

ventral and dorsal hippocampus (vHipp and dHipp)) were microdissected and used in this study.

Finally, since current knowledge supports a relationship between neurobiological as well as psychological aspects of overeating, in **chapter IV** I also investigated the OEA's pro-satiety action in a rat model of BED, which is a prototypical eating-related maladaptive behaviour that may determine fluctuations in body weight and in some instance may cause obesity.

Among the different networks involved in the behavioural effect of OEA, it has been demonstrated that the systemic administration of OEA to obese rodents restores a "normal brain dopaminergic activity", which resulted dampened by the excess of fat intake [433]. Moreover, evidence suggests that OEA attenuates the effect of stress by dampening the hyperactivity of the HPA axis [431]. Since both the abnormal dopaminergic transmission and the hyperactivation of HPA axis are considered mechanisms underlying the pathophysiology of BED, by acting at both the two deregulated conditions OEA might represent a potential novel pharmacological target for controlling aberrant eating patterns occurring in BED [273,388,434].

Based on these premises, in order to test this hypothesis in collaboration with Prof. Carlo Cifani of University of Camerino we evaluated OEA effects in a BED model in which female rats with a history of intermittent food restriction show binge-like palatable food consumption after a 15-minute exposure to the sight of the palatable food (frustration stress) [284,289,435].

In this model, we investigated the anti-bingeing acute effects of OEA (2.5, 5 or 10 mg kg^{-1}, i.p.) on HPF intake and analysed the neurobiological bases of these effects by focusing on the brain pattern of c-Fos expression, on DA release in the shell of the nucleus accumbens (AcbSh), on monoamine concentrations/turnovers in selected brain regions and on both CRF and OXY mRNA in hypothalamic and extra hypothalamic areas.

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Chapter II

Role of the area postrema in the hypophagic effects of oleoylethanolamide

Abstract

The satiety-promoting action of oleoylethanolamide (OEA) has been associated to the indirect activation of selected brain areas, such as the nucleus of the solitary tract (NST) in the brainstem and the tuberomamillary (TMN) and paraventricular (PVN) nuclei in the hypothalamus (HYPO), where noradrenergic, histaminergic and oxytocinergic neurons play a necessary role. Visceral ascending fibres were hypothesized to mediate such effects. However, our previous findings demonstrated that the hypophagic action of peripherally administered OEA does not require intact vagal afferents and is associated to a strong activation of the area postrema (AP). Therefore, we hypothesized that OEA may exert its central effects through the direct activation of this circumventricular organ. To test this hypothesis, we subjected rats to the surgical ablation of the AP (APX rats) and evaluated the effects of OEA (10 mg kg^{-1} i.p.) on food intake, Fos expression, hypothalamic oxytocin (OXY) immunoreactivity and on the expression of dopamine beta hydroxylase (DBH) in the brainstem and HYPO. We found that the AP lesion completely prevented OEA's behavioural and neurochemical effects in the brainstem and the HYPO. Moreover OEA increased DBH expression in AP and NST neurons of SHAM rats while the effect in the NST was absent in APX rats, thus suggesting the possible involvement of noradrenergic AP neurons. These results support the hypothesis of a necessary role of the AP in mediating OEA's central effects that sustain its pro-satiety action.

2.1. Introduction

The central nervous system (CNS) and the gastrointestinal tract are constantly in reciprocal communication, through the so-called "gut-brain axis", a bidirectional system that involves neuronal, hormonal and immunological signals [1]. Many of these signals play a role in the control of energy homeostasis, such as leptin, cholecystokinin (CCK), glucagon-like peptide-1 (GLP-1) and peptide YY (PYY), which have been the best characterized [2,3]. Among these "gut-derived signals" a great deal of attention has also been dedicated to oleoylethanolamide (OEA), an endogenous lipid generated in the intestine upon the ingestion of fat [4,5]. OEA is structurally analogous to the endocannabinoid anandamide, although it does not show any affinity for the cannabinoid receptors, nor does it induce cannabinomimetic effects [6]. Most of the consideration for OEA's biological roles and mechanism of action derived from its potential interest as a novel pharmacological target for the treatment of obesity and eating disorders [5,7]. In fact, as a drug, OEA reduces food intake and body weight gain in both lean and obese rodents [6,8-18], effects that appeared behaviourally selective: not linked to anxiety-like symptoms, visceral malaise, alterations in plasma corticosterone, changes of body temperature, pain threshold, or of glucose, leptin and insulin plasma levels [6].

There is accumulating evidence showing that the OEA-induced satiety state [19,20] is mediated by the activation of the peroxisome proliferator-activated-receptor-alpha (PPAR-α) [21].

This observation is supported by several findings: 1) in vitro experiments of saturation binding showed that OEA binds to the "ligand-binding domain" of PPAR-α receptor with a high affinity with a Kd of approximately 40 nM, and stimulates its transcriptional activity with a median effective concentration (EC50) of about 120 nM [21,22]; 2) OEA's pro-satiety effects are completely prevented in mice lacking a functional PPAR-α gene that still respond to other anorexic agents [21,22]; 3) GW7647 and Wy-14643, two synthetic PPAR-α agonists, mimic OEA-induced food intake suppression [21,23]; 4) feeding evokes an increase of OEA concentration in the intestinal mucosa to levels (300 nM) that are sufficient to fully activate PPAR-α [24,25].

It has been previously demonstrated that the hypophagic effect evoked by systemic OEA administration is associated to the engagement of selected brain areas involved in the control of food intake, such as the nucleus of the solitary tract (NST) and the hypothalamic tuberomamillary (TMN), paraventricular (PVN) and supraoptic nuclei

(SON) [5], where noradrenergic, histaminergic and oxytocinergic neurons play a necessary role [5].

OEA's mechanism of action might also involve the activation of vagal fibres, as suggested by the observation that its effect after intraperitoneal (i.p.) administration is blunted in rats subjected to a complete subdiaphragmatic vagotomy or to a pre-treatment with a neurotoxic dose of capsaicin, which destroys vagal and non-vagal unmyelinated afferent nerve fibres [6,21,26]. These observations, together with the finding that OEA does not reduce food intake when administered by intracerebroventricular (i.c.v.) infusion in rats [6], suggests that its mechanism of action could be peripheral rather than central and that the activation of both brainstem and hypothalamic areas might be indirectly mediated by ascending fibres. However, we recently showed that OEA does not require intact intestinal vagal afferents to reduce food intake [18], as observed in rats that underwent a selective subdiaphragmatic vagal deafferentiation (SDA), a surgery that removes all abdominal vagal afferents, but sparing approximately half of the efferents [27,28]. These findings did not seem to support the hypothesis of a strictly peripheral mechanism and suggest that the blockade of OEA's effects by total subdiaphragmatic vagotomy may be due to the absence of efferent rather than afferent innervation.

Whether OEA signal can directly or indirectly reach the brain remained to be fully elucidated. Although systemically or orally administered OEA quickly reaches the blood stream [19,20], indirect evidence suggests that it does not readily permeate the whole CNS, presumably owing to the high expression of its degrading enzyme, fatty acid amide hydrolase (FAAH), in the blood brain barrier (BBB) [29]. However, these findings do not necessarily exclude a possible action mediated by the circumventricular organs such as the area postrema (AP) in the brainstem. In fact, we recently reported that peripheral OEA induces a significant activation of AP neurons and in the subpostremal nucleus of the NST, as suggested by the increased transcription of c-fos in these areas [12]. The AP lacks a functional BBB by virtue of its lack of tight junctions and the presence of fenestrated capillaries, therefore circulating peptides and other peripheral signals can gain direct access to neurons of the AP. On the basis of these observations, we hypothesized that OEA may exert its central effects through the activation of the AP.

To test this hypothesis, we subjected rats to a surgical lesion of the AP (APX) and evaluated the effects of i.p. OEA administration (10 mg kg^{-1}) on food intake, on Fos expression, on oxytocin (OXY) immunoreactivity at both PVN and neurohypophysial level and on the expression of dopamine beta hydroxylase (DBH) within the brainstem

and PVN. Further, we aimed to assess the phenotype of neuronal populations activated by OEA in the brainstem of SHAM-lesioned and APX rats; to this aim, we assessed, also, whether OEA induced Fos expression co-localized with DBH as marker for noradrenergic neurons. Finally, as last step of our study, we investigated PPAR-α expression within the AP.

2.2. Materials and methods

2.2.1. Animals and housing

Forty-one male Wistar rats (Charles River, Sulzfeld, Germany) were used in this study. In particular, forty rats underwent AP lesion or SHAM surgery, while one drug-naïve rat was used to assess PPAR-α expression within the AP. All animals, weighing 200–250 g upon arrival, were individually housed in acrylic cages under a 12:12 dark-light cycle in a climate-controlled room (22 ± 2 °C and 60% relative humidity). All rats were fed with standard chow pellets (N 3430, Provimi KIiba, Gossau, Switzerland) *ad libitum*, unless otherwise stated. All experiments were performed upon the approval of the Veterinary Office of the Canton of Zurich and according to the European Community directives 2010/63/EU. The experimental timeline is depicted in Fig. 2.1A.
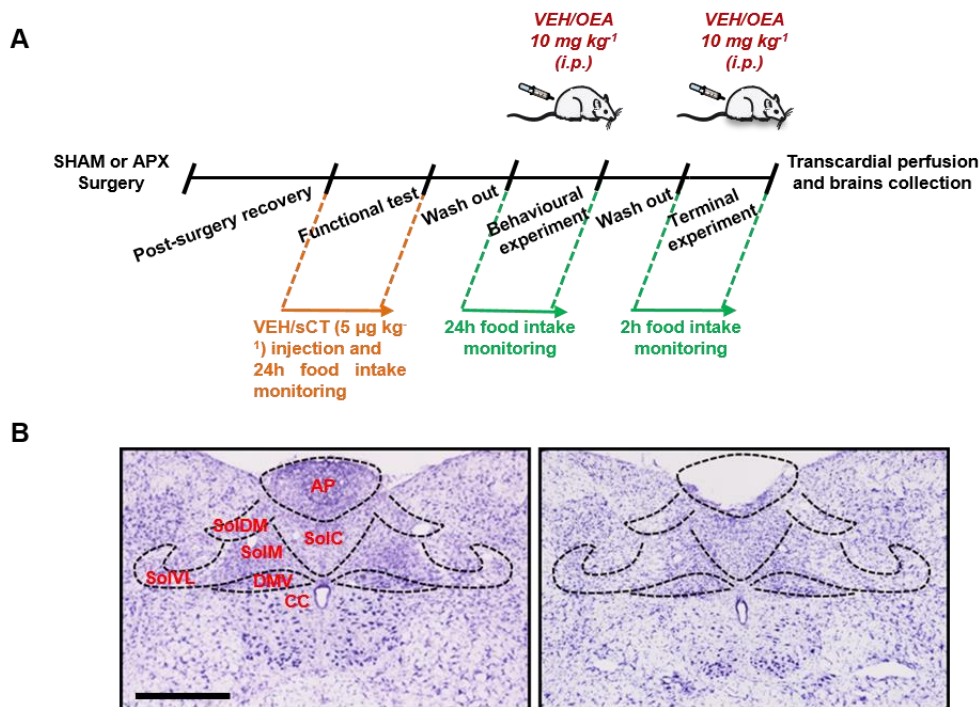
Fig. 2.1: Experimental timeline and AP surgical ablation.
Panel A: Male Wistar rats were subjected to the surgical ablation of the area postrema (APX rats) or to a sham operation (SHAM rats); after a post-surgery recovery period of 2 weeks rats underwent a functional test to verify the lesion. Both APX and SHAM rats were administered with saline or salmon calcitonin (sCT, 5 μg kg^{-1}, i.p.) and cumulative 2-h food intake was monitored. After 2 intervening days for drug washout all animals underwent the behavioural test: they received an i.p. injection of either vehicle (VEH) or OEA (10 mg kg^{-1}) at dark onset, and food intake was measured within 24 h. After 2 days for washout, rats were treated again in a cross-over design with either VEH or OEA (10 mg kg^{-1}); food intake was monitored for 2 h and at the end all animals were euthanized with an overdose of pentobarbital sodium and were subjected to transcardial perfusion. Fixed brains were collected and stored.
Panel B: Representative photomicrographs (scale bar = 500 μm) showing Nissl staining of coronal hindbrain sections obtained from the brain of SHAM-operated rats (left panel) and APX rats (right panel). The superimposed diagrams show the different subnuclei of the nucleus of solitary tract, such as commisural part (SolC), medial part (SolM), dorsomedial part (SolDM) and ventrolateral part (SolVL), and also the dorsal motor nucleus of the vagus (DMV) and the central canal (CC).

2.2.2. Area postrema lesion surgery

Rats were anesthetized using a mixture of ketamine (50 mg kg^{-1}; Narketan, Vetoquinol AG, Ittingen, Switzerland), xylazine (2.5 mg kg^{-1}, Xylazin; Streuli Pharma AG, Uznach, Switzerland) and acepromazine (0.75 mg kg^{-1}, Prequillan; Arovet AG, Dietikon, Switzerland) and placed on a stereotaxic apparatus to be subjected to either APX (N = 20) or SHAM (N = 20) surgery. To this aim, the head of each rat was flexed ventrally at an approximate 110° angle in a stereotaxic frame. Skin and three layers of neck musculature were cut and retracted for visualization of the foramen magnum. Under visual control with a surgical microscope, the cranial dura mater was penetrated and the cerebrospinal fluid was blotted. In the APX procedure, AP lesions were performed by

aspirating the AP with a blunted cannula tip fixed to a flexible tube attached to a vacuum pump [30,31]. In SHAM-operated rats, the AP was exposed but not touched. Rats were given 2 weeks to recover from surgery, receiving post-operative care [32], and were habituated to i.p. injections of saline. One rat subjected to APX died during the recovery period.

As reported in previous studies [32,33], during the course of our experiment it became evident that APX rats were not hyperphagic and they did not gain more weight as compared to SHAM rats. Conversely, after surgery their weight gain was lower than SHAM operated rats and, on the test day, the body weight of APX rats was significantly ($P < 0.001$) lower than SHAM rats (SHAM rats: 363.8 g $\pm$ 5.65 g, APX rats; 265.1 g $\pm$ 8.74 g). Nevertheless, if normalized to body weight (g kg^{-1}), the daily consumption of food intake was similar between the two groups (data not shown).

Success and specificity of the AP lesion was verified both functionally and histologically [30,31,34,35]. In particular, the functional verification was based on the lack of the anorexigenic effects of peripheral salmon calcitonin (sCT, 5 µg kg^{-1}, i.p.), which requires an intact AP to inhibit food intake in rodents [32]. 24 h before the functional test, SHAM and APX rats were administered with saline at dark onset and their 2-h food intake was recorded as amount of food eaten. On the functional test day, at dark onset, rats received sCT (5 µg kg^{-1}, i.p.) and 2h-food intake was recorded. SHAM rats displayed a food intake reduction of minus 19.59 $\pm$ 6.79% after sCT compared to saline; most APX rats (14 out of 19 rats) showed no decrease of food intake after sCT compared to saline; in fact APX rats treated with sCT consumed 4.35 $\pm$ 0.73 g of food, while APX rats administered with saline consumed 3.25 $\pm$ 0.58 g, with no statistical difference between the two groups. Five of 19 APX animals displayed a significant 2-h food intake reduction (minus 79.15 $\pm$ 7.71%) after sCT administration compared to saline, therefore they were suspected to be not properly lesioned. This was then confirmed by the histological verification performed in all animals at the end of the terminal experiment by an experimenter blind to the type of surgery, who evaluated the extent of AP lesion on Nissl-stained brain slices (Fig. 2.1B). Therefore, only data from 14 of 19 APX animals were analyzed, and data from 5 of 19 APX animals were excluded from all further analyses.

2.2.3. Drugs and treatments

OEA was administered (10 mg kg^{-1}) by i.p. injections in a solution (2 ml kg^{-1}) of saline/polyethylene glycol/Tween 80 (90/5/5, v/v/v). Control animals received the

injection of vehicle (VEH). Both VEH and OEA solutions were freshly prepared on each test day and administered about 10 minutes (min) before dark onset.

2.2.4. Food intake experiment

On the day of the feeding experiment, the chow pellets were temporarily removed for 1 hour (h) before the beginning of the dark phase and all animals were treated 10 min before dark onset. Pre-weighted standard chow pellets were returned at dark onset and food intake was measured manually using a digital scale at 5 different time-points (0.5, 1, 2, 20, and 24 h) as the difference between the amount of food given after treatment and the food remaining after food access, with correction for spillage. To minimize the number of animals used in the study, the feeding experiment was carried out on two different days with two intervening days between trials, by using a cross-over design. In particular, VEH and OEA were administered in a randomized, latin square design, so that each animal within the APX and SHAM group was examined under both treatment conditions. The timing schedule, the dosage of OEA, and the route of administration were chosen according to our previous studies [11,14].

2.2.5. Terminal experiment

On the day of the second behavioural test, rats were euthanized after 2 h from dark onset, by transcardial perfusion. The animals were deeply anesthetized with pentobarbital sodium (80 mg kg^{-1}; Kantonsapotheke, Zurich, Switzerland) and transcardially perfused with ice-cold phosphate buffer saline (0.1 M PBS, pH 7.4), followed by fixative solution containing 4% paraformaldehyde. Fixed brains and pituitary glands were collected, postfixed overnight, cryoprotected in 20% sucrose-phosphate buffer (for 48 h at 4 °C), and then snap frozen in dry-ice-cold 2-methylbutane (−60 °C), to be stored at −80 °C until processing. All the brains and the pituitary glands were processed in the immunohistochemistry study, so that the immunohistochemical analyses were performed in the same rats that were used for the behavioural experiment. Moreover, the drug-naïve rat was sacrificed by decapitation and the brain was collected and processed for PPAR-α immunohistochemistry.

2.2.6. Immunohistochemistry study

Brains were cut on a cryostat (model HM550; Thermo Fisher Scientific, Kalamazoo, MI, USA) into five series of 20-μm coronal sections containing hypothalamic and brainstem

structures, and mounted on positively charged microscope slides (SuperFrost Plus, Menzel, Germany) and stored at −20 °C.

- *Fos fluorescent immunostaining*

Fos immunostaining was performed on brain section series containing AP, NST, PVN, and TMN. We adapted the protocol for antigen retrieval described by Shi and collaborators [36] by submerging brain sections in a sodium citrate buffer (10 mM pH 6.0) heated at 95 °C for 5 min. Sections were then rinsed with PBS (0.1 M pH 7.4) and incubated for 30 min in a solution containing 0.3% Triton X-100 (Sigma–Aldrich) and 10% of Normal Goat Serum (Jackson Immunoresearch, Baltimore Pike, Pennsylvania). After additional washes, sections were incubated with a solution containing the primary antibody (rabbit anti-Fos polyclonal primary antibody, 1:500 dilution, Santa Cruz California) overnight at 4 °C. Sections were then incubated with an anti-rabbit Alexa Fluor 594 secondary antibody (1:300 dilution; Invitrogen) for 90 min at room temperature in the presence of Hoechst 33258 (1:5000 dilution; Sigma–Aldrich), used to detect cell nuclei. After final washes slides were cover-slipped with fluoromount (eBioscience).

- *Fos and DBH double fluorescent immunostaining*

A second series of sections containing AP and NST was double-stained for Fos and DBH to assess their co-expression within these brainstem structures. Sections were rinsed with PBS and incubated for 1 h in a solution containing 5% Triton X-100 (Sigma-Aldrich) and 2% of Normal Goat Serum (Jackson Immunoresearch) in PBS. Sections were then incubated with mouse anti-DBH monoclonal primary antibody (1:500 dilution, MAB308, Millipore) for 2 overnights at 4 °C. After 1 overnight incubation at room temperature with goat anti-mouse Alexa Fluor 488 secondary antibody (1:250 dilution, Invitrogen), sections were subjected to the Fos immunostaining procedure following the protocol described in the previous paragraph.

- *DBH and OXY chromogenic immunostaining*

One series containing AP/NST, two series of PVN sections and a series of sagittal sections of the pituitary gland (15 μm thick) were used for DBH or OXY immunostaining using the 3,3′-diaminobenzidine (DAB)-H_2O_2-horseradish peroxidase detection method, following the procedure described in our previous work [14,37]. Briefly, sections were rehydrated in PBS (pH 7.4) and then incubated for 2 h in a solution containing 2% BSA (Sigma Aldrich) in 5% Triton X-100, followed by incubation with the respective primary

antibodies (mouse anti-DBH monoclonal primary antibody 1:500 in BSA/PBST, Millipore or mouse anti-OXY monoclonal primary antibody 1:10000 dilution, MAB 5296, Millipore) at 4 °C. Sections were then incubated with the respective secondary antibodies (Donkey anti-mouse biotinylated secondary antibody; Jackson Immunoresearch, 1:200, for DBH staining, and 1:500, for OXY staining) in 5% PBST overnight at room temperature. After incubation for 1 h with the ABC Kit (Vectastain ABC kit; Vector Laboratories), sections were stained by incubation in DAB (Vector Laboratories) chromogen solution. The slides were then rinsed with PBS, dehydrated in graded alcohol, immersed in xylene and cover-slipped with Eukitt (Sigma–Aldrich).

-   *PPAR- α fluorescent immunostaining*

PPAR-α immunostaining was performed on brain sections containing the AP of a drug-naïve rat, to investigate qualitatively the expression of PPAR-α within the AP. Briefly, sections were rinsed with PBS (0.1 M pH 7.4) and incubated for 1 h in a solution containing 0.3% Triton X-100 (Sigma–Aldrich) and 5% of BSA (Sigma Aldrich) at 37 °C. After additional washes, sections were incubated with a solution containing the primary antibody (rabbit anti-PPAR-α polyclonal primary antibody, 1:200 dilution, Abcam ab8934) overnight at 4 °C. Sections were then incubated with an anti-rabbit Alexa Fluor 594 secondary antibody (1:300 dilution; Invitrogen) for 90 min at room temperature in the presence of Hoechst 33258 (1:5000 dilution; Sigma–Aldrich), used to detect cell nuclei. After final washes, slides were cover-slipped with fluoromount (eBioscience). Moreover, to control for the staining specificity, an additional series of slices containing the AP, underwent the same protocol procedure except for the incubation with the primary antibody.

2.2.7. Brain section analyses

All brain and pituitary sections obtained from SHAM and APX animals included in the analyses were observed under a Nikon Eclipse 80i microscope equipped with a color charge-coupled device camera and controlled by the software NIS-Elements-BR (Nikon). Slices were photographed under epifluorescent conditions or in light field, where appropriate. The rat brain atlas by Paxinos and Watson [38] was used as reference for the localization of the brain areas of interest. Moreover, following the criteria already used in our previous work [12] we referred to the following subdivision of the NST subnuclei: commisural part (SolC), medial part (SolM), dorsomedial part (SolDM), ventrolateral

part (SolVL) (Fig. 2.1 B). The analyses of Fos positive cells were conducted manually by counting each Fos positive cell of the NST, PVN and TMN. Moreover, to assess Fos and DBH co-expression in AP and SolM, the percentage of DBH-positive cells within Fos −positive neurons was determined by counting also the number of double-stained cells. DBH and OXY DAB-immunostaining were measured semi-quantitatively as optical density (OD) by using the Scion Image software and considering, for background normalization, the averaged OD either of non-immunoreactive regions or of white matter structures within the same brain slice. The investigator was blind to animal treatment; measurements were obtained in at least three consecutive tissues sections per animal containing the desired structure. Finally, AP-containing slices, which were immunostained for PPAR- α, were photographed under a FV1000 confocal microscope (Olympus, Tokyo, Japan), using a PlanApo (Olympus) objective 60× oil A.N. 1.42. Excitation light was obtained by a Laser Dapi 408 nm for Hoechst detection and a Diode Laser HeNe (561 nm) for Alexa 597. Hoechst emission was recorded from 415 to 485 nm, Alexa 555 emission was recorded from 583 to 628 nm.

2.2.8. Statistical analyses

Feeding data were statistically analyzed by two-way ANOVA for repeated measures, with "time" and "treatment" as the two factors, followed by Bonferroni's post hoc test for multiple comparisons (IBM SPSS, version 22, IBM Analytics). Immunohistochemical data were statistically analyzed by two-way ANOVA, with "surgery" and "treatment" as the two factors. Tukey's test was used as a post hoc test to perform multiple comparisons. Moreover, because of the difference in the number of slides examined and the high degree of freedom, the error degrees of freedom were kept constant at a value based on the actual number of animals per group used in each experiment. In all instances, the threshold for statistical significance was set at $P < 0.05$. Data obtained from rats that were not properly lesioned were excluded from the analyses.

2.3. Results

2.3.1. APX prevents the hypophagic effect of OEA

We hypothesized that AP neurons are activated by and are necessary for peripherally administered OEA to reduce eating. Therefore, our first aim was to evaluate whether the surgical lesion of the AP would prevent OEA's pro-satiety action. We followed the same

protocol used in our previous studies [11,12,14], according to which OEA was administered i.p. to rats few minutes before dark onset and at a dosage (10 mg kg^{-1}) that does not readily allow penetration into the brain but is able to inhibit food intake in free-feeding animals [11,12,14]. Cumulative food intake was monitored at 0.5, 1, 2, 20 and 24 h after dark onset. The experimental timeline is depicted in Fig. 2.1A and representative photomicrographs showing Nissl staining of coronal hindbrain sections obtained from the brains of SHAM-operated and APX rats are reported in Fig. 2.1B. The two-way ANOVA analyses for repeated measures of the cumulative food intake of SHAM rats showed a significant effect of treatment ($F_{treatment}$ = 18.598 df = 1/8, P < 0.01), a significant effect of time (F_{time} = 1805.834, df = 4/32, P < 0.001) and no interaction between treatment and time ($F_{interaction}$ = 1.556, df = 4/32, P = 0.238). Consistently with our previous observations [5], post hoc analyses revealed that the systemic administration of OEA markedly reduced food intake in SHAM-operated rats at all the time points considered, i.e. without inducing compensatory hyperphagia in response to the early reduction in eating (0.5 h: P < 0.05; 1 h and 2 h; P < 0.001; 20 h; P < 0.01 24 h: P < 0.05; Fig. 2.2, left panel).The two-way ANOVA for repeated measures of the cumulative food intake of APX groups showed no effect of treatment ($F_{treatment}$ = 0.041 df = 1/5, P = 0.847), a significant effect of time (F_{time} = 43.078, df = 4/20, P < 0.001) and no interaction between treatment and time ($F_{interaction}$ = 0.222, df = 4/20, P = 0.709). Hence, as we hypothesized, the AP lesion completely prevented OEA's hypophagic effect, since post hoc analyses revealed that APX rats consumed similar amounts of food, regardless of the acute treatment with VEH or OEA (Fig. 2.2, right panel).
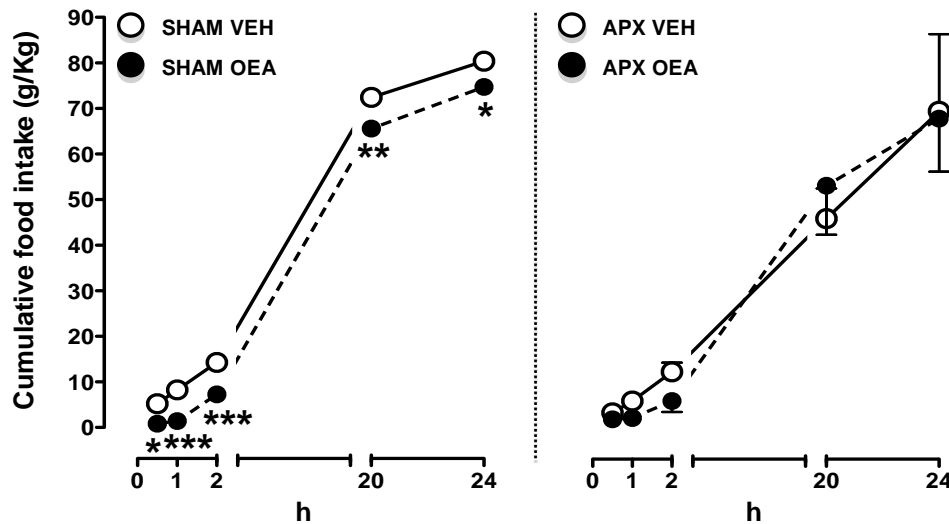
Fig. 2.2. The surgical ablation of the AP prevents OEA's hypophagic action.
Time course of cumulative food intake (normalized to body weight, $g\,kg^{-1}$) at different time-points (0.5, 1, 2, 20 and 24 h) after vehicle (VEH) or OEA (10 mg kg^{-1}, i.p.) administration to SHAM (left panel N = 20) and APX rats (right panel N = 14–15). Data are expressed as mean ± SEM. *P < 0.05; **P < 0.01; ***P < 0.001 vs VEH in the same surgery group (Bonferroni's test).

2.3.2. OEA induces Fos in DBH neurons of the AP

Previous studies demonstrated that OEA's effect on food intake is paralleled by a selective induction of c-fos in specific brain areas [5]; these areas include the AP [12]. We now confirmed this observation by counting Fos-positive neurons in the AP of selected slices obtained from the brain of SHAM rats (Fig. 2.3A). The number of Fos-positive cells in the AP of SHAM rats treated with OEA was about 2.5 fold higher than in SHAM rats treated with VEH (P < 0.001, Fig. 2.3B). Moreover, in our previous study [14], we demonstrated that noradrenergic brainstem-HYPO projections are crucially involved in the activation of the OXY system in the PVN, which, in turn, mediates OEA's pro-satiety action. Therefore, the second aim of our study was to evaluate the effect of peripheral administration of OEA on DBH immunoreactivity of the AP. Double immunohistochemical experiments revealed that OEA-induced Fos expression occurred in DBH-expressing neurons (Fig. 2.3C). In particular, we found that OEA increased (by almost three-fold) the percentage of neurons co-expressing Fos and DBH within the AP (P < 0.05, Fig. 2.3D). Other neuronal populations activated by OEA administration in the AP were not identified in the present study. The semiquantitative densitometric analyses of DBH-immunostaining of the AP (Fig. 2.4A) showed that OEA treatment also significantly increased DBH expression in the AP of SHAM rats (P < 0.05, Fig. 2.4B).
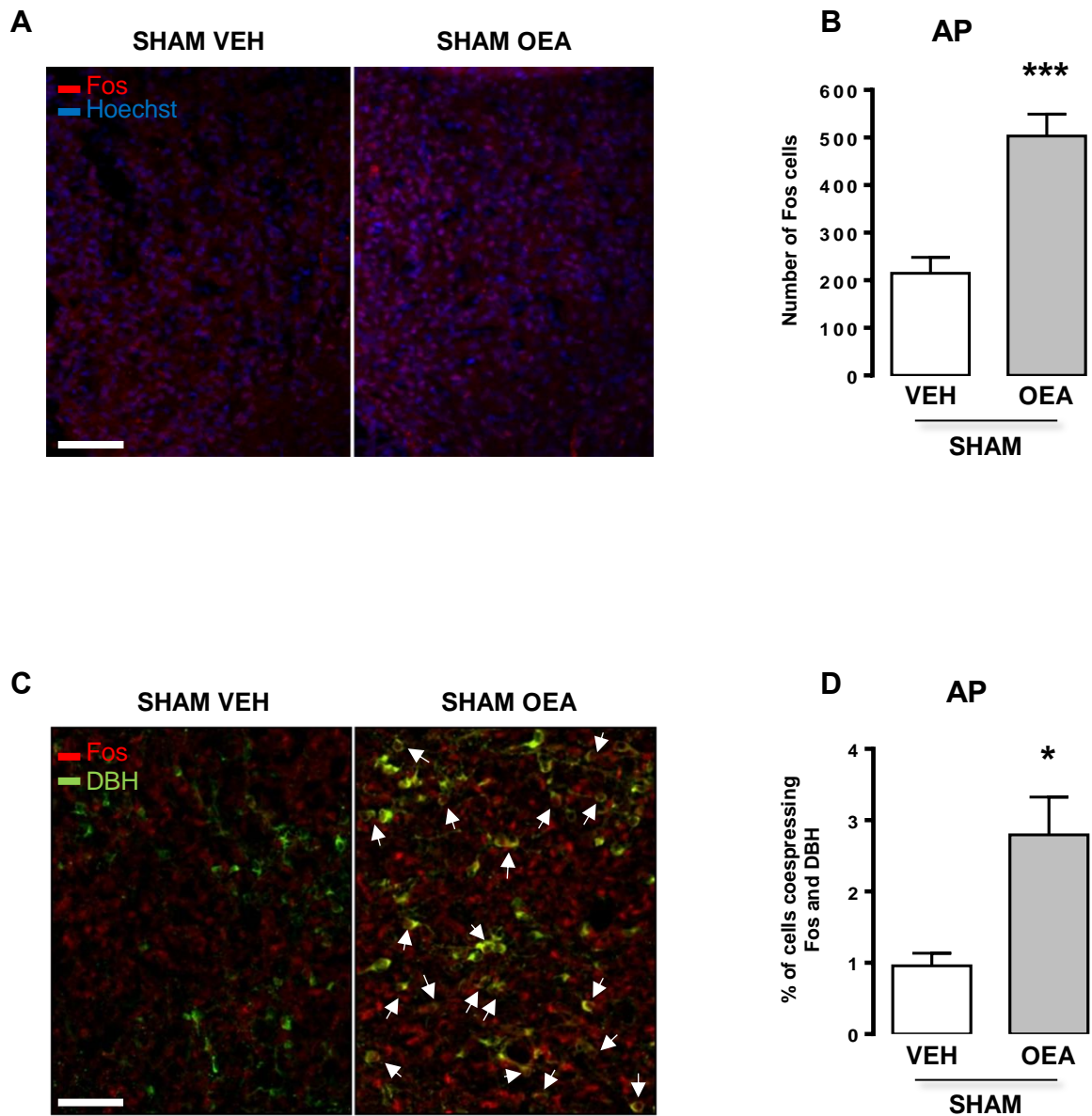
Fig. 2.3. OEA treatment increases Fos immunoreactivity and induces Fos expression in DBH-expressing neurons in the AP of SHAM rats.
Representative fluorescent photomicrographs (scale bar = 100 μm) showing Fos immunostaining/Hoechst (red/blue, **panel A**) and Fos/dopamine beta hydroxylase (DBH) (red/green, **panel C**) double-immunostaining of the area postrema (AP) in brain slices collected from SHAM rats treated with either vehicle (VEH) or OEA (10 mg kg^{-1}, i.p.) and sacrificed 2 h after treatment. Number of Fos positive cells (**panel B**) and percentage of cells co-expressing Fos and DBH (**panel D**) within the AP of SHAM rats treated with either VEH or OEA (10 mg kg^{-1}, i.p.) and sacrificed 2 h after treatment. Data are expressed as mean ± SEM. *$P < 0.05$; ***$P < 0.001$ vs VEH in the same surgery group (Unpaired student t-test; N = 3).

2.3.3. APX prevents OEA-induced DBH and Fos expression in the NST

Neurons from the AP project, among others, to neurons of the NST, which, in turn, project extensively to other key regions involved in the control of food intake, such as the HYPO, the amygdala, and the nucleus accumbens [39–44]. Therefore, we hypothesized that DBH-positive neurons of the AP activated by OEA might, in turn, activate neurons of the NST. To test this hypothesis, we evaluated whether an AP lesion prevents the effects of OEA on DBH and Fos expression in the different subnuclei of the NST. From the semiquantitative densitometric analyses of DBH immunostaining (Fig. 2.4 panels C–F), we found that the signal was different among the different NST subnuclei, with the highest immunoreactivity observed in the SolM (Fig. 2.4D). Moreover, OEA treatment significantly increased DBH expression in most NST subnuclei of SHAM rats, while the AP lesion prevented such increase, without affecting per se DBH immunostaining in VEH-treated rats (Fig. 2.4 panels C–E). The DBH immunostaining of the SolVL of SHAM rats was unaffected by OEA treatment (Fig. 2.4F). Similar results were obtained by counting the number of Fos- positive cells in the different NST subnuclei (Fig. 2.5 panels A–D), with the highest number being found in the SolM (Fig. 2.5B). Moreover, the number of Fos-positive cells was increased in all NST subnuclei of SHAM rats treated with OEA, as compared to SHAM rats treated with VEH. Such increase was completely prevented by the AP lesion (Fig. 2.5 panels A–D). The lesion per se did not affect the baseline number of Fos-positive cells, so that APX-VEH rats did not differ from SHAM-VEH rats. The results from the two-way ANOVA analyses conducted for each of the brain areas and for both DBH and Fos expression levels are reported in Table 2.1; the results from the post-hoc analyses are reported in Fig. 2.4 and 2.5, respectively. Double immunohistochemical experiments revealed that at the level of SolM, OEA-induced Fos expression in SHAM rats included DBH-expressing neurons (Fig. 2.5E).The two way ANOVA of the percentage of neurons co-expressing Fos and DBH in this area, calculated with respect to the total number of neurons showing Fos immunolabeling (Fig. 2.5F), revealed significant effects of treatment, surgery, and their interaction ($F_{treatment} = 50.646$, df = 1/11, P < 0.001; $F_{surgery} = 49.611$, df = 1/11, P < 0.001; $F_{interaction} = 51.121$, df = 1/11, P < 0.001). The post hoc analysis demonstrated that OEA treatment caused a marked increase of Fos-positive neurons expressing DBH only in SHAM rats (P < 0.001 vs their VEH-administered controls) but not in APX rats (P = 0.981; Fig. 2.5F). This observation, together with the strongest DBH and Fos immunostaining of SolM suggests that this

subnucleus might be a putative pathway through which the OEA signal, originating in the AP, can reach higher brain centers. Future studies should address this hypothesis.
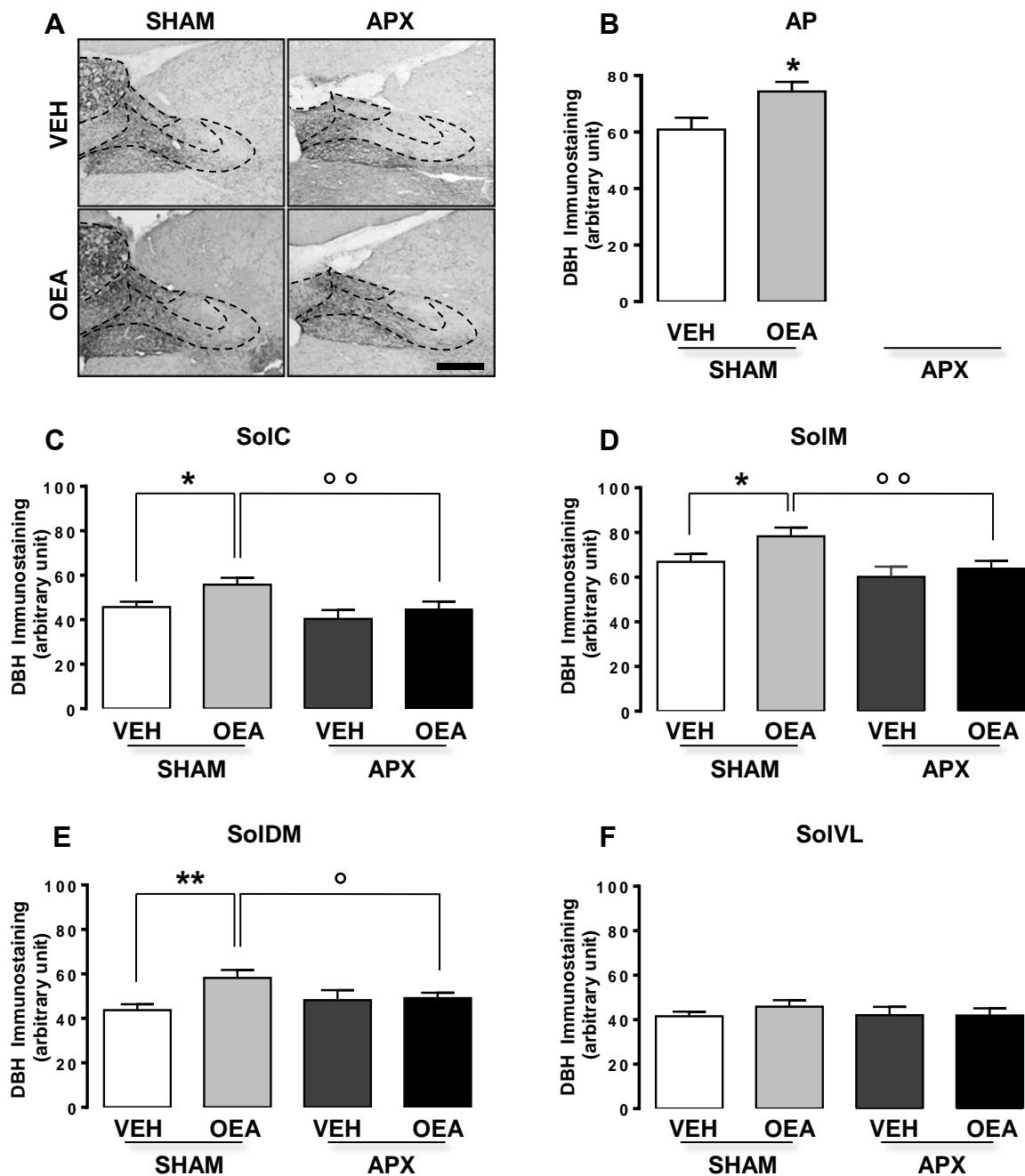
Fig. 2.4. Effects of OEA on DBH expression in the AP and NST of SHAM and APX rats.
Representative photomicrographs (scale bar = 500 μm, **panel A**) showing dopamine beta hydroxylase (DBH) immunostaining within the area postrema (AP) and the nucleus of the solitary tract (NST) in brain slices collected from SHAM and APX rats treated with either vehicle (VEH) or OEA (10 mg kg^{-1}, i.p.) and sacrificed 2 h after treatment. Semiquantitative densitometric analysis of DBH expression within the AP (**panel B**) and the different subnuclei of the NST, such as commisural part (SolC, **panel C**), medial part (SolM, **panel D**), dorsomedial part (SolDM, **panel E**) and ventrolateral part (SolVL, **panel F**), of SHAM and APX rats treated with either VEH or OEA (10 mg kg^{-1}, i.p.) and sacrificed 2 h after treatment. Data are expressed as mean ± SEM. *$P < 0.05$; **$P < 0.01$ vs VEH in the same surgery group; ∘ $P < 0.05$; ∘∘ $P < 0.01$ vs SHAM in the same treatment group (AP: unpaired student t-test; N = 4-6; NST: Tukey's test; SHAM: N = 3–6; APX: N = 3–6).
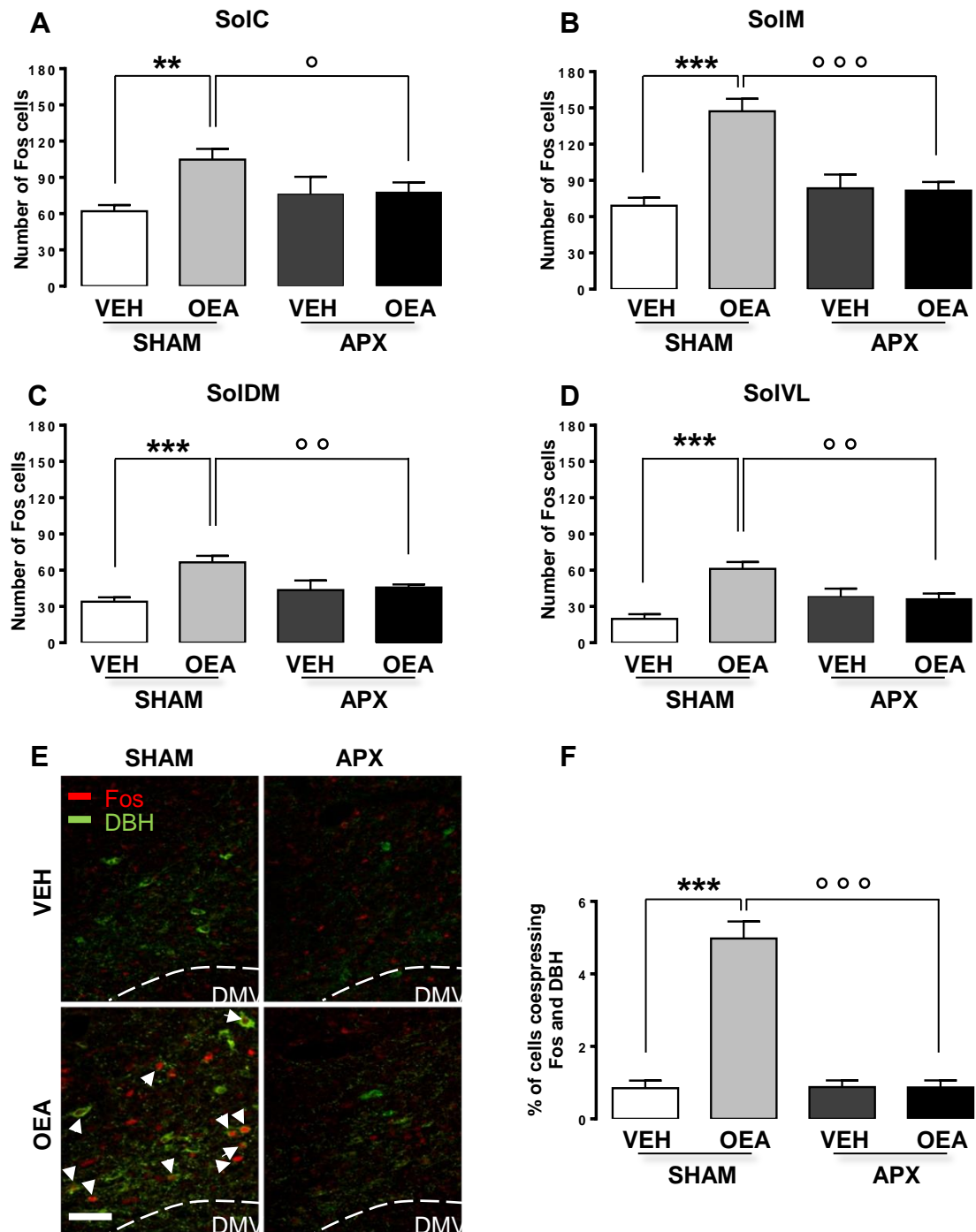
Fig. 2.5. The surgical ablation of the AP prevents OEA-induced increase of Fos immunoreactivity in the NST and reduces the percentage of cells co-expressing Fos and DBH in the SolM. Number of Fos positive cells (**panels A-D**) in the different subnuclei of the nucleus of solitary tract (NST), such as commisural part (SolC, **panel A**), medial part (SolM, **panel B**), dorsomedial part (SolDM, **panel C**) and ventrolateral part (SolVL, **panel D**), and percentage of cells co-expressing Fos and dopamine beta hydroxylase (DBH) within the SolM (**panel F**) in SHAM and APX rats treated with either vehicle (VEH) or OEA (10 mg kg^{-1}, i.p.) and sacrified 2 h after treatment. Data are expressed as mean ± SEM. **P<0.01; ***P<0.001 vs VEH in the same surgery group; ∘P<0.05; ∘∘P<0.01; ∘∘∘P<0.001 vs SHAM in the same treatment group (Tukey's test; SHAM: N = 3; APX: N = 3–4). **Panel E**: representative photomicrographs (scale bar = 100 μm) of double Fos/DBH immunofluorescent staining (red/green) in brain slices containing the SolM, collected from SHAM and APX rats treated with either VEH or OEA (10 mg kg^{-1}, i.p.) and sacrified 2 h after treatment.

Table 2.1. Results of the two-way ANOVA analyses of Fos and DBH expression observed in the NST subnuclei of both SHAM and APX rats after intraperitoneal injection of OEA or vehicle

		$F_{treatment}$	$F_{surgery}$	$F_{interaction}$	df
Fos	**SolC**	5.882 (P < 0.05)	0.487 (P = 0.498)	5.045 (P < 0.05)	1/12
	SolM	17.582 (P < 0.01)	8 (P < 0.05)	19.392 (P < 0.001)	1/12
	SolDM	13.140 (P < 0.01)	1.392 (P = 0.260)	10.105 (P < 0.01)	1/12
	SolVL	14.992 (P < 0.01)	0.429 (P = 0.524)	17.856 (P < 0.01)	1/12
DBH	**SolC**	5.102 (P < 0.05)	6.408 (P < 0.05)	0.779 (P = 0.389)	1/18
	SolM	3.691 (P = 0.071)	7.371 (P < 0.05)	0.995 (P = 0.332)	1/17
	SolDM	5.067 (P < 0.05)	0.429 (P = 0.520)	3.947 (P = 0.06)	1/19
	SolVL	0.478 (P = 0.498)	0.319 (P = 0.579)	0.546 (P = 0.469)	1/18

SolC (Nucleus of Solitary Tract Commissural part), SolM (Nucleus of Solitary Tract Medial part), SolDM (Nucleus of Solitary Tract Dorsomedial part), SolVL (Nucleus of Solitary Tract Ventrolateral part.

2.3.4. APX prevents OEA induced Fos in the PVN and TMN

Our previous work demonstrated that two hypothalamic regions, namely TMN and PVN, participate in OEA's effect on eating [11,45], showing an increase of Fos expression. Therefore, in the present work we evaluated whether this effect could be prevented by the surgical lesion of the AP (Fig. 2.6A, B). By counting Fos-positive neurons in the PVN (Fig. 2.6C) and analyzing the results by two-way ANOVA, we obtained a significant effect of treatment ($F_{treatment}$ = 12.787, df = 1/12, P < 0.01), of surgery ($F_{surgery}$ = 8.443 df = 1/12, P < 0.05) and a significant interaction between the two factors ($F_{interaction}$ = 5.365 df = 1/12, P < 0.05). The results from the two-way ANOVA of the TMN data sets (Fig. 2.6D) revealed significant effects of treatment and surgery, while there was no significant interaction between the two factors ($F_{treatment}$ = 9.998 df = 1/12, P < 0.01; $F_{surgery}$ = 7.036 df = 1/12, P < 0.05; $F_{interaction}$ = 1.586 df = 1/12, P = 0.231).

The results from the test for multiple comparisons showed that, in keeping with our previous observations [11,45], OEA treatment significantly induced Fos expression in the

PVN and TMN of SHAM- operated rats (P < 0.001 and P < 0.01, respectively) but did not exert any effect in both areas of APX rats, thus suggesting that the AP might play a necessary role for the activation of PVN and TMN induced by OEA. The AP lesion, also in this case, did not affect per se the number of Fos-positive neurons at baseline (i.e. in the APX- VEH group versus the SHAM-VEH group) either in the PVN or in the TMN (Fig. 2.6C, D).
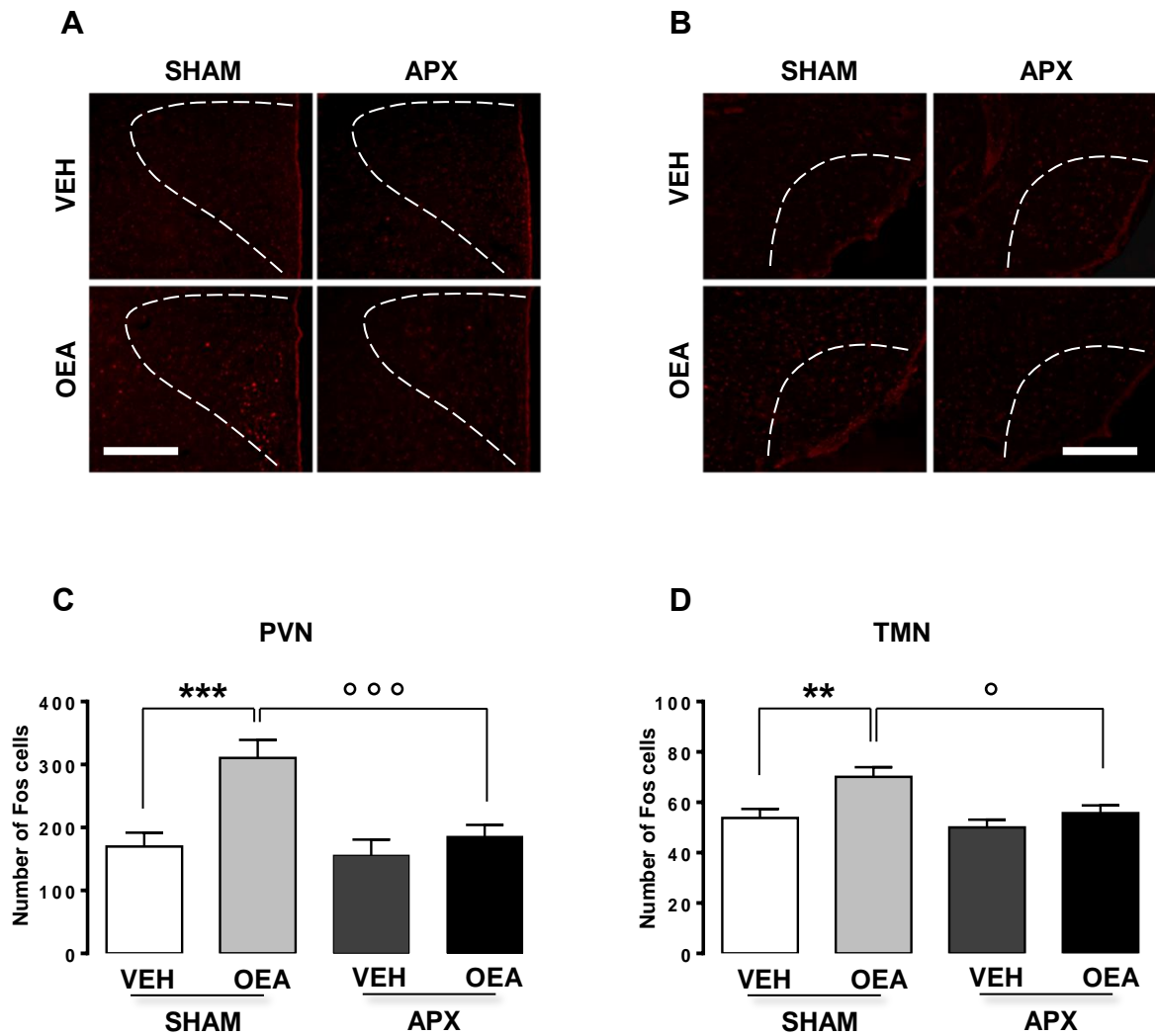
Fig. 2.6. The surgical ablation of the AP prevents the increase of Fos immunoreactivity induced by OEA in the PVN and TMN of the HYPO.
Representative photomicrographs (scale bar = 300 μm) showing Fos immunofluorescent staining of brain slices containing the paraventricular (PVN, **panel A**) and the tuberomamillary (TMN, **panel B**) nuclei collected from SHAM and APX rats treated with either vehicle (VEH) or OEA (10 mg kg^{-1}, i.p.) and sacrificed 2 h after treatment.
Number of Fos positive cells in the PVN (**panel C**) and TMN (**panel D**) in SHAM and APX rats treated with either VEH or OEA (10 mg kg^{-1}, i.p.) and sacrificed 2 h after treatment. Data are expressed as mean ± SEM. **P < 0.01; ***P < 0.001 vs VEH in the same surgery group; ∘ P < 0.05; ∘∘∘ P < 0.001 vs SHAM in the same treatment group (Tukey's test; PVN, SHAM: N = 3; APX: N = 3–4. TMN, SHAM: N = 3–4; APX: N = 3).

2.3.5. APX prevents OEA-induced DBH and OXY expression in the PVN

OXY neurons of the PVN receive a direct excitatory input arising from the A2 noradrenergic cell group in the NST [46]. We previously demonstrated that these noradrenergic projections mediate OEA's effects on eating and on hypothalamic OXY transmission [14]. Therefore, the next step of the present work was to evaluate whether the surgical lesion of the AP prevented the effects of OEA on OXY and DBH expression in the PVN (Fig. 2.7A, B). Both OXY and DBH immunostaining were semiquantitatively evaluated by optical densitometry and data are reported in Fig. 2.7, panels C–D. The results obtained from the two-way ANOVA of OXY OD revealed a significant effect of the treatment ($F_{treatment}$ = 4.531 df = 1/28, P < 0.05), a significant interaction between surgery and treatment ($F_{interaction}$ = 19.584 df = 1/28, P < 0.001) and no effect of the surgery ($F_{surgery}$ = 1.927 df = 1/28, P = 0.176). A significant interaction between the surgery and treatment was also found by analyzing the OD of DBH staining ($F_{interaction}$ = 10.907 df = 1/26, P < 0.01), while no significant effect was found either for the treatment ($F_{treatment}$ = 0.098 df = 1/26, P = 0.756) or for the surgery ($F_{surgery}$ = 0.139 df = 1/26, P = 0.712).

Post hoc analyses revealed that OEA treatment significantly increased the expression of both OXY and DBH within the PVN in SHAM rats (Fig. 2.7C, D; P < 0.001 and P < 0.05 respectively). The AP lesion completely prevented such increases without per se altering the baseline levels found in VEH-treated rats.

Finally, we investigated whether the AP lesion would prevent the effects of OEA on OXY immunoreactivity of the posterior pituitary gland (Fig. 2.7E) [11,14]. The results of the two-way ANOVA of the OD measured in the pituitary glands immunostained for OXY revealed a significant effect of treatment ($F_{treatment}$ = 11.013, df = 1/22, P < 0.01) a significant effect of the surgery ($F_{surgery}$ = 5.033, df = 1/22, P < 0.05) and a significant interaction between the two factors ($F_{interaction}$ = 12.301, df = 1/22, P = 0.01). The post hoc test for multiple comparisons demonstrated that OEA caused a significant increase of OXY immunostaining in the posterior pituitary gland of SHAM rats (Fig. 2.7F, P < 0.001 vs their VEH-administered controls) and that the AP lesion completely prevented such increase without per se altering the baseline OXY expression found in VEH-treated rats.
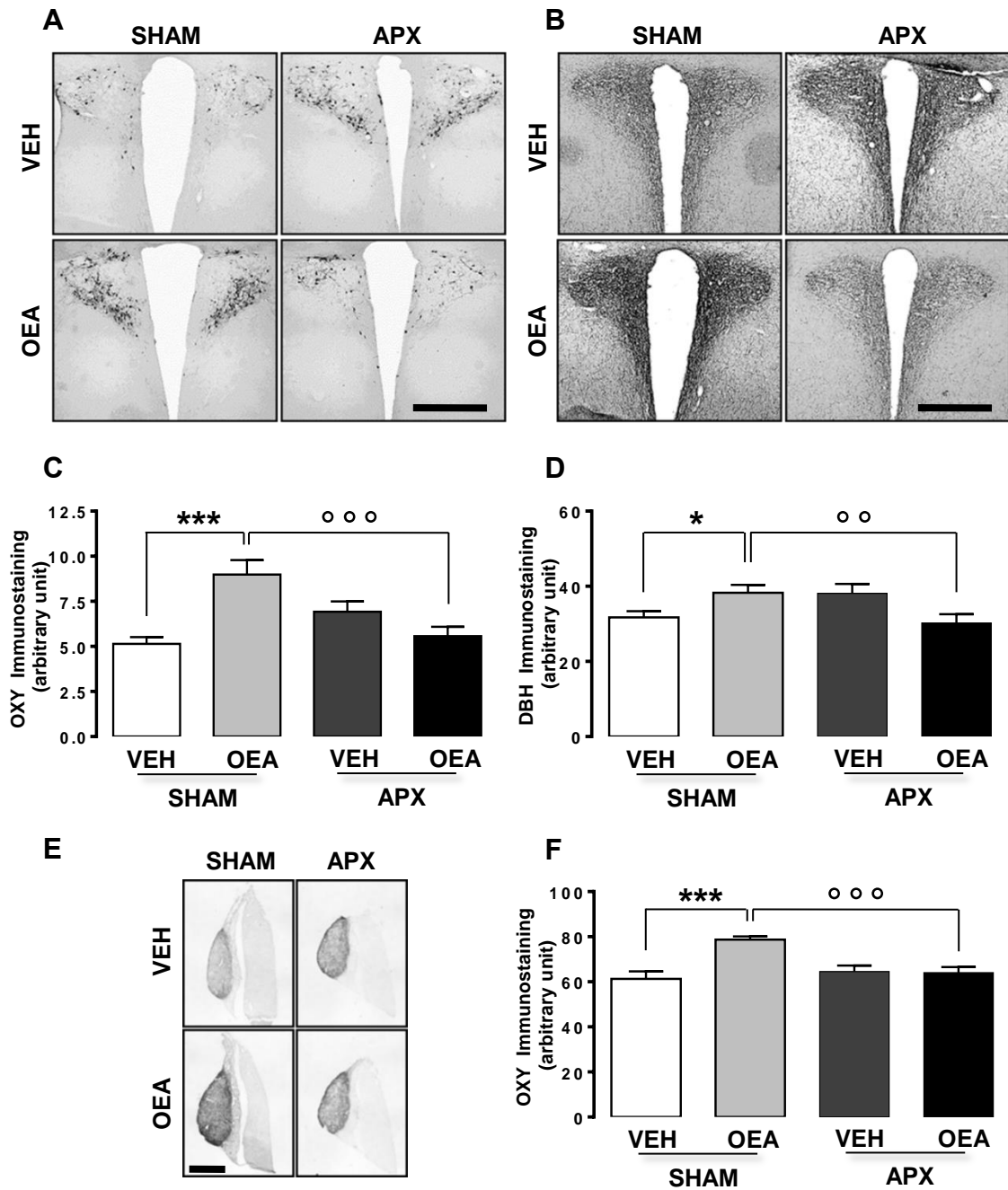
Fig. 2.7. The surgical ablation of the AP prevents the increase of OXY immunoreactivity induced by OEA at PVN and pituitary gland level and inhibits DBH immunoreactivity induced by OEA in the PVN. Representative photomicrographs of oxytocin (OXY, **panel A**) and dopamine beta hydroxylase (DBH, **panel B**) immunostaining (scale bar = 300 μm) of brain slices containing the paraventricular nucleus (PVN), collected from SHAM and APX rats treated with either vehicle (VEH) or OEA (10 mg kg^{-1}, i.p.) and sacrificed 2 h after treatment. Semiquantitative densitometric analysis of OXY (**panel C**) and DBH (**panel D**) immunostaining within the PVN of SHAM and APX rats treated with either VEH or OEA (10 mg kg^{-1}, i.p.). Data are expressed as mean ± SEM. *$P < 0.05$; ***$P < 0.001$ vs VEH in the same surgery group; ∘∘ $P < 0.01$; ∘∘∘ $P < 0.001$ vs SHAM in the same treatment group (Tukey's test; OXY, SHAM: N = 8–10; APX: N = 5–6. DBH, SHAM: N = 8–9; APX: N = 4–6). Representative photomicrographs (scale bar = 500 μm, **panel E**) and semiquantitative densitometric analysis (**panel F**) of OXY immunostaining of slices containing the posterior pituitary gland collected from SHAM and APX rats treated with either VEH or OEA (10 mg kg^{-1}, i.p.) and sacrificed 2 h after treatment. Data are expressed as mean ± SEM. ***$P < 0.001$ vs VEH in the same surgery group; ∘∘∘ $P < 0.001$ vs SHAM in the same treatment group (Tukey's test; SHAM: N = 3–9; APX: N = 4-7).

2.3.6. PPAR-α receptors are expressed at AP level

Finally, we qualitatively investigated the expression of PPAR-α within the AP. Our results show for the first time that a large number of PPAR-α −immunoreactive cells are present in the AP (Fig. 2.8A). The specificity of the immunostaining was assessed by the absence of signal in control brain sections containing the AP that underwent the same protocol procedure except for the incubation with the primary antibody (Fig. 2.8B).
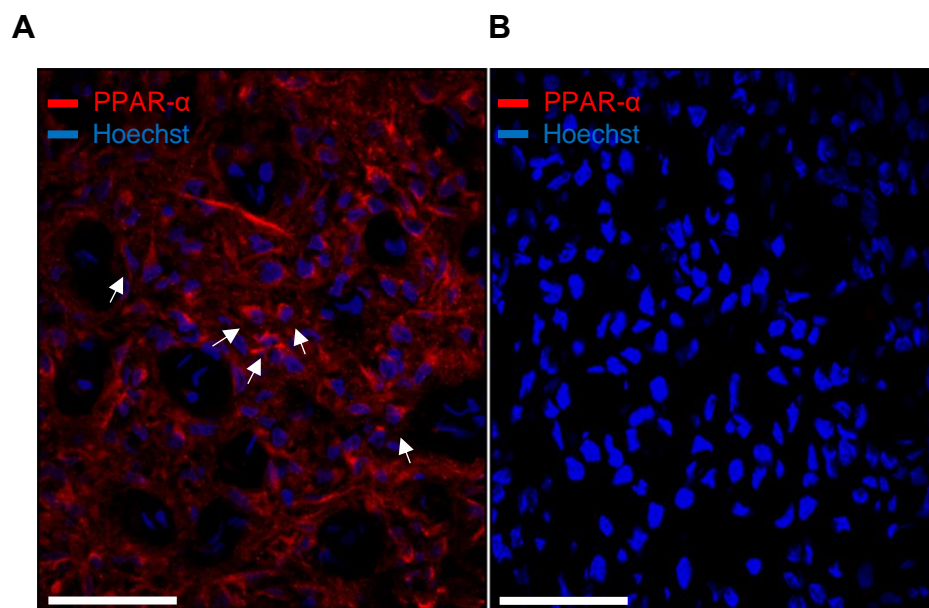
Fig. 2.8. PPAR-α expression in the AP.
Representative fluorescent photomicrographs (scale bar = 50 μm) showing PPAR-α immunostaining/Hoechst (red/blue) of the area postrema (AP, **panel A**) and negative control photomicrographs (slices processed without primary antibody) (**panel B**) in brain slices collected from a drug-naïve rat. White arrows in **panel A** represent PPAR-α −immunoreactive cells of the AP.

2.4. Discussion

The AP is a critical circumventricular organ that conveys information from the periphery to the CNS; projections from the AP/NST to the HYPO are involved in the control of eating and energy metabolism [47–50]. Both physiological and anatomical evidence reveal that the AP displays a weak BBB and is located in close contact with the cerebral ventricles [51]. These properties allow the permeation of blood-born substances, which do not readily reach other areas of the brain parenchyma across the BBB [47–50,52]. Therefore, we hypothesized that the AP might represent a receptive region for circulating OEA and that AP neurons might be the first central target of this lipid signal.

The present study reveals for the first time a necessary role for the AP in mediating the anorexigenic effects of peripherally administered OEA. In particular, our results suggest that neurons of the AP are a necessary component of the circuit responsible for the stimulation of brainstem noradrenergic neurons projecting to the hypothalamic OXY system, which, in turn, sustains the inhibitory control of food intake exerted by OEA. The evidence supporting these conclusions was collected by comparing the effects induced by peripherally administered OEA in rats subjected to a surgical lesion of the AP, which we defined as APX rats, and in control rats that were SHAM operated.

In our previous studies we demonstrated that selected brainstem areas, including the AP and different NST subnuclei, respond to systemic OEA administration by increasing the transcription of c-fos, an early gene widely used as a marker of cellular activation [6,11,12]. In accordance with these findings, peripheral OEA administration induced an increase of Fos expression in the AP and in all the NST subnuclei analyzed in SHAM rats, an effect that was completely absent in APX rats. Moreover, by double immunofluorescence, we demonstrated that, within the AP, OEA induced Fos in neurons expressing the noradrenaline synthesizing enzyme DBH, whose expression was also increased. A similar increase of DBH immunostaining was observed in almost all the NST subnuclei of SHAM rats, but it was completely prevented by the surgical ablation of the AP. These results are in accordance with our previous findings demonstrating the necessary role played by NST-PVN noradrenergic projections in mediating OEA anorexigenic and neurochemical effects in vivo [14].

Anterograde labeling experiments, performed by Van der Kooy and Koida in 1983 [51], through the injection of wheat germ agglutinin conjugated to horseradish peroxidase into the AP, demonstrated that AP neurons are connected with brain structures participating

to the transmission of visceral enteroceptive informations, including the NST [51]. Moreover results obtained by Armstrong and collaborators in 1981 [53], from experiments of immunocytochemical localization of DBH, suggest the presence of a reciprocal noradrenergic connection between the AP and the SolM of the NST [53]. Based on these previous observations, we hypothesize that the ability of OEA to activate brainstem neurons might be the result of a direct activation of noradrenergic neurons located in the AP. Moreover, we suggest that the activation of these neurons might be synaptically transmitted to noradrenergic neurons of the NST, particularly SolM neurons. This idea is raised by the observations that Fos and DBH expression in the NST, which are particularly evident in the SolM, were induced by OEA administration to SHAM rats, whereas they were completely abolished in APX rats. Moreover, double immunofluorescent staining revealed that, within the SolM, OEA induced a five-fold increase of Fos expression in DBH- positive neurons and this effect was completely prevented by the AP lesion.

This latter finding is particularly significant for at least two reasons: 1) the SolM is the subnucleus of the NST mostly innervated by neurons of the AP [54]; 2) A2 noradrenergic neurons are mostly prevalent within the SolM [54] and send projections to the PVN and the TMN [54], two hypothalamic nuclei involved in the pro-satiety effects of OEA [5]. A2 NST-PVN projections mediate the effects of OEA on oxytocinergic transmission in the HYPO, which, in turn, is responsible for the inhibition of food intake, as demonstrated by our previous observations in rats subjected to a saporin-mediated lesion of these fibers [14]. In keeping with these latter results, in the present study AP ablation completely abolished the ability of OEA to increase Fos, DBH and OXY expression within the PVN. Moreover, OEA increased the OXY immunoreactivity of the pituitary gland of SHAM rats, while the AP lesion completely prevented such increase. On the basis of this latter result, we hypothesize that the AP lesion prevents OXY accumulation in the neurohypophysis, which, in turn, might lead to a decreased OXY release into the blood stream. Finally, Provensi and his collaborators [45] demonstrated that OEA recruits also the hypothalamic histaminergic system, which, in turn, is able to stimulate oxytocinergic neurons of the PVN to mediate OEA anorexiant actions. In our study, the absence of an intact AP completely prevented the increase of Fos expression in the TMN of rats treated with OEA and this result raises the possibility that secondary noradrenergic neurons from the NST could project to OXY neurons of the PVN either directly or indirectly, through the activation of histaminergic neurons of the TMN.

Altogether, the results from the present study suggest that all the three central pathways associated so far to the anorexigenic effects of OEA, namely the brainstem noradrenergic and the hypothalamic oxytocinergic and histaminergic systems, require an intact AP, which likely acts as a primary site of action of an AP/NST- HYPO circuit, although the specific molecular target of OEA at AP level deserves further studies.

To this regard several lines of evidence support that PPAR-α activation mediates the hypophagic effect of OEA [21–25].

Even though previous findings demonstrated the expression of PPAR-α within most of the brain areas included in the present study [55], the scientific literature is still scarce on PPAR-α expression in the AP. Preliminary results obtained in the present study after PPAR-α immunostaining in the AP clearly show the expression of this receptor at this site. Therefore, we interpret our findings suggesting a direct recruitment of AP neurons exerted by OEA, through the activation of PPAR-α, although we cannot exclude the interaction with other eating signals acting at this brain structure such as amylin, PYY, GLP-1, apolipoprotein A-IV, CCK and adiponectin [11,16,33,40,56]. In fact, we might also hypothesize that the ablation of the AP could interfere with the activity of these signals and therefore blunt OEA's hypophagic action. However, this hypothesis is not supported by previous findings demonstrating that OEA suppresses feeding without altering circulating plasma levels of several peripheral signals, including insulin, leptin, ghrelin, PYY, and apolipoprotein A-IV, or the brain levels of different neuromodulators, such as CCK, vasopressin, and thyrotropin releasing hormone [11,16,56]. Of note, the eating inhibitory effect of OEA in free feeding animals differs from that of CCK or amylin because the latter hormones reduce eating by a meal size effect whereas OEA seems to influence mainly the latency to eat [19]. Future studies, with siRNA and/or optogenetic gene expression manipulation might allow a deeper exploration of this aspect, thus extending the current novel findings that pave the way for the understanding of the key role of the AP in the central effects of OEA.

Furthermore, because the AP plays a critical role in the formation of conditioned taste aversion (CTA) [57], our results might suggest that the hypophagic response induced by peripherally administered OEA could be, at least in part, the manifestation of a CTA that can be prevented by the surgical ablation of the AP. However, this hypothesis is confuted by previous findings demonstrating that OEA does not induce a CTA after its peripheral administration to rats, even at a two-fold higher dose than the ones used in the present study [6]. In keeping with this conclusion, OEA was also previously found unable to

increase stress hormone levels [6] or to induce a conditioned place preference or a conditioned place aversion in rodents [58].

The pivotal role of the AP in mediating the behavioural effect of OEA is also supported by our previous observation that OEA reduces feeding in rats subjected to SDA; these rats have both an intact and functional brainstem and an intact 50% vagal efferent innervation, whereas they lack functional vagal afferent fibers ascending from the small intestine [18]. We recently demonstrated that these rats still respond to the anorexigenic effects of OEA, a result that appears in contrast with pioneering similar studies on vagotomised rats or on capsaicine-deafferentiated rats [6,21]. In fact, the results of the former studies demonstrated that the anorexigenic effects of OEA were blunted in rats subjected to a total subdiaphragmatic vagotomy (which also lesions all vagal efferents) or to a pre-treatment with a neurotoxic dose of capsaicin, which destroys vagal, but also non-vagal unmyelinated afferent nerve fibers [6,21]. These data, together with the findings that OEA was unable to inhibit food intake when injected into the brain ventricles [6], suggested at that time the conclusion that its mode of action might involve peripheral, rather than central mechanisms.

This hypothesis was weakened by the opposite findings on SDA rats, although a possible explanation for the discrepant findings remained elusive. Because the total vagotomy removes both vagal afferents and efferents, the first more simplistic interpretation for the opposite results might lay on the possible effects of OEA on gastrointestinal secretory and motor functions that could be destroyed in totally vagotomised rats, whereas remain intact in SDA rats. Furthermore, in light of the current novel set of information, the apparent contradictory findings on SDA vs capsaicine-deafferentiated rats can be reconciled by considering that capsaicin treatment exerts neurotoxic effects also on neurons of the NST and AP, which receive projections from unmyelinated primary sensory neurons destroyed by the capsaicin treatment [59,60] and being left intact by the SDA procedure. Last, but not least, why i.c.v. administration of OEA did not inhibit food intake remained unexplored. One possible explanation might be based on the high expression of the enzyme FAAH found in the rat brain ventricles, in particular at the choroid plexus level [29,61,62]. This enzyme is responsible for the hydrolysis of N-acylethanolamides, including OEA. Therefore its high expression in the rat choroid plexus probably reduced the cerebral availability of i.c.v. administered OEA, hence precluding its accumulation to a critical concentration in the AP to inhibit food intake. Interestingly, FAAH levels were reported to be undetectable in both neurons and glia of

the AP [62], thus supporting the conclusions from the current study that circulating OEA might reach the neurons of this area, which lack a BBB, and trigger the central cascade of effects that inhibit food intake. We previously suggested that OEA might represent a novel and safer anti-obesity alternative to cannabinoid receptors antagonists [13] like rimonabant, which failed mostly due to its significant central side effects. Although OEA shares similar powerful effects on food intake with rimonabant, it presents a different impact on feeding behaviour and on the brain circuits involved, thanks to its pharmacodynamics properties. In fact, differently to rimonabant, OEA does not block cannabinoid receptors and is not accountable for the wide spectrum of unwanted effects typically observed with CB1 antagonists/reverse agonists. Conversely, by acting as a functional antagonist, OEA might counteract the biological actions of endocannabinoids on appetite stimulation and on the control of energy balance.

2.5. Conclusions

Although further studies are necessary, the results collected over the last 20 years clearly support that OEA might represent a promising candidate for the development of more effective anti-obesity therapies. The present findings suggesting the AP as the primary central target for this lipid, expand our knowledge on the central mechanism of action of OEA in the control of feeding behaviour and might contribute to open novel perspectives for the potential clinical use of this compound.

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Chapter III

Evaluation of the brain distribution of oleoylethanolamide and its analogues after acute systemic administration in rats

Abstract

The gut-derived satiety signal oleoylethanolamide (OEA), which belongs to the family of N-acylethanolamides (NAEs) is a potent activator of the alpha type peroxisome proliferator-activated receptor (PPAR-α) and is able to inhibit food intake and control lipid metabolism both in rodents and humans. The hypophagic action of OEA has been associated to the activation of selected brain areas, particularly in the brainstem and hypothalamus, although how OEA signal reaches the brain remained to be fully elucidated. Recent findings demonstrated a necessary role of the area postrema (AP), a circumventricular organ with a weak blood brain barrier (BBB) and a high density of PPAR-α receptors, for i.p. injected OEA to reduce eating, suggesting that this brain area could represent a receptive region for circulating OEA.

Based on these premises, in order to fully elucidate whether systemically administered OEA might directly reach and permeate the brain parenchyma through circumventricular organs, such as the AP and the median eminence (ME), male rats were sacrificed at different time points (2.5, 5, 15, 30, 60 minutes) after acute administration of OEA (10 mg kg^{-1}, i.p.); plasma and different brain areas (AP, ME, nucleus of the solitary tract, ventral and dorsal hippocampus) were microdissected and NAEs, including OEA, were extracted and measured by UPLC-MS/MS. Vehicle treated animals were used as controls.

Our results revealed that OEA was able to permeate all the brain areas analysed as early as 5 minutes after its systemic administration. OEA levels resulted considerably higher than those of vehicle-treated animals at 15 minutes in all the brain areas studied and particularly in the AP, where it reached the highest increase. In accordance, plasma OEA levels increased as early as 2.5 minutes after i.p. administration with a maximum increase registered at 15-30 minutes. OEA administration did not significantly affect the levels of other NAEs in the brain, whereas in the plasma we observed an increase of anandamide (AEA), palmitoylethanolamide (PEA), stearoylethanolamide (SEA), and linoleoylethanolamide (LEA), which deserves further studies.

Overall, these results showed that OEA is able to reach and permeate the brain parenchyma as an intact molecule within few minutes after i.p administration.

3.1. Introduction

Obesity and eating disorders are currently considered as major worldwide public health issues. Moreover, the limited and poorly efficacious anti-obesity therapies highlight the urgency to find novel pharmacological targets for controlling the so called "obesity epidemic". In this background, growing attention has been focused on the N-acylethanolamides (NAEs), a family of bioactive lipid mediators which are synthesized "on demand" by the fatty acid amidation of membrane phospholipid precursors (N-acylphosphatidylethanolamines, NAPEs) [1].

Among NAEs, anandamide (AEA), oleoylethanolamide (OEA), palmitoylethanolamide (PEA), stearoylethanolamide (SEA) and linoleoylethanolamide (LEA) are the most widely characterized and, in addition to feeding [2–7], they have been shown to participate in many physiological and pathological conditions including inflammation, pain and memory consolidation [8,9]. Besides cannabinoid receptors, NAEs have been found to interact with different receptor targets (depending on the nature of the N-linked acyl chain), including also the transient receptor potential vanilloid 1 (TRPV1) and peroxisome proliferator activated-receptor alpha (PPAR-α) [7,10–13].

Within these lipid mediators, OEA has been the most studied for its pivotal role as a key regulator of food intake [4,5,7]. OEA is a gut-derived compound generated by enterocytes of the proximal small intestine from oleic acid, which is released upon the absorption of dietary fat [4]. Although OEA shares biosynthesis and degradation pathways with its monounsaturated analogue, AEA, it exerts opposite effects in the control of feeding behaviour and lipid metabolism [4,14]. In fact, growing evidence accumulated over the last 20 years demonstrated that OEA acts as a satiety signal since it is able to significantly reduce food intake and body weight in obese rodents [5,19–30] and humans [27] through the activation of PPAR-α, for which OEA shows a high affinity [10].

A large body of observations revealed that the pro-satiety effect evoked by OEA requires the involvement of selected brain regions, which actively participate in the control of energy homeostasis and feeding behaviour, such as the nucleus of solitary tract (NST) and area postrema (AP) of brainstem [5,19,22,24], and the paraventricular (PVN) and supraoptic (SON) hypothalamic nuclei [5,19,22,24].

Although OEA has been demonstrated to induce satiety by activating key brain hypothalamic and hindbrain areas, the mechanism by which OEA reaches the central nervous system (CNS) from the periphery is still debated. After being systemically or

orally administered, OEA quickly reaches the bloodstream, however it cannot readily permeate the brain parenchyma because in the blood brain barrier (BBB) there is a high expression of its main degrading enzyme, fatty acid amide hydrolase (FAAH) [28].

In this context, prior knowledge considering vagus nerve as the primary responsible for conveying OEA's effects from the periphery to the CNS [10], failed after the observation that the anorexic effect of OEA is still present in animals lacking functional vagal afferent fibres [21].

Moreover, a novel study revealed that after the ablation of the AP, a circumventricular organ devoid of a functional BBB, both behavioural and neurochemical effects of OEA were completely prevented [18], thus suggesting the crucial role of this brain area in mediating OEA pro-satiety effect.

In this background, the AP and other circumventricular organs such as the median eminence (ME), are attracting a great deal of attention for their privileged properties: 1) the weakness of the BBB, by virtue of the absence of tight junctions and the presence of fenestrated capillaries, and 2) the location in close contact with the cerebral ventricles.

These observations, together with very recent findings demonstrating the high expression of PPAR-α in the AP [18] and in the ME (not published data), point to a possible role of these circumventricular organs as receptive brain regions for OEA and other peripheral signals.

On the basis of these premises, we hypothesized that the AP and the ME could represent the first central targets of this lipid signal in the brain.

Based on this background, the goal of the present study was to fully elucidate whether systemically administered OEA might directly reach and permeate the brain parenchyma through circumventricular organs devoid of a functional BBB, such as the AP and the ME.

OEA tissue levels in the brain have been already quantified by Oveisi and collaborators after an acute oral administration of this gut-derived lipid mediator (50 mg kg^{-1}) [29]. The authors hypothesized that OEA was not able to penetrate the brain since, after being orally administered, cerebral OEA levels were comparable to those observed in vehicle (veh)-treated rats used as controls [29]. However, it is important to mention that in this experiment, rats were sacrificed 4 hours after treatment, therefore we cannot exclude an increase in central OEA levels at shorter time-points.

To this purpose, male Wistar rats were sacrificed at different time points (2.5, 5, 15, 30, 60 minutes) after acute administration of OEA (10 mg kg^{-1}, i.p.). Plasma and different

brain areas were collected for UPLC-MS/MS quantification of the main NAEs (including OEA, AEA, PEA, SEA, and LEA), and 2-arachidonoyl-glycerol (2-AG). In particular, in order to selectively investigate OEA concentrations within a variety of PPAR-α-expressing cerebral regions, selected brain areas of interest (AP, ME, NST, ventral and dorsal hippocampus (vHipp and dHipp)) were microdissected and used in this study. Vehicle treated animals were used as controls.

3.2. Materials and Methods

3.2.1. Animals and housing

Eighty male Wistar-Han rats (Janvier Labs, Le Genest-Saint-Isle, France), weighing 275–325 g upon arrival, were individually housed in wire mesh cages under a 12:12 dark-light cycle in a climate-controlled room ($22 \pm 2°C$ and 60% relative humidity) and received *ad libitum* standard chow pellets (N 3430, Provimi KIiba, Gossau, Switzerland) and water. All experiments were performed upon the approval of the Veterinary Office of the Canton of Zurich and according to the European Community directives 2010/63/EU.

3.2.2. Drugs and treatments

OEA was synthesized as previously described [30], dissolved in a veh solution (2 ml kg^{-1}) of saline/polyethylene glycol/Tween 80 (90/5/5, v/v/v), and administered 10 mg kg^{-1} via i.p. injections. Veh treated animals were used as controls. Both veh and OEA solutions were freshly prepared on test day and administered about 30 minutes before dark onset.

3.2.3. Tissue collection

After the injection, animals were deeply anesthetized with isoflurane and sacrificed at the following time points: 2.5, 5, 15, 30 and 60 min. From these animals, blood was withdrawn from the heart and collected in glass tubes pre-coated with K_3-EDTA and immediately centrifuged at 4°C, 3000 rcf for 15 minutes for plasma separation. Brain areas of interest (AP, NST, dHipp, vHipp and the periventricular area of the hypothalamus including the ME) were freshly microdissected and snap frozen in liquid nitrogen. The experimental timeline is depicted in Fig. 3.1.
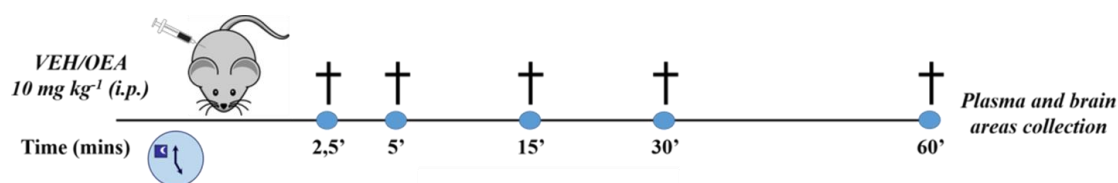
Fig. 3.1: Experimental timeline.

3.2.4. N-acylethanolamides extraction

All the tissues collected were subjected to the lipid extraction procedure. Briefly, as previously described [31] brain frozen tissues were homogenized in cold dichloromethane (CH_2Cl_2, 8 mL), followed by the addiction of: internal standards (5 pmol of d4-OEA, d4-AEA, d4-PEA, d4-SEA and 15 pmol of d5-2-AG), 4 mL of cold methanol (MeOH, + 0.175% BHT) and 2 mL of bi-distilled water (H_2O, + EDTA 7mM). The extracting vials were then subjected to vigorous mixing and sonication in ice-cold water, prior to be centrifuged for 10 min, 700xg at 4 °C. The organic layer was then collected and evaporated to dryness under a stream of nitrogen. A pre-purification of the resulting lipid fraction was obtained by solid-phase extraction (SPE) over silica (as stationary phase), using 6 mL of hexane-isopropanol (99:1, v/v) to elute highly lipophilic compounds and cholesterol. 3.6 mL of hexane-isopropanol (7:3, v/v) were then added to the SPE columns in order to allow NAEs (OEA, AEA, PEA, SEA, and LEA) and 2-AG elution. The fraction of interest was dried under a nitrogen stream and the dry residue reconstituted with 30μL of MeOH for the UPLC-MS/MS analysis.

Plasma samples were extracted with the same procedure, by adding 100 μL of sample in each extraction vial.

3.2.5. UPLC-MS/MS analyses

The resulting lipid extracts were analyzed by UPLC-MS/MS using a Xevo TQ-S mass spectrometer (Waters, Ireland, UK) coupled to an Acquity UPLC® class H (Waters, Ireland, UK). The analytes were separated using an Acquity UPLC BEH C18 column (1.7 μm, 2.1 × 50 mm; Waters, Ireland, UK) connected to an in-line filter unit and maintained at 40°C. Mobile phases A and B were composed of MeOH-H_2O-acetic acid (75:24.9:0.1, v/v/v) and MeOH-acetic acid (99.9:0.1, v/v), respectively. The injection volume was 1 μl. The gradient (0.2 mL/min) was designed as follows: transition from 100% A to 0% A linearly over 2.3 min, followed by a 4.7 minutes plateau before re-

equilibration at 100% A during 3 min. An electrospray ionization (ESI) source in positive mode was used. Precursor ions, product ions, multiple reaction monitoring (MRM) transitions, optimal cone voltage and collision energies were established for each analyte. The MRM mode was used to profile two transitions per compound, one quantitative (Q) and one qualitative (q). The MRM transitions used were: 326,5->61,9 (Q) and 326,5->309,2 (q) for OEA; 348,2->61,9 (Q), 348,2->90,9 (q) and 348,2->287,3 (q) for AEA; 300,3->61,9 (Q) and 300,3->283 (q) for PEA; 328,2->61,9 (Q) and 328,2->311 (q) for SEA; 324,5->61,9 (Q) and 324,5->306,5 (q) for LEA; 379,2->287,3 (Q), 379,2->269,3 (q), 379,2->90,9 (q) for 2-AG; 330,5->65,9 (Q) and 330,5->313,2 (q) for d_4-OEA; 352,2->65,9 (Q) and 352,2->287,3 (q) for d_4-AEA; 304,3->65,9 (Q) and 304,3->287 (q) for d_4-PEA; 332,2->65,9 (Q) and 332,2->315 (q) for d_4-SEA; 384,3->287,3 (Q) and 384,3->269,3 (q) for d_5-2-AG.

ESI conditions were as follows: capillary voltage at 2800 V; desolvation temperature at 550 °C; desolvation and cone gas flow at 1100 and 170 L/H, respectively; nebuliser gas flow at 7.0 bar.

During the analysis, the autosampler was maintained at 5 °C. For data acquisition and processing, the MassLynx® software (Waters, Ireland, UK) was used.

NAEs were identified based on their mass and by retention time matching (by using a reference solution containing deuterated standards and pure NAE standards injected at the beginning and at the end of each sequence of injections).

A calibration curve for each compound of interest was established by preparing standard solutions, containing an appropriate amount for each selected compound, which were then diluted to obtain 10 calibration points (0, 2.5, 5, 10, 25, 50, 100, 200, 400, 800 fmol on column for NAEs and 0, 50, 100, 200, 500, 1000, 2000, 4000, 8000, 16000 fmol on column for 2-AG). For OEA 3 additional points were added (1600, 3200, 6400 fmol on column). The ranges for the calibration curves were chosen for each standard based on preliminary data obtained in rat brain test tissues. Both deuterated standards and diluted solutions of non-deuterated standards were then subjected to the same procedure as the biological samples.

The amounts of the compounds analysed were determined by linear interpolation from calibration curves; data were analysed by normalizing the pmol amount of each compound to the weight of the brain tissue samples (pmol/g) and to the volume of plasma samples (pmol/mL).

3.2.6. Statistical analyses

All data were expressed as mean ± SEM and statistically analysed by two-way ANOVA with "time" and "treatment" as the two factors. Tukey's test was used as a post hoc to perform multiple comparisons (IBM SPSS, version 22, IBM Analytics). Additionally, a one-way ANOVA followed by Dunnett's post hoc test was performed in order to evaluate differences among the veh-treated rats (by using as control the group of rats sacrificed 2.5 minutes after administration of veh). In all instances, the threshold for statistical significance was set at $P < 0.05$.

3.3. Results

3.3.1. Effects of OEA systemic administration on the levels of OEA and its analogues in selected brain areas

- *OEA distribution in the brain*

The data obtained by UPLC-MS/MS analysis revealed, for the first time, that OEA was able to reach the CNS and to permeate all the brain areas analysed within few minutes of its administration. In particular, OEA levels were significantly higher as early as 2.5 minutes after treatment in the ME ($P<0.05$), dHipp ($P<0.01$) and vHipp ($P<0.05$) of rats acutely treated with OEA, as compared to those administered with veh (Fig. 3.2 C-E); whereas, in the AP and NST OEA reached significant levels compared to those evaluated in control rats as early as 5 minutes after its systemic administration (Fig. 3.2 A, B; AP: $P<0.05$, NST: $P<0.01$).

The maximum increase of OEA was registered 15 minutes after its acute injection. In fact, at this time point OEA levels (expressed as pmol/g of wet tissue) resulted considerably higher in OEA- than in veh-treated animals in all the brain areas studied (Fig. 3.2 B-E; $P<0.001$) and particularly in the AP, where it reached the highest increase (Fig. 3.2 A; $P<0.001$) supporting our hypothesis concerning the crucial role of this area in conveying OEA signal to the brain. This finding is more clearly shown by expressing OEA levels as a percentage of those evaluated in the respective controls (Fig. 3.2 F-J). These graphs show that 15 minutes after its systemic administration, OEA levels were up to 3-fold higher in the AP and 1.5-fold higher in the other brain areas (ME, NST, dHipp and vHipp) in OEA animals vs veh-treated controls (Fig. 3.2 F-J).

Moreover, OEA endogenous levels in veh- treated rats appeared to be not significantly different over the time in all the brain areas analysed except for the AP where they were statistically higher at 30 minutes compared to those quantified at 2.5 minutes (Fig. 3.2 A; P<0.01). The results obtained from the two-way ANOVA analyses of OEA levels (expressed as pmol/g and as percentage of controls) are reported in Table 3.1; while significant differences among groups evaluated by the post hoc analysis are indicated in Fig. 3.2.
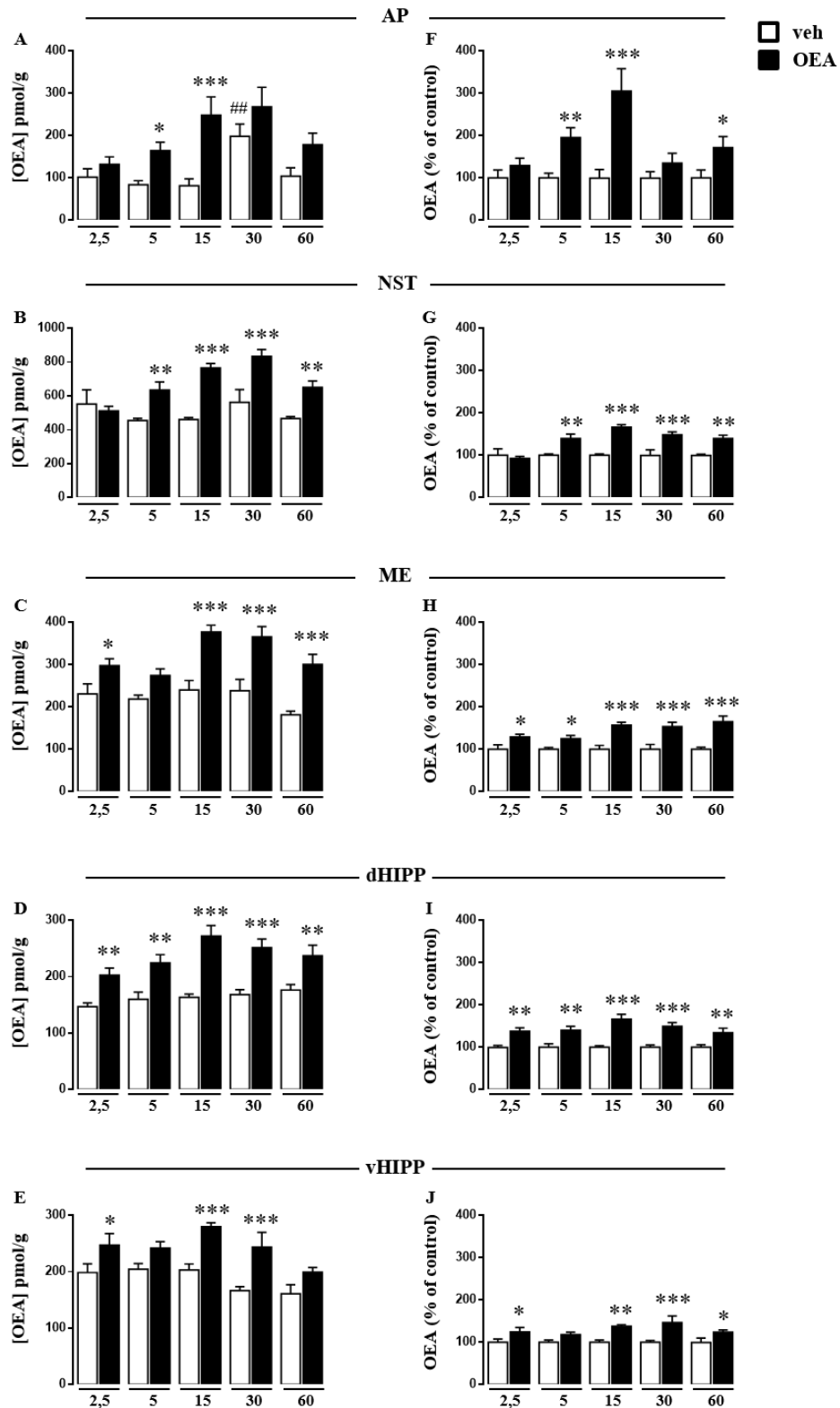
Fig. 3.2: OEA Concentration expressed in pmol/g and OEA levels expressed as % of control in the area postrema (AP; **panels A, F**), nucleus of the solitary tract (NST; **panels B, G**), median eminence (ME; **panels C, H**), dorsal and ventral hippocampus (dHipp (**panels D, I**) and vHipp (**panels E, J**)) of rats sacrificed at different time points (2.5, 5, 15, 30, 60 minutes) after acute administration of OEA (10 mg kg^{-1}, i.p.) or vehicle (veh). Data are expressed as mean ± SEM. (N=6-8). * $P<0.05$, ** $P<0.01$, *** $P<0.001$ vs veh in the same time point (Tukey's test). ## $P<0.01$ vs veh 2.5 minutes (Dunnett's test).

Table 3.1: Results of the two-way ANOVA analyses of NAEs and 2-AG quantified in different brain areas and in the plasma of rats sacrificed at different time points after intraperitoneal injection of OEA or vehicle.

		F treatment	F time	F interaction	df
	OEA (%)	29,330 (p<0,001)	3,800 (p<0,01)	3,800 (p<0,01)	1/77
	OEA (pmol/g)	23,702 (p<0,001)	5,724 (p<0,001)	1,653 (p=0,171)	1/77
	AEA ″	0,012 (p=0,912)	0,368 (p=0,830)	0,179 (p=0,948)	1/78
AP	PEA ″	0,015 (p=0,904)	0,255 (p=0,906)	0,114 (p=0,977)	1/74
	LEA ″	0,003 (p=0,959)	1,391 (p=0,248)	0,108 (p=0,979)	1/68
	SEA ″	0,023 (p=0,879)	0,380 (p=0,822)	0,232 (p=0,920)	1/73
	2-AG ″	0,081 (p=0,777)	1,601 (p=0,184)	0,822 (p=0,561)	1/78
	OEA (%)	49,027 (p<0,001)	4,822 (p<0,01)	4,823 (p<0,01)	1/75
	OEA (pmol/g)	41,433 (p<0,001)	4,811 (p<0,01)	4,280 (p<0,01)	1/75
NST	AEA ″	1,790 (p=0,186)	1,528 (p=0,207)	0,175 (p=0,950)	1/64
	PEA ″	1,668 (p=0,201)	2,240 (p=0,074)	2,346 (p=0,063)	1/76
	LEA ″	0,298 (p=0,587)	4,091 (p<0,01)	0,409 (p=0,801)	1/74
	SEA ″	0,006 (p=0,941)	1,106 (p=0,361)	1,418 (p=0,238)	1/74
	2-AG ″	8,306 (p<0,01)	2,774 (p<0,05)	0,429 (p=0,788)	1/77
	OEA (%)	67,384 (p<0,001)	2,032 (p=0,099)	2,033 (p=0,099)	1/79
	OEA (pmol/g)	65,676 (p<0,001)	5,036 (p<0,01)	1,793 (p=0,140)	1/79
ME	AEA ″	0,650 (p=0,423)	1,730 (p=0,156)	0,416 (p=0,797)	1/67
	PEA ″	1,325 (p=0,254)	0,893 (p=0,473)	1,394 (p=0,245)	1/77
	LEA ″	2,667 (p=0,107)	0,364 (p=0,834)	0,402 (p=0,807)	1/74
	SEA ″	0,000 (p=0,987)	8,132 (p<0,001)	1,563 (p=0,195)	1/75
	2-AG ″	1,658 (p=0,202)	3,700 (p<0,01)	0,153 (p=0,961)	1/78
	OEA (%)	82,702 (p<0,001)	1,311 (p=0,274)	1,311 (p=0,274)	1/79
	OEA (pmol/g)	81,631 (p<0,001)	3,371 (p<0,05)	1,386 (p=0,248)	1/79
dHipp	AEA ″	8,068 (p<0,01)	2,922 (p<0,05)	2,241 (p=0,073)	1/79
	PEA ″	0,000 (p=0,999)	3,482 (p<0,05)	0,129 (p=0,971)	1/79
	LEA ″	2,458 (p=0,121)	4,854 (p<0,01)	0,135 (p=0,969)	1/79
	SEA ″	0,425 (p=0,517)	5,227 (p<0,01)	0,496 (p=0,739)	1/79
	2-AG ″	3,539 (p=0,064)	0,523 (p=0,719)	0,936 (p=0,448)	1/79
	OEA (%)	34,950 (p<0,001)	1,018 (p=0,404)	1,018 (p=0,404)	1/79
	OEA (pmol/g)	36,707 (p<0,001)	5,044 (p<0,01)	0,932 (p=0,451)	1/79
vHipp	AEA ″	3,140 (p=0,081)	2,630 (p<0,05)	0,839 (p=0,505)	1/78
	PEA ″	10,223 (p<0,01)	2,896 (p<0,05)	0,163 (p=0,956)	1/78
	LEA ″	1,329 (p=0,253)	1,341 (p=0,264)	1,308 (p=0,276)	1/79
	SEA ″	14,781 (p<0,001)	0,455 (p=0,768)	0,302 (p=0,876)	1/78
	2-AG ″	0,011 (p=0,918)	2,932 (p<0,05)	0,207 (p=0,934)	1/79
	OEA (%)	645,049 (p<0,001)	16,473 (p<0,001)	16,473 (p<0,001)	1/78
	OEA (pmol/ml)	635,248 (p<0,001)	12,713 (p<0,001)	12,866 (p<0,001)	1/78
Plasma	AEA ″	15,847 (p<0,001)	2,334 (p=0,064)	2,682 (p<0,05)	1/79
	PEA ″	59,285 (p<0,001)	3,790 (p<0,01)	1,966 (p=0,110)	1/77
	LEA ″	290,397 (p<0,001)	11,309 (p<0,001)	11,804 (p<0,001)	1/78
	SEA ″	419,094 (p<0,001)	41,454 (p<0,001)	30,815 (p<0,001)	1/78
	2-AG ″	0,429 (p=0,515)	2,667 (p<0,05)	2,935 (p<0,05)	1/79

Abbreviations: 2-AG, 2-arachidonoyl-glycerol; AEA, anandamide; AP, area postrema; dHipp, dorsal Hippocampus; LEA, linoleoylethanolamide; ME, median eminence; NST, nucleus of solitary tract; OEA, oleoylethanolamide; PEA, palmitoylethanolamide; SEA, stearoylethanolamide; vHipp, ventral Hippocampus.

- *N-acylethanolamides distribution in the brain*

Whole tissue concentrations of NAEs (including AEA, PEA, LEA, and SEA), and 2-AG were measured in selected brain regions such as AP, ME, NST, dHipp and vHipp of rats treated with either OEA (10 mg kg^{-1}, i.p.) or veh and sacrificed at different time-points after treatment. All data, expressed as pmol/g of wet tissue, are reported in Table 3.2.

Overall, our results revealed that OEA systemic administration did not significantly affect the levels of other NAEs and 2-AG in all the brain areas analyzed, except for the Hipp.

In fact, in the dHipp OEA acute injection induced a significant decrease of AEA levels in OEA- than in veh-treated rats 30 minutes after treatment ($P<0.01$). Whereas in the vHipp we observed a decrease of both PEA ($P<0.05$) and SEA ($P<0.05$) few minutes after OEA administration compared to animals treated with veh.

Results obtained from two-way ANOVA analyses conducted for each parameter are reported in Table 3.1, whereas the results from the post hoc analyses (Tukey's and Dunnett's test) are reported in Table 3.2.

Table 3.2: NAEs and 2-AG concentration expressed in pmol/g in selected brain areas of interest in rats after intraperitoneal injection of OEA or vehicle.

Area	Analyte	2.5 min VEH	2.5 min OEA	5 min VEH	5 min OEA	15 min VEH	15 min OEA	30 min VEH	30 min OEA	60 min VEH	60 min OEA
AP	AEA	123,3 ± 21,43	112,9 ± 25,12	104,7 ± 18,02	96,35 ± 13,43	115,4 ± 9,720	118,1 ± 26,27	118,1 ± 27,14	109,2 ± 19,12	91,86 ± 14,19	109,8 ± 16,98
	PEA	1160 ± 208,3	1180 ± 328,7	1211 ± 250,5	1091 ± 171,1	1033 ± 93,74	1123 ± 281,2	1282 ± 232,0	1241 ± 295,9	961,6 ± 148,7	1123 ± 230,9
	LEA	58,50 ± 10,80	50,00 ± 6,736	57,42 ± 9,195	60,03 ± 8,034	48,64 ± 4,832	53,44 ± 14,93	79,03 ± 15,15	76,28 ± 20,92	61,69 ± 10,60	63,67 ± 12,41
	SEA	179,6 ± 41,28	152,9 ± 54,43	164,6 ± 44,80	163,7 ± 34,32	174,8 ± 19,22	163,4 ± 29,65	171,4 ± 42,56	189,3 ± 51,73	114,9 ± 17,87	154,9 ± 36,66
	2-AG	4120 ± 460,0	3696 ± 516,6	3767 ± 307,3	4083 ± 294,9	4261 ± 459,0	3801 ± 547,3	3343 ± 373,1	3122 ± 307,2	2944 ± 309,7	3559 ± 453,6
NST	AEA	122,7 ± 40,77	88,08 ± 15,44	117,1 ± 24,59	92,34 ± 19,88	123,4 ± 10,51	113,7 ± 15,99	123,1 ± 16,57	116,9 ± 27,39	85,36 ± 6,837	77,11 ± 7,696
	PEA	2812 ± 296,3	1721 ± 131,2	2035 ± 58,58	1982 ± 128,7	1997 ± 68,67	2032 ± 99,74	2413 ± 325,2	2560 ± 261,9	2156 ± 86,99	2156 ± 71,24
	LEA	44,39 ± 14,38	59,24 ± 17,80	38,52 ± 10,70	50,23 ± 7,541	31,58 ± 3,873	38,47 ± 2,537	78,70 ± 18,75	69,42 ± 16,26	34,81 ± 9,316	30,15 ± 4,898
	SEA	419,5 ± 44,69	300,0 ± 60,39	373,7 ± 53,00	380,8 ± 46,48	382,8 ± 15,27	435,4 ± 35,85	349,1 ± 67,55	407,7 ± 28,38	430,5 ± 16,44	441,2 ± 24,51
	2-AG	15120 ± 2371	11909 ± 1528	14238 ± 2189	11503 ± 827,3	12524 ± 829,2	9473 ± 1086	13684 ± 654,1	11351 ± 620,3	9981 ± 233,2	9648 ± 554,8
ME	AEA	77,30 ± 7,907	62,59 ± 7,913	81,02 ± 5,052	81,75 ± 13,88	80,71 ± 10,67	74,48 ± 9,919	89,92 ± 8,631	80,55 ± 8,880	60,88 ± 3,951	66,65 ± 6,652
	PEA	739,3 ± 86,20	718,7 ± 38,79	789,7 ± 73,49	601,0 ± 29,28	690,8 ± 72,18	756,0 ± 65,28	709,5 ± 99,02	572,3 ± 41,25	618,6 ± 48,45	659,1 ± 70,84
	LEA	22,47 ± 4,841	24,04 ± 5,469	12,81 ± 2,007	22,34 ± 3,568	19,75 ± 4,866	20,91 ± 1,750	15,02 ± 2,957	25,83 ± 4,123	18,31 ± 3,417	21,16 ± 9,584
	SEA	101,5 ± 13,00	94,69 ± 11,79	116,6 ± 15,03	72,30 ± 10,44	78,79 ± 15,70	112,5 ± 22,56	154,1 ± 16,57#	153,4 ± 9,825§§	138,0 ± 12,63	149,5 ± 9,303§§
	2-AG	26660 ± 4070	26174 ± 2593	26560 ± 2515	24821 ± 2651	26337 ± 2656	22564 ± 1609	29630 ± 3351	26045 ± 2926	19163 ± 2044	18018 ± 1309
dHipp	AEA	142,5 ± 10,36	156,8 ± 10,31	163,5 ± 8,616	136,6 ± 4,314	158,7 ± 15,27	132,6 ± 9,416	170,7 ± 13,17	126,2 ± 6,099*§	125,8 ± 14,37	115,4 ± 6,141§§
	PEA	593,0 ± 42,13	615,5 ± 44,45	601,0 ± 54,11	586,5 ± 43,73	641,8 ± 24,35	665,9 ± 74,18	685,4 ± 68,60	647,6 ± 42,04	762,1 ± 40,17	768,1 ± 62,32
	LEA	11,54 ± 0,6578	13,13 ± 0,4788	12,17 ± 0,8385	14,94 ± 1,328	13,54 ± 1,315	15,02 ± 0,7960	18,06 ± 2,498#	19,57 ± 2,575§	14,37 ± 1,575	14,83 ± 1,997
	SEA	80,51 ± 9,880	91,00 ± 8,295	83,87 ± 9,594	78,39 ± 9,017	88,83 ± 8,813	94,82 ± 12,50	111,1 ± 19,47	104,0 ± 6,922	114,7 ± 8,453	133,4 ± 12,26§
	2-AG	8817 ± 258,4	7589 ± 313,6	7957 ± 434,9	8414 ± 687,1	8211 ± 548,9	7170 ± 299,6	8506 ± 571,3	8119 ± 472,5	8418 ± 414,3	7729 ± 383,3
vHipp	AEA	232,1 ± 16,33	227,8 ± 13,33	237,7 ± 9,996	214,1 ± 15,61	244,6 ± 14,64	215,0 ± 14,37	242,3 ± 9,202	211,0 ± 20,11	187,3 ± 14,22	197,7 ± 8,673
	PEA	718,5 ± 60,18	598,7 ± 53,01	763,1 ± 52,83	622,6 ± 39,64*	697,8 ± 89,25	617,2 ± 42,27	582,1 ± 44,61	501,2 ± 39,52	634,5 ± 44,90	554,8 ± 36,82
	LEA	18,36 ± 1,148	19,56 ± 1,742	16,75 ± 1,227	21,97 ± 2,716	19,73 ± 1,758	19,62 ± 1,347	16,54 ± 1,077	19,27 ± 2,924	17,29 ± 1,952	14,81 ± 0,7246
	SEA	132,3 ± 11,70	102,5 ± 10,36*	137,2 ± 11,36	104,4 ± 5,641*	119,0 ± 12,22	101,1 ± 9,564	120,2 ± 8,479	102,0 ± 8,843	126,0 ± 9,490	108,4 ± 4,701
	2-AG	14175 ± 549,5	13389 ± 861,5	12196 ± 818,7	13302 ± 741,6	13567 ± 938,1	12939 ± 721,3	13976 ± 1341	13832 ± 1396	16177 ± 1468	15986 ± 1178

Data are expressed as mean ± SEM. *P<0.05 vs veh in the same time point (Tukey's test); # P<0.05 vs veh 2.5 minutes (Dunnett's test); § P<0.05; §§ P<0.01 vs OEA 2.5 minutes (Dunnett's test). N = 5-8.
Abbreviations: 2-AG, 2-arachidonoyl-glycerol; AEA, anandamide; AP, area postrema; dHipp, dorsal Hippocampus; LEA, linoleoylethanolamide; ME, median eminence; NST, nucleus of solitary tract; OEA, oleoylethanolamide; PEA, palmitoylethanolamide; SEA, stearoylethanolamide; veh, vehicle; vHipp, ventral Hippocampus.

3.3.2. Effects of OEA systemic administration on the levels of OEA and its analogues in the plasma

Plasma concentration of NAEs (including OEA, AEA, PEA, SEA, and LEA), and 2-AG were expressed as pmol/ml and are reported in Fig. 3.3.

In keeping with the results obtained in the different brain areas, plasma OEA levels significantly increased within minutes of its administration (as early as 2.5 minutes after injection; $P<0.001$) with a maximum increase registered at 15-30 minutes (60-fold higher than controls; $P<0.001$) (Fig. 3.3 A). Conversely from what observed in the brain, OEA systemic injection significantly affected the levels of other NAEs in the plasma of OEA-treated rats as compared to control animals (Fig. 3.3 B-E). In particular, we observed that OEA treatment was able to significantly increase the amount of AEA, PEA, LEA and SEA with different kinetics compared to control rats. The maximum increase was registered at 15 minutes for AEA ($P<0.01$), PEA ($P<0.001$) and LEA ($P<0.001$) whereas SEA reached the maximum levels 30 minutes after OEA systemic administration ($P<0.001$). Overall, no change was observed for 2-AG levels except for those measured at 15 minutes after treatment, when OEA administration induced a significant reduction of 2-AG amount as compared to controls (Fig. 3.3 F; $P<0.01$).

Results obtained from two-way ANOVA analyses conducted for each parameter are reported in Table 3.1, while the results from the post hoc analyses (Tukey's test) are reported in Figure 3.3.
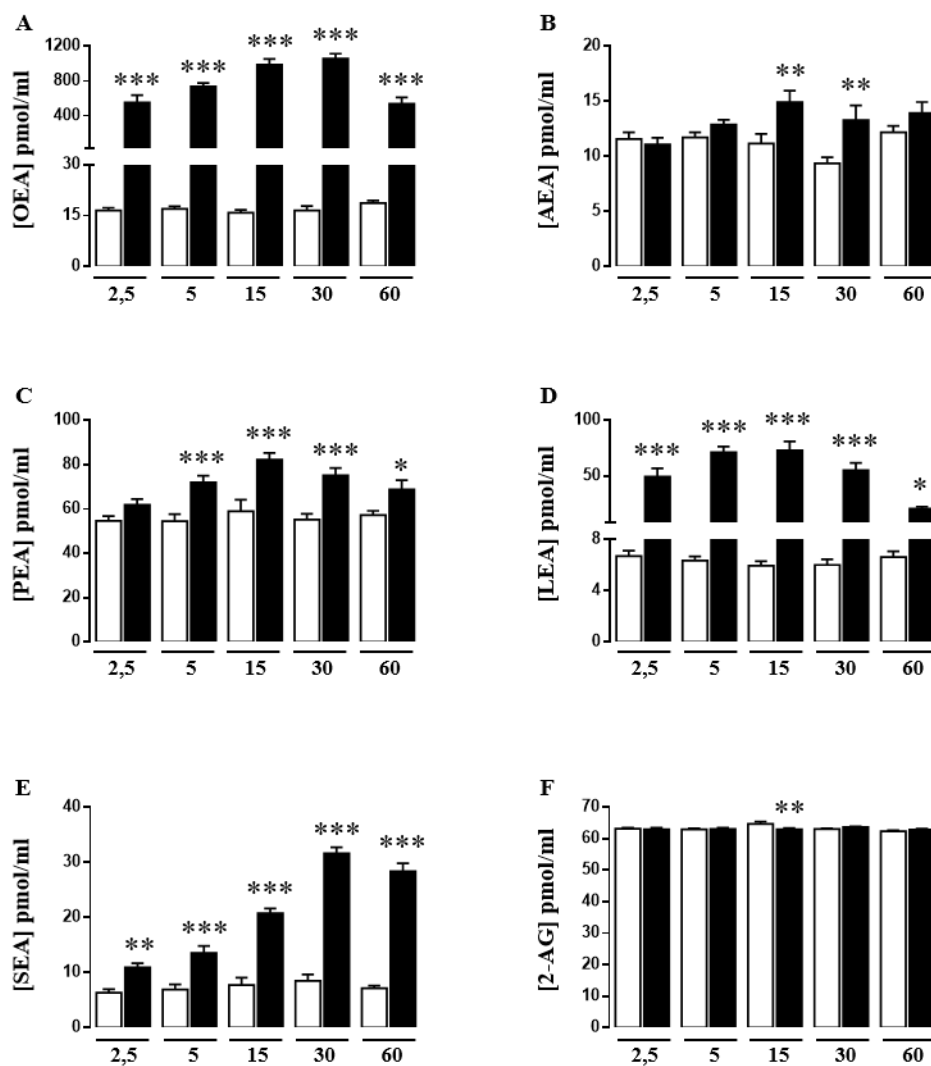
Fig. 3.3: Concentration of OEA (**panel A**) and other NAEs (AEA, **panel B**; PEA, **panel C**; LEA, **panel D**; SEA, **panel E**) and 2-AG (**panel F**) expressed in pmol/ml in the plasma of rats sacrificed at different time points (2.5, 5, 15, 30, 60 minutes) after acute administration of OEA (10 mg kg^{-1}, i.p.) or vehicle (veh). Data are expressed as mean ± SEM. (N=7-8). * $P<0.05$, ** $P<0.01$, *** $P<0.001$ vs veh in the same time point (Tukey's test).

3.4. Discussion

OEA is a gut-derived satiety lipid signal that has gained a great deal of attention over the last two decades for its ability to evoke consistent and sustained food intake suppression in both rodents and humans [5,15,18,22,27,32] through the activation of intestinal PPAR-α receptors [10]. It is now well recognised that the hypophagic action of this lipid mediator is associated to the involvement of selected brain areas, particularly in the brainstem and hypothalamus [18,19,22,24,33], although how OEA signal reaches the brain remained to be fully elucidated.

Recent findings demonstrated a necessary role of the AP, a circumventricular organ with a weak BBB and a high density of PPAR-α receptors, for i.p. injected OEA to reduce eating [18], suggesting that this brain area could represent a receptive region for circulating OEA.

However, previous findings suggested that OEA was not able to penetrate the brain parenchyma [29] thus proposing that its pro-satiety mechanism of action could be peripheral rather than central.

In particular, the authors revealed that OEA levels, measured in total brain of animals acutely administered with OEA (50 mg kg^{-1}, oral capsules) and sacrificed 4 hours after treatment, were comparable to those quantified in veh-treated rats [29]. However, these results cannot exclude a possible increase in central OEA levels at shorter time-points.

In accordance with this latter hypothesis and as confirmed by previous reports, OEA inhibitory effect on food intake appeared evident within minutes of its administration [18,21].

These observations further supported the possibility that OEA signal could reach the CNS and exert its neurochemical effects very quickly, thus suggesting also a non-transcriptional mechanism involved in such a rapid action for OEA.

With these concepts in mind, in this study we measured OEA levels in rat brain by UPLC-MS/MS system at shorter time-points after its systemic administration (at a dose that causes a significant inhibition of eating, 10 mg kg^{-1}), such as 2.5, 5, 15, 30, 60 minutes after treatment.

In particular, in order to selectively investigate the tissue distribution of exogenous OEA within a variety of PPAR-α-expressing cerebral regions, we here quantified OEA and other NAEs levels in selected brain areas of interest such as AP, ME, NST, vHipp and dHippo.

Surprisingly, our results demonstrated, for the first time, that OEA is able to directly reach and permeate all the brain areas analysed as early as 5 minutes after being acutely injected.

Overall, the maximum increase of OEA in the brain was registered 15 minutes after its acute administration and this is in accordance with our previous behavioural findings demonstrating that OEA significantly suppresses cumulative food intake in rats as early as 30 minutes following injection [18,21].

The rapidity through which OEA is able to exert its pro-satiety effect reveals the existence of an additional non-transcriptional mechanism for PPAR-α, whose transcriptional changes, conversely, unfold over a period of hours [4].

Accordingly, Gaetani and collaborators in 2003 demonstrated that plasma concentration of OEA, after i.p. administration of a single dose (5 mg kg^{-1}), were significantly higher than control at 15 and 30 minutes after the injection [32]. However, to date, no pharmacokinetics studies have been performed to test OEA (10 mg kg^{-1}) absorption and distribution in the brain in such short time points.

As already described in the text, brain distribution of OEA have been analysed by Oveisi research team only 4 hours after its oral administration [29]. Moreover, at this time point, the authors also investigated tissue distribution of orally administered OEA, and they found increased OEA concentration in the initial segment of the gastrointestinal tract (stomach, duodenum, and jejunum) and in the liver in OEA- than in veh-treated rats [29].

Interestingly, in our study we found that 15 minutes after treatment, OEA amount reached the highest increase in the AP, where it was up to 3-fold higher in OEA- than in veh-treated rats, as compared to the other brain areas analysed, such as ME, NST, dHipp and vHipp, where OEA levels were only 1.5 fold higher than controls.

This result further supports the crucial role of the AP in mediating OEA mechanism of action, and is in line with our previous study in which, after the surgical ablation of the AP, both behavioural and neurochemical effects of OEA were completely prevented [18].

Additional evidence confirming the idea of a pivotal role of the AP in OEA-induced satiety comes from the observation of physiological OEA. In fact, OEA endogenous levels in veh- treated rats appeared to be not significantly different over the time in all the brain areas analysed except for the AP, where they were statistically higher at 30 minutes than those quantified at 2.5 minutes.

OEA endogenous levels are, indeed, strictly dependent on the nutritional status of the animals [2,4]. For instance, it has been observed that fasting decreases OEA content in the proximal segment of the small intestine, while feeding stimulates OEA mobilization from the mucosal layer of the duodenum and jejunum [3,34].

Based on this premises, since the experiments have been conducted during the dark phase (when rats are active), the increased endogenous levels of OEA measured in the AP of rats treated with veh, are strictly dependent on the fact that animals are consuming food.

The observation that endogenous OEA reaches a statistically significant increase during feeding around 30 minutes after the beginning of the dark phase, selectively in the AP, corroborates our hypothesis of the necessary function of this hindbrain area in regulating feeding behaviour and in mediating OEA anorexic effect.

Moreover, the considerable increase of OEA levels in the AP and ME, following its systemic administration, could be explained by the decreased activity of FAAH enzyme in these regions of the CNS [35]. FAAH is the enzyme mainly responsible for the hydrolysis of NAEs, including OEA, and highly expressed in the BBB [28].

The BBB, formed by the brain capillary endothelial cells, represents the physical protective barrier between the blood and the CNS [36], a property that is allowed by the presence of highly developed tight junction complexes and a lack of fenestrations [37]. Because of its intrinsic structure, the BBB restricts the diffusion of molecules and solutes into the brain, including fatty acids.

In this scenario, the AP and the ME are defined as circumventricular organs since they lack a functional BBB and they are highly vascularised, being in close contact with cerebral ventricles [38]. This property, together with their abundant expression in PPAR-α [18], further suggests their crucial role as receptive brain regions for circulating OEA and confirms our results showing OEA capability to reach and permeate the brain parenchyma.

Moreover, we were not surprised that OEA was able to penetrate the CNS by reaching high levels also in the NST, since this is a nucleus in close contact with the AP [22] and with a high density of PPAR-α. Therefore, by virtue of the strict connection and reciprocal communication between AP and NST neurons [18,39], we propose that the AP might also be a putative pathway through which OEA can diffuse to the nearby NST.

However, the high levels of OEA measured in the dHipp and vHipp as early as 2.5 minutes after its systemic administration, led us to hypothesise that OEA increase in

these brain regions is not the consequence of OEA entrance into the brain through the AP.

In this regard, the research is currently shedding light on possible protein-mediated transport mechanisms that could allow fatty acid import into the brain [36].

Mitchell and collaborators demonstrated that the precursor of OEA, oleic acid, is transported across confluent cultures of primary human brain microvessel endothelial cells through the engagement of fatty acid transport proteins. The authors showed that RNAi knockdown of fatty acid transport protein 1 (FATP-1) or fatty acid translocase (FAT/CD36), which are specific fatty acid transporters, significantly decreased oleic acid transport through these microvessel endothelial cells [40].

Both FATP-1 and FAT/CD36, together with an additional class of fatty acid transporter (fatty acid-binding protein (FABPs)), have been shown to be specific for long-chain fatty acids [41] and their genes are regulated by PPAR-α [42].

Moreover, CD36, along with FABP5 subtype of FABPs family, has been found to be highly expressed also within the endothelial cells of the brain microvasculature [43,44].

These observations, together with the finding that FABP5 is expressed (both gene and protein) in the lateral ventricles of both developing and adult mice CNS, led us to speculate that OEA penetration within the dHipp and vHipp might be mediated by the involvement of this fatty acid transporter, being the Hipp a region of the CNS in close contact with the lateral ventricles [45]. Further studies should test such hypothesis.

Interestingly, the rapidity trough which OEA reaches and permeates the brain parenchyma could be determinant not only in allowing a rapid inhibition of food intake but also in sustaining the cognitive properties of this lipid compound. Studies, in fact, reveal the ability of OEA (10 mg kg^{-1} i.p.) to enhance memory consolidation, after behavioural training, in two aversively motivated task: the inhibitory avoidance task [8] and in the contextual fear conditioning [46]. Moreover, OEA has also been shown to improve spatial cognitive deficits in a rat model of acute cerebral ischemic injury through enhancing the neurogenesis in the Hipp [47].

In keeping with these findings, we can speculate that the rapid increase of OEA concentration that we registered in all the brain areas analysed and, particularly in the Hipp, as early as 2.5 minutes after its systemic administration, could be crucial for OEA ability in improving memory retention, and in regulating memory processes (two conditions which require the involvement of both the Hipp and amygdala).

As second aim of our study, we also investigated the levels of other NAEs (including AEA, PEA, SEA, and LEA), and 2-AG in the different brain areas following OEA systemic administration.

Among their biological functions, PEA, SEA, and LEA have been proposed to play also a role in the regulation of feeding behaviour, effect mediated by their ability to activate PPAR-α.

However, OEA has been found to be the most active in reducing energy intake. In fact, when administered i.p. to laboratory rats, PEA, SEA and LEA are less active than OEA in inducing satiety [5,13,48], thus suggesting the structural selectivity of OEA anorexic effect.

In line with these observations, although NAEs share the same biosynthetic and catabolic pathways with OEA, our results demonstrated overall that OEA administration did not significantly affect the levels of other NAEs in all the brain areas analyzed except for AEA in the Hipp.

In the dHipp OEA systemic administration appeared to reduce AEA levels in OEA-treated rats 30 minutes after treatment. The same trend was observed within the ventral part of the Hipp, although in this brain structure OEA-induced reduction of AEA levels did not reach the statistical significance.

The present result is in line with the scientific literature showing that AEA is an endocannabinoid compound, which binding to cannabinoid receptors type 1 (CB1) and 2 (CB2) exert opposite effects in the control of feeding behavior as compared to OEA [48]. Thereby, OEA-induced reduction of AEA levels in the dHipp might have an effect in lowering food intake since the hippocampal formation is enriched with CB1 receptors [49,50]. The Hipp is not directly involved in the control of feeding, however it has been demonstrated to play important roles in hedonic aspects of eating [51].

Similarly to what observed for NAEs, OEA did not significantly affect the levels of 2-AG in all the brain areas analysed in the present study.

In keeping with the results obtained in the brain, plasma OEA levels increased within minutes of its administration and, in particular, as early as 2.5 minutes after i.p. injection with a maximum increase registered at 15-30 minutes (60-fold higher than controls).

Interestingly, 60 minutes after treatment, OEA levels were still up to 30-fold higher than those measured in control rats treated with veh. This is supported by previous findings

demonstrating that 4 hours after its oral administration, the levels of OEA in plasma were still 2 fold higher than controls [29].

Although OEA has a prolonged pro-satiety action [18,29], many of its effects are rather considered relatively short-lived [29,32]. In this regard, Astarita and colleagues demonstrated that plasmatic OEA levels, following either its systemic (10 mg kg^{-1}) or oral (100 mg kg^{-1}) administration, are characterized by a rapid clearance since they sharply increased within few minutes after treatment (reaching a maximum concentration at 15 minutes) returning to baseline levels within 4 hours (i.p.) or 6 hours (per os) of administration [52]. This time course probably reflects a rapid distribution and metabolism of OEA [17,52], which is known to be hydrolyzed to oleic acid and ethanolamine by FAAH [53–55].

Conversely form what observed in the brain, OEA systemic injection significantly increased also the amount of the other NAEs in the plasma.

The maximum increase was registered at 15 minutes for AEA, PEA and LEA whereas SEA reached the maximum levels 30 minutes after OEA systemic administration.

The OEA-induced increase of circulating NAEs levels could be a result of the "entourage" effect of this lipid bioactive mediator, which consists on the ability to increase the amount of AEA and other members of this family, by competing with them for FAAH, thereby reducing their hydrolysis [56–58].

The discrepancy of these findings compared to what we observed in the brain, where NAEs levels were not affected by OEA injection, could be explained by a lower activity in the brain of calcium-dependent N-acyltransferase (NAT), the enzyme involved in the first step of NAEs synthesis.

This is supported by the literature since it has been demonstrated that two structurally distinct PPAR-α agonists, GW-7647 and PEA, cause a rapid decrease in the amplitude of evoked calcium transients in mouse sensory neurons [59] after binding to PPAR-α. In line with this observation, we can speculate that OEA-induced PPAR-α activation is responsible for the reduction of intracellular calcium concentration in the brain thus resulting in the attenuation of cerebral NAT activity, with a consequence reduction in endogenous NAEs synthesis in the CNS. This could be the reason why OEA systemic administration does not induce an "entourage" effect in the brain.

This PPAR-α mediated mechanism has been investigated by many electrophysiological studies supporting PPAR-α ability to influence membrane ion-channel activities by

recruiting intracellular signal transduction pathways that are gene expression independent [60].

Since there is no evidence supporting the existence of this mechanism in plasma, we can speculate that OEA-induced reduction of endogenous NAEs synthesis, which is mediated by PPAR-α activation, might be selectively restricted to the brain. However, this hypothesis deserves further analyses.

Overall, the findings obtained in the present study suggest that OEA is able to directly reach and permeate the brain parenchyma as an intact molecule within few minutes after i.p administration. Furthermore, our data indicate that OEA has a very extensive brain penetration capability, and its effect on feeding behavior following systemic administration may be mediated by either the brain regions in close proximity to the circumventricular organs or sites outside of the BBB, including AP and ME.

Finally, our results further support the crucial role played by the AP in the complex circuitry underlying OEA pro-satiety effect.

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Chapter IV

Satiety factor oleoylethanolamide prevents binge-like palatable food consumption induced by stress in female rats with a history of food restriction

Abstract

Binge-eating disorder (BED) is a proto-typical eating-related maladaptive behaviour characterized by compulsive and uncontrollable overeating of highly palatable food (HPF). A large body of evidence suggests that the neurobiological mechanisms of BED converge on the activation of the mesocorticolimbic dopamine (DA) system, as well as on the impairment of brain serotonin (5-HT) and noradrenaline (NA) signalling. Moreover, a number of observations revealed that the combination of dieting and stress is a common trigger for people suffering from BED, who displays a hyperactivation of the hypothalamic-pituitary-adrenal (HPA) axis and higher cortisol levels, as compared to obese people not affected by BED. Current BED therapies lack sufficient efficacy and are complicated by high relapse rates and a wide range of side effects. In this background, the satiety signal oleoylethanolamide (OEA), an endogenous gut-derived lipid compound released from enterocytes during meal consumption, might represent a better pharmacological target for the treatment of BED. OEA reduces food intake and body weight gain in obese rodents and humans through the activation of the peroxisome proliferator-activated-receptor-alpha (PPAR-α). Besides its pro-satiety effect, OEA has been also demonstrated to: 1) restore a normal brain dopaminergic response (when it is deregulated by an excessive dietary fat intake); 2) exert anti-depressant-like effect in rodents by regulating 5-HT and NA levels in the brain; 3) attenuate the effect of stress by dampening the hyperactivity of the HPA axis. Based on these premises, in order to test our hypothesis that OEA might be a novel target for BED treatment, in this study we used a BED model in which female rats with a history of intermittent food restriction show binge-like palatable food consumption after a 15-minute exposure to the sight of HPF. In this model, we investigated the anti-bingeing acute effects of OEA (2.5, 5 or 10 mg kg^{-1}, i.p.) on HPF intake and analysed the neurobiological bases of these effects by focusing on the brain pattern of c-Fos expression, on DA release in the shell of the nucleus accumbens (AcbSh), on monoamine concentrations/turnovers in selected brain regions and on both corticotropin-releasing factor (CRF) and oxytocin (oxy) mRNA. Our results showed that OEA treatment was able to selectively prevent binge eating in BED rats. This effect was associated to an increase of hypothalamic oxy mRNA levels and to a decrease of CRF mRNA levels within the CeA. Moreover, we found that OEA decreased both DA efflux and DA tissue concentration in the Acb. We finally observed that OEA enhanced 5-HT transmission in most of the brain areas

analysed selectively in BED rats. Overall, these results support the hypothesis that OEA might represent a novel potential pharmacological target for the treatment of BED.

4.1. Introduction

Binge-eating disorder (BED) is the most frequent eating disorder occurring in 2-5% of the adult population, with a higher prevalence among women than man (10:1 ratio) [1–3]. BED is characterized by uncontrollable and compulsive episodes of excessive consumption of high palatable food (HPF) accompanied by a strong sense of loss of control, feeling of shame, guilt, disgust and anxiety. Although BED is associated to an increased responsivity to food-associated cues [4] inducing food cravings, overeating and obesity [5,6], a significant part of the people affected by BED has a normal body-weight [7,8].

Different factors might contribute to the development of BED, including genetic and biological factors, psychological disturbances and the occurrence of other eating disorders [9]. A large body of evidence suggests that the combination of dieting and stress is a common trigger for people suffering from BED, who displays a hyperactivation of the hypothalamic-pituitary-adrenal (HPA) axis and higher cortisol levels, as compared to obese people not affected by BED [10,11]. Additionally, both animal and human studies have identified the involvement of different neurotransmitter signalling pathways in the pathophysiology of BED [12–15]. In particular, it has been introduced the concept of addiction to explain the compulsive basis and neural mechanisms of BED [16,17]; in fact, BED shares a variety of commonalities with drug addiction, including physiological and emotional-affective dependence, cue-induced craving, escalation of consumption, and high relapse rate [17].

Palatable food is a strong activator of the dopaminergic reward system and may induce addictive behaviour leading to eating disorders [18,19]; on this line, a large body of evidence suggests that the neurobiological mechanisms of BED converge on the activation of the mesocorticolimbic dopamine (DA) system [20,21], as well as on the impairment of brain serotonin (5-HT) and noradrenaline (NA) signalling [22–25]. In keeping with this observation, drugs increasing 5-HT content (e.g. fluoxetine and d-fenfluramine) [26–29] and lorcaserin, a selective $5-HT_{2c}$ receptor agonist that stimulates central dopaminergic neuronal activity [30], have been demonstrated to suppress binge eating.

Nevertheless treatments for BED lack sufficient efficacy and are complicated by high relapse rates and a wide range of side effects [31,32]. Lisdexamfetamine (LDX), a pro-drug of d-amphetamine, is the first medication approved for the treatment of BED in the

United States [33] that acts primarily by enhancing brain dopaminergic and noradrenergic neurotransmission [33], however it causes several adverse effects [34–36]. Several observations have been accumulated by our and other research groups suggesting that the lipid-derived messenger oleoylethanolamide (OEA) might represent a better pharmacological target for the treatment of BED [37–39]. OEA reduces food intake and body weight gain in obese rodents and humans [40–53] mainly through the activation of the peroxisome proliferator-activated-receptor-alpha (PPAR-α), for which OEA shows a high affinity (EC50= 120.0 ± 10.7 nM) [54]. The hypophagic effect of OEA is associated to the induction of a satiety state [55] and appears behaviourally selective: i.e. not linked to anxiety, malaise, changes of body temperature, pain, motor impairment, or HPA axis activation [40]. Moreover, this effect has been associated to the indirect activation of selected brain areas, such as the nucleus of the solitary tract (NST) and the area postrema in the brainstem [44,50], as well as the tuberomammillary (TMN) [56] and paraventricular (PVN) [47] nuclei in the hypothalamus (HYPO), where noradrenergic [51], histaminergic [57] and oxytocinergic [58] neurons play a necessary role. Interestingly, OEA seems to modulate feeding behaviour acting not only within the caloric-homeostatic control system but also within the hedonic non-homeostatic circuits, as reported by Tellez and co-authors [59], who focused on the hypofunction of the dopaminergic reward system induced by excessive dietary fat intake. In particular, these authors reported that the systemic treatment with OEA can re-establish a normal sensitivity to the rewarding properties of fat and suppresses fat ingestion in diet-induced obese mice [59]. Moreover, it has been demonstrated that OEA administration is able to exert anti-depressant-like effects in different animal models [60], by regulating BDNF [61], 5-HT and NA levels in the brain [62].

In recent years different animal models of BED have been developed and validated offering important research tools with face, predictive and construct validity to investigate the neurobiological bases of this disorder and to search for novel efficacious and safe pharmacological targets [63–65]. In this study, we used a rat model of binge-like palatable food consumption [66–68] to test the hypothesis that OEA might be a novel target for BED treatment.

In our binge model adult female rats, which underwent three 8-day cycles of intermittent food restriction with limited access to HPF, show binge-like HPF consumption (i.e. intake of a large amount of HPF within the first few minutes (min) of access) after the exposure to a 15-min "frustration stress", consisting of the sight and

smell of HPF placed out of reach [66]. We will refer in the text to dietary restricted (R) vs non restricted (NR) rats and exposed to stress (S) vs non exposed to stress (NS) rats (Fig. 4.1A).

In this model, the putative interaction of dieting and negative-valence states plays a key role in stimulating binge eating behaviour and inducing neuro-endocrine alterations that present elements of similarity with the human symptomatology [67,69].

To evaluate whether OEA might represent a potential target for novel BED treatment, we investigated on this model the anti-bingeing effects of OEA (2.5, 5 or 10 mg kg^{-1}, i.p.) on HPF intake and analysed the neurobiological bases of these effects.

To this second aim, we first evaluated whether the interaction between dietary regimen and stress exposure is characterized by a specific pattern of brain activation (assessed by the neuronal activity marker c-Fos) and whether OEA treatment is able to modulate such pattern.

Secondly, we investigated the effect of OEA administration on DA release in the shell of the nucleus accumbens (AcbSh), a ventral striatal brain structure receiving dopaminergic projections from the ventral tegmental area (VTA) and involved in the motivational/reinforcing properties of addictive drugs and food [70,71]. Furthermore, we analysed the tissue concentrations of monoamines (DA, 5-HT, NA) and their main metabolites to evaluate monoamine turnover in the principal neural nodes that control different aspects of food intake in the brain. Specifically, we focused on limbic structures, such as nucleus accumbens (Acb) and caudate putamen (CPu), which regulate the habituation and hedonic value of food; VTA and substantia nigra (SN) crucial for the salience and motivation; cortical structures involved in the decision making, such as the medial prefrontal cortex (mPFC); HYPO, which coordinates arousal with energy homeostasis; amygdala (AMY) that, together with the hippocampus (HIPP), regulates emotional or memory processes and conditioned responses; the two brainstem nuclei, dorsal raphe (DR) and locus coeruleus (LC), which send, respectively, serotonergic and noradrenergic inputs to the VTA [72].

Finally, we evaluated the impact of OEA administration on the corticotropin-releasing factor (CRF) system, which plays a critical role in stress-induced binge eating in our rat model [67] and on the hypothalamic oxytocinergic system playing a necessary role in the hypophagic action of OEA [47,58].

4.2. Methods and Materials

### 4.2.1. Experimental procedure for Binge-eating induction

Female Sprague Dawley rats were purchased from Charles River and were used when their body weight was 200–225 g at the beginning of the experiments. Rats were group housed under a 12 h light/dark cycle (lights on at 8:00 A.M.), at constant temperature (20 –22°C) and humidity (45–55%) and with access to food and water *ad libitum* for 2 weeks before the experiments. All experiments were carried out in accordance with the European directive 2010/63/UE governing animal welfare and with the Italian Ministry of Health guidelines for the care and use of laboratory animals. According to the dietary schedule, the rats were given standard food pellets (4RF18; Mucedola; 2.6 kcal/g) or HPF (3.63 kcal/g) consisting in a paste prepared by mixing Nutella (Ferrero[R]) chocolate cream (5.33 kcal/g; 56, 31, and 7% from carbohydrate, fat, and protein, respectively), ground food pellets (4RF18), and water in the following w/w/w percent ratio: 52% Nutella, 33% food pellets, and 15% water. The procedure for binge eating induction was performed according to our previous studies [66–68]. Briefly, two groups of female rats were housed individually in metal cages (30 x 30 x 30 cm) and exposed (or not exposed) for 24 days to three 8-day cycles of intermittent food restriction (66% of chow intake on days 1–4 and free feeding on days 5–8 of each cycle), during which they were given access to HPF for 2 h during the light cycle between 10:00 A.M. and 12:00 A.M. (2 h after the onset of the light cycle) on days 5–6 and 13–14 of the first two cycles (total of four exposures) [67]. We will refer in the text to dietary restricted (R) and non restricted (NR) rats. On the test day (day 25), at 10 A.M., half of the animals in each group was subjected to a 15 min frustration stress, consisting of the exposure to HPF placed out of reach [66]. During this 15 min period, the rats could smell and see the HPF and repeatedly attempted to reach it. The second half of animals in each group was not exposed to the stress manipulation, therefore we will refer in the text to stressed (S) and non stressed (NS) rats. After 15 min stress exposure, the HPF cup was placed inside the cage. In accordance with our previous studies [66–68], binge eating behaviour occurred in R+S rats (Fig. 4.2), as demonstrated by the immediate and persistent consumption of a large amount HPF within the first 15 min access. Vaginal smears were collected at the end of experiments to exclude from the results those rats resulting in the estrous phase [69].

The experimental paradigm is depicted in Fig. 4.1A. This paradigm was used in four different experiments, in which the consumption of the HPF was allowed for 2 h, 1 h, or 0 h, depending on the endpoints analyzed (Fig. 4.1B).

4.2.2. Experiment 1: Effects of OEA on stress-induced binge-eating

The first set of rats (n = 115) was divided into 16 groups (n = 6-8 per group) in a 2 (history of intermittent food restriction: no, yes, R or NR rats) × 2 (stress during testing: no, yes, S or NS rats) × 4 (OEA dose: 0, 2.5, 5 and 10 mg kg^{-1}) factorial design, to evaluate the behavioural effects of OEA during the test day. To this aim OEA or vehicle (veh) were administered 60 min before the access to HPF; rats were exposed (or not exposed) to the 15-min frustration stress; and once they gained access to the HPF, the intake was measured at the following time points (15, 30, 60, and 120 min). The experimental paradigm is depicted in Fig. 4.1B (EXP.1).
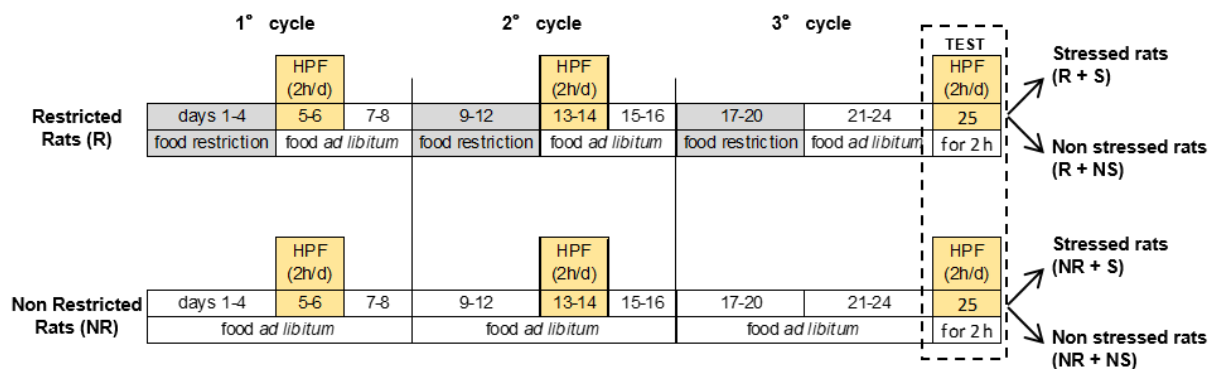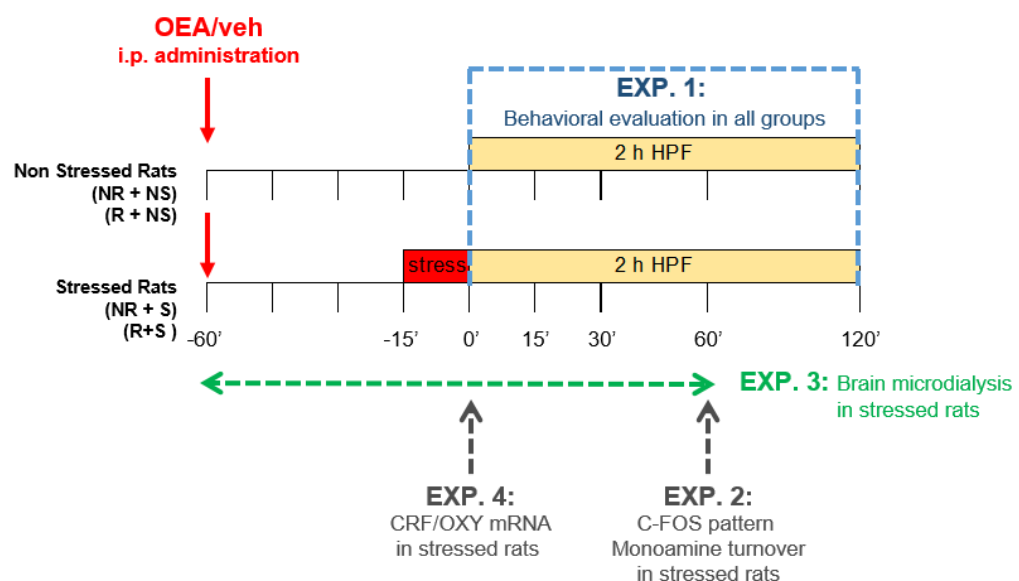
Fig. 4.1: (**Panel A**) Different groups of female rats were exposed (Restricted Rats, R) or not exposed (Non restricted Rats, NR) for 24 days to three 8-day cycles of food restriction (66% of chow intake) on days 1–4 and free feeding on days 5–8 of each cycle. During the "free feeding" exposure, both NR and R rats were given access to high palatable food (HPF) for 2 hours (h) during the light cycle on days 5–6 and 13–14 of the first two cycles. On the day 25 both R and NR rats were exposed (R+S and NR+S) or not exposed (R+NS and NR+NS) to frustration stress. (**Panel B**) **EXP. 1**: on test day (day 25), after the third cycle, both NR and R rats were administered with vehicle (veh) or 3 different doses of OEA (2.5, 5 and 10 mg kg^{-1} i.p.). 45 min after the treatments, both NR and R rats were exposed (stressed: NR+S and R+S) or not exposed (not stressed: NR+NS and R+NS) to a 15 min stress procedure. 1 h after the respective treatments rats were given free access to HPF and food intake was monitored for 2 h. **EXP. 2**: NR+S and R+S rats were administered with veh or OEA (10 mg kg^{-1} i.p.) and were allowed to consume the HPF only for 1 h. At the end of this procedure rats were sacrificed, their brains immediately collected for immunohistochemical evaluation of the pattern of Fos expression and HPLC analyses of monoamine turnover. **EXP. 3**: NR+S and R+S rats were administered with veh or OEA (10 mg kg^{-1}, i.p.) and underwent brain microdialysis in the shell of the nucleus accumbens (AcbSh) for the analysis of dopamine (DA) extracellular levels (the detailed paradigm of the microdialysis experiment is described in the legend of the Fig. 4.5). **EXP. 4**: NR+S and R+S rats were administered with veh or OEA (10 mg kg^{-1}, i.p.) and immediately sacrificed at the end of stress procedure. Their brains were collected for in situ hybridization experiment.

130

4.2.3. Experiment 2: Effects of OEA on the pattern of c-Fos expression and on monoamine turnover in selected brain areas

- *Brain dissection and sample preparation*

Based on the results obtained from the behavioural study, a second experiment (EXP. 2) was conducted focusing on the NR+S and R+S groups of rats treated with either veh or OEA at the dose of 10 mg kg^{-1}. In this experiment we evaluated whether the interaction between dietary regimen and stress exposure is characterized by a specific pattern of brain activation (assessed by the neuronal activity marker c-Fos) and whether OEA treatment is able to modulate such pattern. Moreover, as a further aim of this experiment we analyzed the effects of OEA on the tissue concentrations of monoamines (DA, 5-HT, NA) and their main metabolites in the principal neural nodes that control different aspects of food intake in the brain. 30 rats were subjected to the same procedure used in EXP.1, but were allowed to consume the HPF only for 1 hour (Fig. 4.1B). At the end of this procedure all rats were sacrificed, their brains immediately collected, snap frozen in 2-metylbutane (-60°C) and stored at -80°C until analysis. The experimental paradigm is depicted in Figure 4.1B (EXP.2). The brain analyses included immunohystochemical evaluation of the pattern of c-Fos expression and HPLC analyses of monoamine turnover. Each brain was sliced into 20 μm serial coronal sections by using a cryostat (model HM550; Thermo Fisher Scientific, Kalamazoo, MI, USA). The coronal slices were partly mounted on positively charged slides (SuperFrost Plus, Menzel, Germany) and stored at -20°C until further analysed for immunohystochemistry, partly microdissected into ten regions of interest that were collected in microtubes (pooled from both hemispheres), weighed to a high degree of accuracy by using a microbalance and stored at -80°C until the HPLC analysis for monoamine turnover. These regions included mPFC, striatum (comprising CPu and Acb), HYPO, AMY, HIPP, SN, VTA, DR and LC.

- *Immunohistochemistry*

c-Fos immunohistochemistry was performed in brain areas controlling different aspects of eating and eating-related behaviour that included the AMY and the PVN, which are crucially involved in the control of stress and hedonic/homeostatic feeding respectively, different limbic areas (Acb, CPu, VTA and SN), which regulate the reinforcement and

the motivational aspects of feeding, the ventral pallidum (VP) and the pedunculopontine nucleus (PP), which regulate the activity of DA neurons within the VTA [73].

The immunohistochemistry protocol was based on our previous study [44]. Briefly, brain slices containing the desired brain areas underwent antigen retrieval procedure by submerging selected slides in a sodium citrate buffer (10 mM pH 6.0) heated at 95°C for 5 min [44]. Sections were then rinsed with PB (0.1 M pH 7.4) and incubated for 1 h in a solution containing 0.1% Triton X-100 (Sigma–Aldrich) and 2% of Normal Donkey Serum (Jackson Immunoresearch, Baltimore Pike, Pennsylvania). After additional washes, sections were incubated with a solution containing the primary antibody (rabbit anti-c-Fos polyclonal primary antibody, 1:5000 dilution, Santa Cruz California) for 2 overnight at 4°C. The sections were then incubated with biotinylated donkey anti-rabbit IgG (1:500 in 0.3% PBT, Jackson Immunoresearch, Baltimore Pike, Pennsylvania) for 2 h at room temperature. After incubation for 1 h with the ABC Kit (Vectastain ABC kit; Vector Laboratories), sections were stained by incubation in DAB (Vector Laboratories) chromogen solution. The slides were then rinsed with PBS, dehydrated in graded alcohol, immersed in xylene and cover-slipped with Eukitt (Sigma–Aldrich). For the semi-quantitative analysis of the slices processed by immunohystochemistry, all brain sections were observed under a Nikon Eclipse 80i microscope equipped with a colour charge-coupled device camera and controlled by the software NIS-Elements-BR (Nikon). Slices were photographed in light field using a 4× objective, and the rat brain atlas by Paxinos and Watson [74] was used as reference for the localization of the brain areas of interest. c-Fos DAB-immunostaining was measured semi-quantitatively as optical density (OD) by using the Scion Image J software and considering, for background normalization, the averaged OD either of non-immunoreactive regions or of white matter structures within the same brain slice. For this analysis, the investigator was blinded to experimental groups and measurements were obtained in at least five consecutive tissues sections per animal containing the desired structure.

- *Monoamine- and monoamine metabolite-assay in brain tissues*

Brain samples obtained by microdissections from NR+S and R+S rats, treated with either OEA or veh and sacrificed 1 h after gaining access to the HPF, were ultrasonicated in ice-cold 0.1 M perchloric acid and then centrifuged at $15000 \times g$ for 20

min at 4°C as previously described [75]. Supernatants were collected and used for HPLC analyses to determine monoamine- and monoamine metabolite concentrations.

- *HPLC analysis*

DA, NA, 5-HT and both 5-HT and DA metabolites, 5-Hydroxyindoleacetic acid (5-HIAA), homovanillic acid (HVA) and 3,4-dihydroxyphenylacetic acid (DOPAC) were detected and quantified by HPLC, as previously described by [75,76]. Monoamines and their metabolites were analysed by microbore HPLC; the detection was accomplished with a Unijet cell (BAS) with a 6-mm diameter glassy carbon electrode set at +650 mV vs an Ag/AgCl reference electrode, connected to an electrochemical amperometric detector (INTRO, Antec Leyden, Netherlands). The analytes were separated using a SphereClone 150-mm × 2-mm column (3-µm packing) and a mobile phase composed of 85 mM of sodium acetate, 0.34 mM EDTA, 15 mM sodium chloride, 0.81 mM of octanesulphonic acid sodium salt, 6% methanol (v/v) (pH = 4.85) delivered at a flow rate of 800 µl/min for a total runtime of 35 min. For each analysis, a set of standards containing various concentrations of each compound (monoamines and their metabolites) was prepared in the acid solution to obtain appropriate calibration curves. The concentrations of neurotransmitters were determined by linear interpolation from standard curves; for tissue monoamines we normalized their concentration to the weight of the wet tissue sample. Concentrations of monoamines were reported in pg ml^{-1} for DA extracellular levels and in ng mg^{-1} for tissue monoamines. DA and 5-HT turnover were calculated as the ratio between the metabolite and the monoamine (DOPAC+HVA/DA for the DA and 5HIAA/5-HT for the 5-HT).

Samples of selected brain regions (mPFC, Acb, CPu, HYPO, AMY, HIPP, SN, VTA, DR and LC) of all animal groups were processed simultaneously to minimize experimental errors.

4.2.4. Experiment 3: Effects of OEA on dopamine transmission in the AcbSh

- *In vivo microdialysis*

NR and R (15-20 per group) rats were deeply anaesthetized with equithesin (3 ml kg^{-1}, i.p.), placed on a stereotaxic apparatus (David Kopf Instruments) and implanted with a guide cannula (MAB4.15.IC Microbiotech/se AB, Stockholm, Sweden) for microdialysis probes (cod. MAB 4.15.2.CU Microbiotech/se AB, Stockholm, Sweden)

placed vertically 2mm above the left or the right AcbSh, according to the following coordinates [74]: (AP = + 2.7 mm from bregma, ML = ± 1.2 mm from midline and DV = - 5.6 mm from skull; Fig. 4.5A). When the animals had fully recovered from the surgery, a probe was inserted in the guide cannula and the microdialysis was carried out in freely moving rats, according to our previous study [77]. Each microdialysis probe was perfused with a KrebsRinger phosphate (KRP) buffer as previously described [78] at a constant flow rate of 1.5 µl min^{-1} and dialysate samples were collected every 15 min in minivials containing 5 µl of 10% acetic acid. After an initial wash out period (at least 1h) three baseline samples (no more than 10% difference among four consecutive samples) were collected before treating rats. Thereafter, rats were administered with OEA (10 mg kg^{-1}, i.p.) or veh and after 45 min underwent the stress procedure for 15 min. At the end of this procedure, they gained access to HPF for 1h (Fig. 4.1B, EXP.3). After 30 min from the end of the HPF exposure, rats were acutely challenged with amphetamine (0.5 mg kg^{-1}) administered subcutaneously. Microdialysates were collected up to 1.5 h after amphetamine treatment. Each microdialysis sample was analysed by HPLC, that was performed as described in the previous section. The correct placement of the probe was verified histologically with a post mortem Nissl staining procedure (data not shown) and data from animals with incorrect probe implant were excluded from the statistical analyses.

4.2.5. Experiment 4: effects of OEA on frustration stress exposure

- *In situ hybridization of oxy and CRF mRNA*

A third set of 33 rats from the NR and R group was administered with veh or OEA (10 mg kg^{-1}, i.p.) 45 min before the frustration stress exposure and immediately sacrificed at the end of stress. Their brains were collected, immediately snap frozen in 2-methylbutane (-60°C) and stored at -80°C until they were cut in 20 µm serial coronal sections. On these sections we performed *in situ* hybridization with an antisense [^{35}S]-labelled riboprobe of rat oxy [47,50] or with an antisense [^{35}S]-labelled riboprobe of rat CRF that were both used in our previous studies [47,50,79], to detect oxy mRNA in the PVN and CRF mRNA in both PVN and central AMY (CeA).

Briefly, oxy and CRF riboprobes were generated from linearized vector constructs, by in vitro transcription using SP6 RNA polymerases (Roche Diagnostic, Monza, Italy), respectively in the presence of both [35S]-CTP and [35S]-UTP. Brain sections were then hybridized at 60°C for 16 h in a buffer containing [35S] cRNA (~45,000 dpm

ml−1), 10% dextran sulfate, 50% formamide, 1× Denhardt's solution, 100 μg ml^{-1} denatured salmon sperm DNA, 0.15 mg ml^{-1} tRNA and 40 mM dithiothreitol as previously described [47]. After hybridization, brain sections were exposed to Kodak Biomax film (Sigma-Aldrich) for 16 or 4 h to the detect CRF or oxy mRNA, respectively.

The effects of OEA on oxy and CRF mRNA were determined by a semi-quantitative autoradiography analysis, as previously described [47,50,79]. For semi-quantitative autoradiography analysis, films were first scanned (Epson perfection 3200 PHOTO) at high resolution (1200 dpi). A brain atlas [74] was used to define the localization of the brain structures of interest. Quantitative analyses of hybridized signals were performed using the Scion Image software. Optical densities were converted into radioactivity concentrations by densitometric analysis of 14C-microscale standards (American Radiolabeled Chemicals), so as to create for each film a calibration curve with a linear coefficient r2 > 0.9. In every brain section, the OD of the corpus callosum was used as background and an integrated OD value was calculated as radioactivity per extension of hybridized area.

For this analysis the investigator was blinded to animal treatment, measurements were obtained in at least five consecutive tissues sections per animal containing the desired structure.

4.2.6. Statistical Analyses

All data were expressed as mean ± SEM. Feeding data were statistically analysed by two-way ANOVA for repeated measures, with time as within-subject factor and treatment as between-subject factor, followed by Bonferroni's post hoc test for multiple comparisons (IBM SPSS, version 22, IBM Analytics).

Data obtained from immunohistochemistry, HPLC analysis of tissue monoamines and in situ hybridization were statistically analysed by two-way ANOVA, with "diet regimen" and "treatment" as the two factors. Tukey's test was used as a post hoc to perform multiple comparisons. Moreover, for analysis of data obtained from the semi-quantitative densitometric analyses, because of the difference in the number of slices examined and the high degree of freedom, the error degrees of freedom were kept constant at a value based on the actual number of animals per group used in each experiment [44,80].

The results from microdialysis experiments resulted homoscedastic and were analysed by two-way ANOVA for repeated measures, with time as the within variable and treatment as the between variable, followed by Dunnett's and Bonferroni's *post hoc* tests for multiple comparisons. Overall, DA extracellular levels were calculated as percentages of baseline, which was defined as the average of the first three consecutive samples with stable level of neurotransmitters. Unpaired t-test was used to evaluate a difference between the marginal means of the first three dialysate samples (basal values). In all instances, the threshold for statistical significance was set at $P < 0.05$.

4.3. Results

4.3.1. The combination of caloric restriction and stress exposure induced BED

In keeping with our previous studies [66–68], we found that, in rats with a history of intermittent food restriction, the exposure to 15 min frustration stress increased HPF consumption at all the time point of the feeding test conducted on day 25; this stress manipulation had no effect on food intake in rats that were not food restricted (Fig. 4.2). Three-way ANOVA for repeated measures showed a significant effect of diet regimen ($F_{diet\ regimen} = 14.601$ df=1/26, P<0.01), a significant effect of stress ($F_{stress} = 7,336$, df = 1/26, P<0.05) and a significant effect of time ($F_{time} = 44,017$, df = 3/78, P<0.001); no interaction between the three factors was observed ($F_{interaction\ (time\ x\ diet\ regimen)} = 0.214$, df = 3/78, P=0.728; $F_{interaction\ (time\ x\ stress)} = 0.869$, df = 3/78, P=0.392; $F_{interaction\ (diet\ regimen\ x\ stress)} = 2.287$, df = 1/26, P=0.142; $F_{interaction\ (time\ x\ diet\ regimen\ x\ stress)} = 1.401$, df = 3/78, P=0.254). The results obtained by *Bonferroni post hoc test* are indicated in Fig. 4.2.
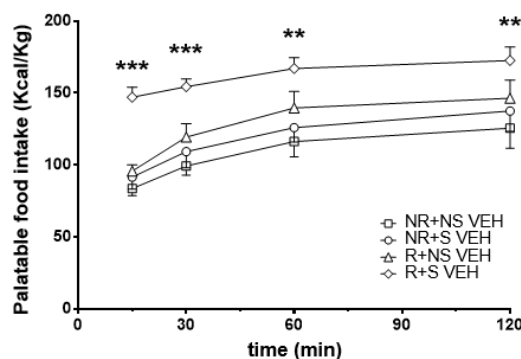
Fig. 4.2: Time course of cumulative HPF intake (kcal kg^{-1}) at different time-points (15, 30, 60, 120 min) in NR+NS (non restricted – non stressed), NR+S (non restricted – stressed), R+NS (restricted – non stressed) and R+S (restricted – stressed) rats (N=30) after vehicle (veh) administration. Data are expressed as mean ± SEM. **P<0.01; ***P<0.001 vs NR+NS veh at the same time point (Bonferroni's test for between groups comparisons).

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

We found that an acute treatment with OEA, systemically administered to rats 60 min before giving free access to HPF (Fig. 4.1B, EXP. 1), was able to selectively prevent binge-like eating in animals with a history of intermittent food restriction and exposed to stress (R+S rats, Fig. 4.3D), without significantly altering feeding behaviour in the other experimental groups (Fig. 4.3 A, B and C). although a trend toward decreased food intake, which did not reach statistical significance, was observed in NR+NS rats treated with the highest dose of OEA (Fig. 4.3A).

In particular, OEA decreased frustration stress-induced HPF overconsumption in a dose- and time-dependent manner, with the strongest and long-lasting effect observed at the dosage of 10 mg kg^{-1} i.p. (R+S group, Fig. 4.3D), which was effective at all the time points considered (i.e. 15, 30, 60 and 120 min). The intermediate dose of OEA (5 mg kg^{-1} i.p.) was effective only during the first 30 min, while no effect was detected in rats administered with the lowest dose of OEA, 2.5 mg kg^{-1} i.p..

The reduction of HPF intake displayed by R+S rats treated with 10 mg kg^{-1} OEA ranged from a percentage of -62,7%, at the earliest time-point, up to -34,02%, at 120 min (Fig. 4.3D). Conversely, the initial decrease of HPF consumption observed in R+S rats treated with 5 mg kg^{-1} OEA (-36,3% and –29,8% at 15 and 30 min, respectively), was compensated at later time points (Fig. 4.3D).

The two-way ANOVA for repeated measures of the cumulative food intake of R+S group showed a significant effect of treatment ($F_{treatment}$=15.602 df=1/27, P<0.001), a significant effect of time (F_{time} = 48.741, df = 3/81, P < 0.001) and a significant interaction between treatment and time ($F_{interaction}$ = 4.167, df = 9/81, P < 0.01). Significant differences among groups evaluated by the *post hoc* analysis are indicated in Fig. 4.3.
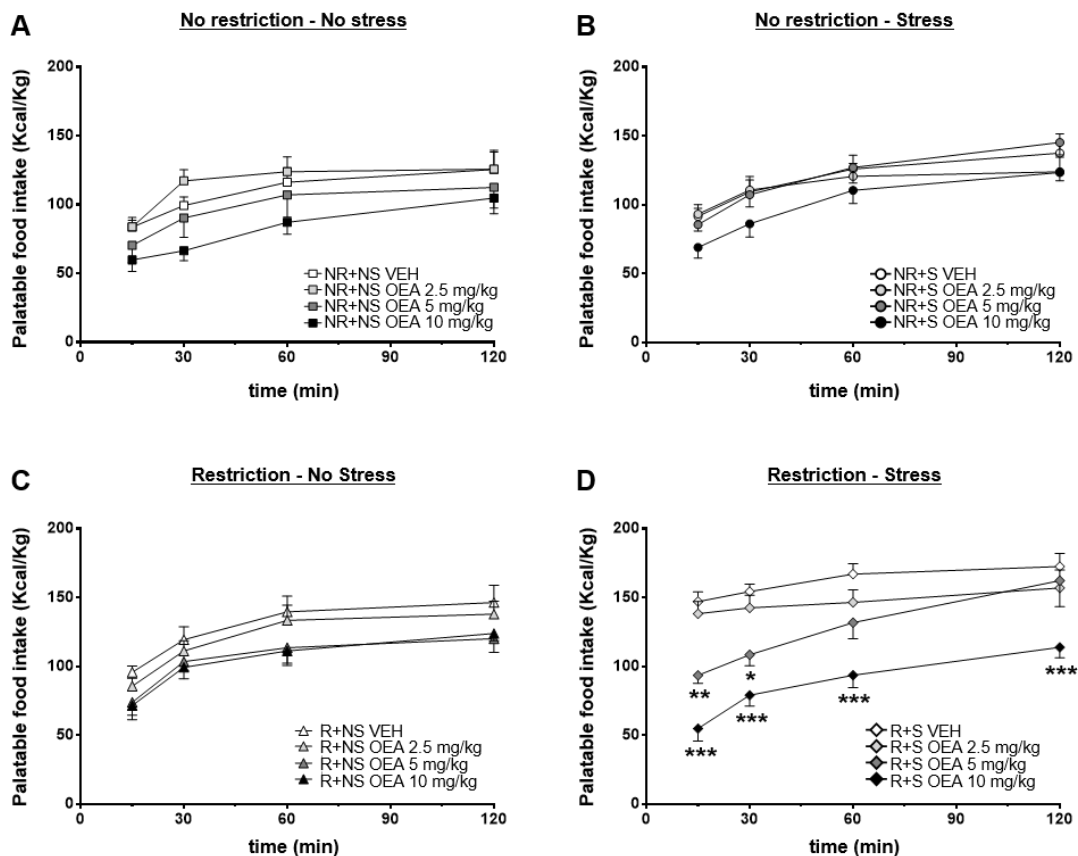
Fig. 4.3: Time course of cumulative HPF intake (kcal kg^{-1}) at different time-points (15, 30, 60, 120 min) after vehicle (veh) or 3 different doses of OEA (2.5, 5 and 10 mg kg^{-1} i.p.) administration to NR+NS (**panel A**, non restricted - non stressed, N = 30), NR+S (**panel B,** non restricted – stressed, N = 26), R+NS (**panel C**, restricted - non stressed, N = 28) and R+S (**panel D**, restricted – stressed N = 31) rats. Data are expressed as mean ± SEM. *P<0.05; **P<0.01; ***P<0.001 vs R+S veh at the same time point (Bonferroni's test for between groups comparisons).

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

Previous studies demonstrated that OEA's effect on food intake is paralleled by a selective induction of c-Fos at the level of the HYPO and brainstem [44,47,50], key brain regions involved in the control of feeding [81]. In the present work we have expanded our previous findings by examining the impact of OEA treatment on the brain pattern of c-Fos immunostaining in response to 60 min-HPF consumption in female rats with different diet histories and exposed to acute stress (R+S vs NR+S, Fig. 4.1B, EXP. 2). In particular, we focused our attention on brain areas (Fig. 4.4A) controlling different aspects of eating and eating-related behaviour that included the AMY and the PVN, which are crucially involved in the control of stress and hedonic/homeostatic feeding respectively, different limbic areas (Acb, CPu, VTA and SN), which regulate the

138

reinforcement and the motivational aspects of feeding, the VP and PP, which regulate the activity of DA neurons within the VTA [73].

The results obtained from the semiquantitative analyses of immunostaining optical densities revealed that the interaction between intermittent food restriction and stress exposure induced an increase of c-Fos expression in the Acb, CPu, AMY, and SN (Fig. 4.4 C, D, F, and H) of bingeing rats (R+S veh), with respect to non-bingeing rats (NR+S veh), and that OEA treatment (10 mg kg^{-1} i.p.) was able to completely prevent such increase, in accordance with the behavioural effects on binge eating. Conversely, c-Fos expression within the PVN, PP and VTA (Figure 4.4 E, G and I) resulted unchanged in bingeing rats (R+S veh), with respect to non-bingeing rats (NR+S veh), but significantly increased by OEA treatment (R+S OEA vs R+S veh), which induced a similar effect also in the AMY and PP of NR rats (Fig. 4.4 F, and G, respectively). No difference was observed within the VP nucleus among all animal groups (Fig. 4.4B).

The results from the two-way ANOVA analyses of c-Fos expression levels conducted for each of the brain areas considered are reported in Table 4.1; the results obtained from the *post-hoc* analyses are reported in Fig. 4.4.
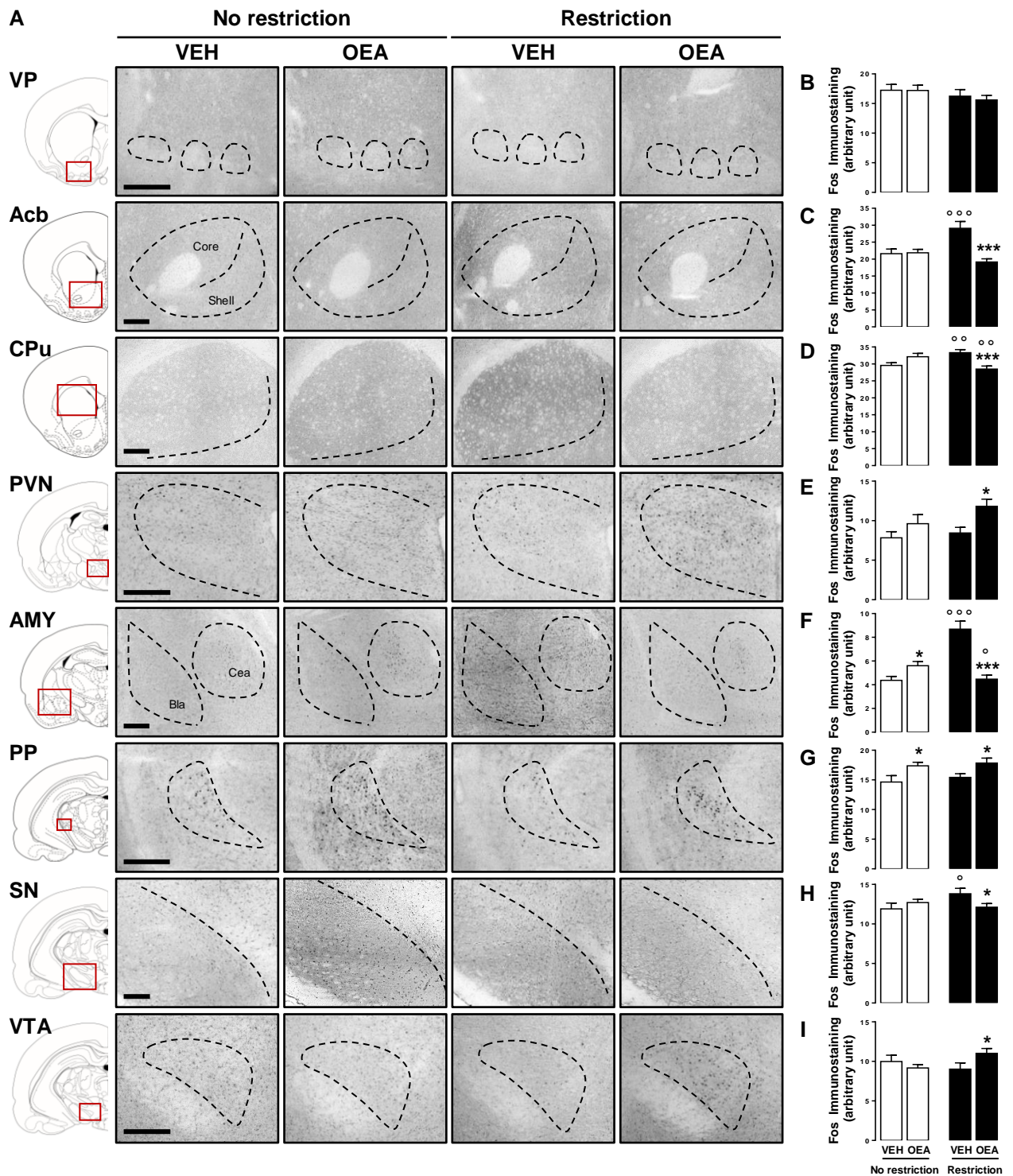
Fig. 4.4: Representative photomicrographs (scale bar = 500 μm, panel A) showing c-Fos immunostaining within the ventral pallidum (VP), nucleus accumbens (Acb), caudate putamen (CPu), paraventricular nucleus (PVN), amygdala (AMY), pedunculopontine nucleus (PP), substantia nigra (SN) and ventral tegmental area (VTA) in brain slices collected from both NR+S (non restricted – stressed) and R+S (restricted – stressed) rats treated with either vehicle (veh) or OEA (10 mg kg $^{-1}$, i.p.) and sacrificed 2 h after treatment. Semiquantitative densitometric analysis of c-Fos expression within the VP (**panel B**), Acb (**panel C**), CPu (**panel D**), PVN (**panel E**), AMY (**panel F**), PP (**panel G**), SN (**panel H**) and VTA (**panel I**) of NR+S and R+S rats treated with either veh or OEA (10 mg kg $^{-1}$, i.p.) and sacrificed 2 h after treatment. Data are expressed as mean ± SEM. *$P<0.05$; ***$P<0.001$ vs veh in the same diet regimen group; °$P<0.05$; °°$P<0.01$; °°°$P<0.001$ vs NR+S in the same treatment group (Tukey's *post hoc* test; N = 3).

Table 4.1: Results of the two-way ANOVA analyses of c-Fos expression observed in different brain areas of both NR+S and R+S rats after intraperitoneal injection of OEA or vehicle

	F diet regimen	F treatment	F interaction	df
VP	1,84 (p=0,202)	0,124 (p=0,73)	0,094 (p=0,76)	1/11
Acb	3,291 (p=0,097)	13,462 (p<0,01)	14,821 (p<0,01)	1/11
CPu	0,005 (p=0,945)	1,538 (p=0,241)	16,172 (p<0,01)	1/11
PVN	2,281 (p=0,159)	7,668 (p<0,05)	0,721 (p=0,414)	1/11
AMY	14,812 (p<0,01)	12,933 (p<0,01)	43,259(p<0,001)	1/11
PP	0,622 (p=0,447)	10,574 (p<0,01)	0,039 (p=0,847)	1/11
SN	1,329 (p=0,273)	0,613 (p=0,450)	4,627 (p=0,054)	1/11
VTA	0,409 (p=0,536)	0,709 (p=0,418)	4,038 (p=0,069)	1/11

Abbreviations: Acb, nucleus accumbens; AMY, amygdala; CPu, caudate putamen; PP, pedunculopontine nucleus; PVN, paraventricular nucleus; SN, substantia nigra; VP, ventral pallidum; VTA, ventral tegmental area.

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

To investigate whether OEA was able to attenuate binge eating by affecting the central dopaminergic response to appetitive/reinforcing stimuli, in a third experiment (Fig. 4.1B, EXP. 3), 15-min consecutive microdialysate samples were collected from NR and R rats through a vertical probe stereotaxically placed in the left or the right AcbSh (Fig. 4.5A). After an initial wash out period, three baseline samples were collected before treating rats with OEA (10 mg kg^{-1}, i.p.) or veh; 45 min later rats underwent the stress procedure for 15 min and then they gained access to HPF for 1h; 30 min from the end of the HPF exposure, rats were subcutaneously administered with amphetamine (0.5 mg kg^{-1}), to burst DA release, and microdialysis was terminated 90 min later. Each microdialysate sample was analysed by HPLC and results were expressed as pg/ml for basal values (Fig. 4.5B) and as % of basal levels for the time-course of extracellular DA levels (Fig. 4.5C and D).

The basal values were pooled as average of dialysate samples 1–3, and no significant difference was detected between the two experimental groups (p=0.7089, unpaired student t-test, Fig. 4.5B). OEA administration did not alter DA basal levels, but was able to prevent or significantly dampen DA efflux evoked by frustration stress or by amphetamine challenge, independently from the history of caloric restriction (Fig. 4.5C and D). The results obtained by the two-way ANOVA for repeated measures performed in R+S and NR+S rats revealed a significant effect of time, treatment and a significant interaction between the two factors (R+S: F_{time} = 17.252 df = 18/234, P < 0.001, $F_{treatment}$ = 27.407 df = 1/13, P < 0.001, $F_{interaction}$ = 5.018 df = 18/234, P < 0.01; NR+S: F_{time} = 15.216 df = 18/324, P < 0.001, $F_{treatment}$ = 6.154 df = 1/18, P < 0.05, $F_{interaction}$ = 3.142 df = 18/234, P < 0.05). The results obtained by *post hoc* tests are reported in Fig. 4.5.
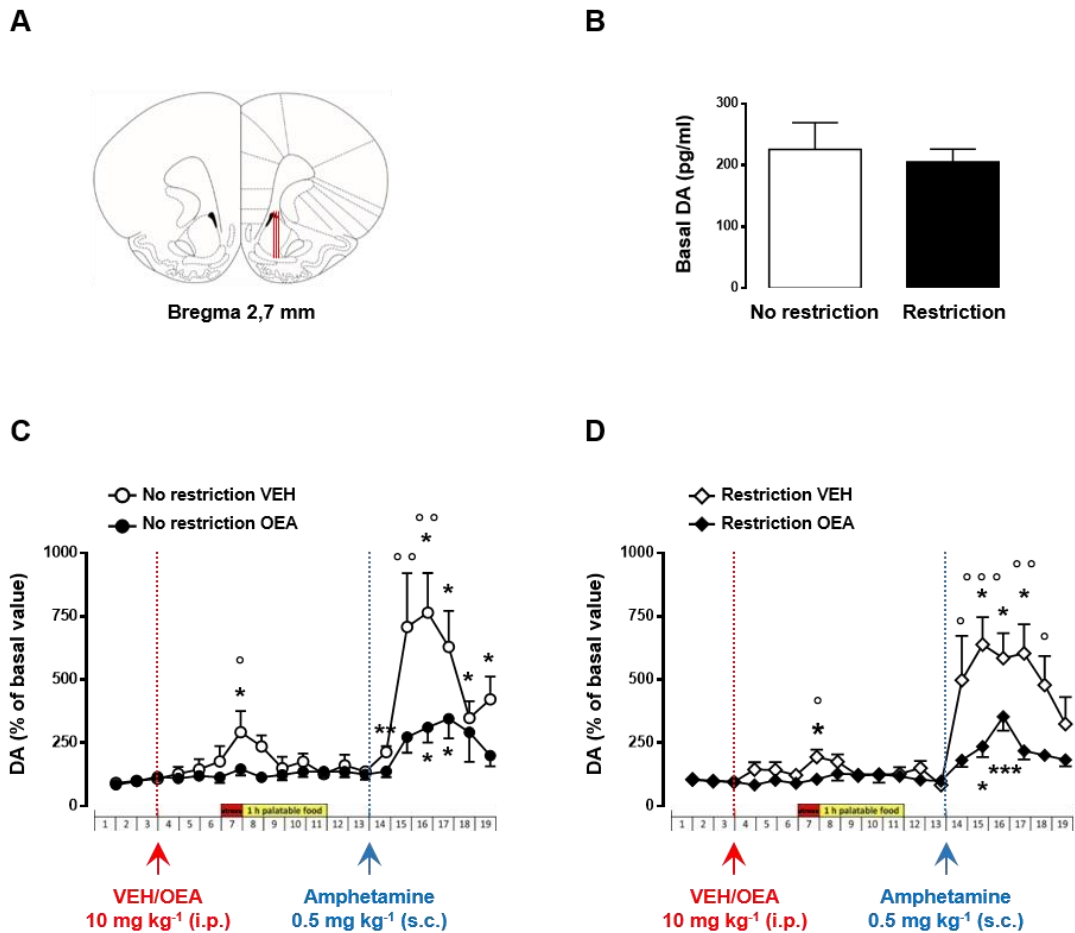
Fig. 4.5: Brain diagram (**panel A**) illustrating the average sites (red lines) where microdialysis probe was implanted. Basal dopamine (DA) extracellular levels (**panel B**, calculated as marginal means of the first three dialysate samples) in the Shell of the nucleus accumbens (AcbSh) of NR+S (non restricted – stressed) and R+S (restricted – stressed) rats during microdialysis experiment. Data are expressed as mean ± SEM (Unpaired student *t-test*; N=16-22).

Time course of extracellular DA levels (expressed as % of basal values) of NR+S (**panel C**, N = 9-11) and R+S (**panel D**, N = 6-9) rats during microdialysis experiment. The first three samples were collected before treating rats with vehicle (veh) or OEA (10 mg kg^{-1}, i.p.) and used as baseline; 45 min after treatment rats were subjected to the stress procedure for 15 min and subsequently exposed to HPF for 1 h. 30 min after the HPF exposure, the rats were given a challenge of amphetamine (0.5 mg kg^{-1}, s.c.).

Data are expressed as mean ± SEM. *$P<0.05$; **$P<0.01$; ***$P<0.001$ vs the mean of the first three samples (basal values) within the same group (Dunnett's multiple comparison test). °$P<0.05$; °°$P<0.01$; °°°$P<0.001$ vs OEA in the same time point (Bonferroni's test for between group comparisons). Red arrow: veh or OEA (10 mg kg^{-1}, i.p.) administration; blue arrow: amphetamine challenge (0.5 mg kg^{-1}, s.c.).

4.3.5. OEA treatment affected monoaminergic system in bingeing rats

Whole tissue concentrations of DA, NA, 5-HT and the metabolites DOPAC, HVA and 5-HIAA were measured in selected brain regions of both R+S and NR+S animals treated with either OEA or veh and consuming the HPF for 1 h (Fig. 1B, EXP. 2), to evaluate possible effects on monoaminergic systems. Specifically, we focused not only

on those areas where c-Fos expression was affected by OEA treatment, such as Acb, CPu, VTA, SN, VTA, and HYPO, but also on structures involved in the decision making, mood tone, memory processes and conditioned responses such as the medial prefrontal cortex (mPFC), the hippocampus (HIPP), and the two brainstem nuclei, dorsal raphe (DR) and locus coeruleus (LC), which send, respectively, serotonergic and noradrenergic inputs to the VTA [72]. These brain areas were microdissected and monoamines and metabolites were extracted and analysed by HPLC; the results were expressed as ng mg^{-1} of wet tissue, as reported in Table 4.2. DA and 5-HT turnovers were calculated as the concentration ratios DOPAC+HVA/DA and 5HIAA/5-HT, respectively (Table 4.2). Results obtained from two-way ANOVA analyses conducted for each parameter are reported in Table 4.3, whereas the results from the *post hoc* analyses (Tukey's test) are reported in Table 4.2. Overall, the results revealed that OEA treatment affected mainly monoaminergic tissue concentration/turnover in bingeing rats, rather than in NR+S rats, and that the baseline differences between the two experimental groups resulted quite limited (NR+S veh vs R+S veh). In fact, in NR+S rats the effects of OEA treatment included only an increase of NA and DA concentration within the HYPO and VTA, respectively, and an increase of 5-HT turnover in the Acb. When compared to NR+S rats administered with veh, bingeing rats (R+S veh) showed per se an increased DA turnover in the mPFC and AMY, as well as an increased 5-HT turnover and 5-HT tissue concentration in the AMY and HYPO, respectively. These increased turnovers were completely prevented by OEA treatment, which was able to increase both DA and 5-HT concentration in the mPFC, causing also a decrease of 5-HT turnover in R+S rats. DA tissue concentrations were affected by OEA treatment also in Acb (where it decreased) and VTA (where it increased) of R+S rats, without producing any effect on DA turnover. Similarly, OEA administration to R+S rats caused a marked increase of 5HT tissue concentrations also in Acb, HYPO, HIPP, VTA, and LC, without affecting 5-HT turnover in these areas. Finally, OEA treatment caused a significant increase of NA concentration in the CPu, HYPO, VTA, and LC.

Table 4.2: concentrations of tissue monoamines (expressed in ng mg^{-1} of wet tissue) and their turnover in the brain regions of interest in NR+S and R+S rats after intraperitoneal injection of OEA or vehicle.

Region	Measure	No restriction VEH	No restriction OEA	Restriction VEH	Restriction OEA	
mPFC	DA	0,05 ± 0,01	0,05 ± 0,005	0,03 ± 0,002	0,06 ± 0,01 **	↑
	DOPAC+HVA/DA	3,07 ± 0,16	2,69 ± 0,52	4,48 ± 0,41 ° ↑	2,11 ± 0,19 ***	↓
	NA	0,20 ± 0,03	0,20 ± 0,02	0,20 ± 0,02	0,19 ± 0,03	
	5HT	0,08 ± 0,01	0,09 ± 0,01	0,07 ± 0,01	0,14 ± 0,03 *	↑
	5HIAA/5HT	4,13 ± 0,67	3,54 ± 0,80	5,15 ± 1,11	2,44 ± 0,31 *	↓
Acb	DA	1,40 ± 0,28	0,81 ± 0,16	1,29 ± 0,18	0,64 ± 0,16 *	↓
	DOPAC+HVA/DA	0,74 ± 0,23	0,96 ± 0,13	1,02 ± 0,16	0,86 ± 0,22	
	NA	0,12 ± 0,03	0,12 ± 0,03	0,12 ± 0,02	0,12 ± 0,02	
	5HT	0,07 ± 0,02	0,06 ± 0,01	0,06 ± 0,01	0,13 ± 0,03 *, °	↑
	5HIAA/5HT	0,63 ± 0,08	0,99 ± 0,17 * ↑	0,68 ± 0,06	0,42 ± 0,08 °°	↓
CPu	DA	2,56 ± 0,52	2,74 ± 0,64	2,34 ± 0,36	2,18 ± 0,52	
	DOPAC+HVA/DA	0,59 ± 0,14	0,65 ± 0,14	0,69 ± 0,08	0,48 ± 0,06	
	NA	0,07 ± 0,01	0,10 ± 0,01	0,10 ± 0,01	0,14 ± 0,01 *, °	↑
	5HT	0,15 ± 0,02	0,11 ± 0,02	0,12 ± 0,02	0,12 ± 0,02	
	5HIAA/5HT	2,43 ± 0,51	3,21 ± 0,62	2,87 ± 0,65	2,77 ± 0,76	
HYPO	DA	0,28 ± 0,03	0,39 ± 0,11	0,25 ± 0,06	0,35 ± 0,03	
	DOPAC+HVA/DA	0,52 ± 0,10	0,48 ± 0,06	0,50 ± 0,07	0,36 ± 0,05	
	NA	0,90 ± 0,07	1,45 ± 0,11 * ↑	1,06 ± 0,14	1,56 ± 0,21 *	↑
	5HT	0,24 ± 0,03	0,32 ± 0,04	0,44 ± 0,06 ° ↑	0,59 ± 0,07 °°	↑
	5HIAA/5HT	3,13 ± 0,61	2,30 ± 0,88	2,17 ± 0,24	1,23 ± 0,18	
AMY	DA	0,40 ± 0,13	0,43 ± 0,07	0,59 ± 0,24	0,36 ± 0,09	
	DOPAC+HVA/DA	0,59 ± 0,09	0,46 ± 0,08	0,92 ± 0,16 ° ↑	0,47 ± 0,07 **	↓
	NA	0,24 ± 0,05	0,23 ± 0,04	0,28 ± 0,08	0,19 ± 0,03	
	5HT	0,10 ± 0,03	0,16 ± 0,04	0,07 ± 0,02	0,10 ± 0,03	
	5HIAA/5HT	0,94 ± 0,41	1,39 ± 0,36	3,16 ± 1,22 ° ↑	1,16 ± 0,37 *	↓
HIPP	DA	0,02 ± 0,01	0,03 ± 0,01	0,02 ± 0,01	0,03 ± 0,01	
	DOPAC+HVA/DA	3,11 ± 0,75	3,86 ± 1,06	3,67 ± 1,00	2,88 ± 0,84	
	NA	0,22 ± 0,04	0,20 ± 0,02	0,23 ± 0,04	0,27 ± 0,03	
	5HT	0,11 ± 0,02	0,12 ± 0,03	0,09 ± 0,02	0,22 ± 0,05 *	↑
	5HIAA/5HT	4,27 ± 0,73	3,85 ± 0,51	4,83 ± 0,75	4,08 ± 0,42	
SN	DA	0,18 ± 0,03	0,17 ± 0,03	0,13 ± 0,03	0,15 ± 0,01	
	DOPAC+HVA/DA	0,93 ± 0,09	0,94 ± 0,07	1,07 ± 0,10	0,73 ± 0,10	
	NA	0,17 ± 0,03	0,23 ± 0,05	0,19 ± 0,03	0,24 ± 0,02	
	5HT	0,19 ± 0,05	0,26 ± 0,07	0,14 ± 0,04	0,24 ± 0,03	
	5HIAA/5HT	3,11 ± 0,90	3,60 ± 0,86	2,90 ± 0,55	1,30 ± 0,21 °	↓
VTA	DA	0,17 ± 0,02	0,34 ± 0,07 * ↑	0,18 ± 0,03	0,34 ± 0,06 *	↑
	DOPAC+HVA/DA	0,90 ± 0,11	0,79 ± 0,09	0,95 ± 0,08	0,73 ± 0,08	
	NA	0,24 ± 0,02	0,28 ± 0,07	0,20 ± 0,03	0,34 ± 0,03 *	↑
	5HT	0,13 ± 0,02	0,16 ± 0,04	0,11 ± 0,02	0,24 ± 0,04 **	↑
	5HIAA/5HT	1,78 ± 0,59	1,92 ± 0,47	1,72 ± 0,41	1,43 ± 0,29	
DR	DA	0,05 ± 0,01	0,09 ± 0,01	0,07 ± 0,01	0,09 ± 0,03	
	DOPAC+HVA/DA	0,31 ± 0,04	0,17 ± 0,03	0,33 ± 0,08	0,33 ± 0,06 °	↑
	NA	0,15 ± 0,02	0,21 ± 0,02	0,18 ± 0,05	0,19 ± 0,05	
	5HT	0,04 ± 0,01	0,08 ± 0,01	0,11 ± 0,03	0,19 ± 0,06 °	↑
	5HIAA/5HT	1,07 ± 0,47	1,30 ± 0,68	1,67 ± 0,74	1,13 ± 0,43	
LC	DA	0,07 ± 0,01	0,08 ± 0,01	0,06 ± 0,01	0,10 ± 0,01	
	DOPAC+HVA/DA	0,87 ± 0,14	0,71 ± 0,16	0,64 ± 0,06	0,70 ± 0,08	
	NA	0,32 ± 0,06	0,29 ± 0,07	0,22 ± 0,05	0,40 ± 0,05 *	↑
	5HT	0,07 ± 0,03	0,10 ± 0,04	0,06 ± 0,01	0,16 ± 0,04 *	↑
	5HIAA/5HT	2,76 ± 0,95	2,41 ± 0,37	2,63 ± 0,34	2,98 ± 0,53	

Data are expressed as mean ± SEM. *P<0.05; **P<0.01 and ***P<0.001 vs veh in the same diet regimen group, °P<0.05; °°P<0.01 vs NR+S in the same treatment group (Tukey's post hoc test; N = 4-8). Abbreviations: Acb, nucleus accumbens; AMY, amygdala; CPu, caudate putamen; DR, dorsal raphe; HIPP, hippocampus; HYPO, hypothalamus; LC, locus coeruleus; mPFC, medial prefrontal cortex; OEA (10 mg kg $^{-1}$, i.p.); SN, substantia nigra; veh, vehicle; VTA, ventral tegmental area.

Table 4.3: Results of the two-way ANOVA analyses of brain tissue monoamines and their turnover in NR+S and R+S rats after intraperitoneal injection of OEA or vehicle.

		F diet regimen	F treatment	F interaction	df
mPFC	DA	0,009 (p=0,927)	5,930 (p<0,05)	3,705 (p=0,069)	1/23
	DOPAC+HVA/DA	1,332 (p=0,262)	14,739 (p<0,01)	7,648 (p<0,05)	1/23
	NA	0,045 (p=0,834)	0,041 (p=0,842)	0,001 (p=0,978)	1/24
	5HT	1,242 (p=0,281)	4,512 (p<0,05)	2,719 (p=0,118)	1/20
	5HIAA/5HT	0,003 (p=0,960)	4,361 (p=0,051)	1,818 (p=0,194)	1/21
Acb	DA	0,449 (p=0,511)	8,902 (p<0,01)	0,023 (p=0,882)	1/22
	DOPAC+HVA/DA	0,191 (p=0,666)	0,021 (p=0,886)	0,890 (p=0,356)	1/25
	NA	0,000 (p=0,984)	0,007 (p=0,933)	0,041 (p=0,842)	1/27
	5HT	2,211 (p=0,152)	2,175 (p=0,155)	4,308 (p=0,050)	1/24
	5HIAA/5HT	7,405 (p<0,05)	0,209 (p=0,654)	10,034 (p<0,01)	1/18
Cpu	DA	0,596 (p=0,447)	0,000 (p=0,984)	0,107 (p=0,747)	1/29
	DOPAC+HVA/DA	0,103 (p=0,751)	0,444 (p=0,511)	1,600 (p=0,217)	1/29
	NA	5,602 (p<0,05)	6,349 (p<0,05)	0,622 (p=0,438)	1/28
	5HT	0,119 (p=0,733)	1,279 (p=0,269)	1,032 (p=0,320)	1/27
	5HIAA/5HT	0,000 (p=0,998)	0,262 (p=0,613)	0,450 (p=0,508)	1/29
HYPO	DA	0,307 (p=0,585)	2,715 (p=0,114)	0,009 (p=0,926)	1/25
	DOPAC+HVA/DA	0,931 (p=0,345)	1,570 (p=0,223)	0,512 (p=0,482)	1/25
	NA	0,729 (p=0,402)	11,151 (p<0,01)	0,019 (p=0,891)	1/25
	5HT	17,355 (p<0,01)	3,940 (p=0,062)	0,362 (p=0,554)	1/22
	5HIAA/5HT	3,842 (p=0,065)	2,911 (p=0,104)	0,012 (p=0,913)	1/22
AMY	DA	0,146 (p=0,706)	0,468 (p=0,501)	0,696 (p=0,413)	1/26
	DOPAC+HVA/DA	2,699 (p=0,113)	7,952 (p<0,01)	2,542 (p=0,124)	1/27
	NA	0,000 (p=0,999)	0,826 (p=0,373)	0,589 (p=0,451)	1/25
	5HT	2,458 (p=0,131)	2,062 (p=0,165)	0,270 (p=0,608)	1/25
	5HIAA/5HT	2,094 (p=0,163)	1,285 (p=0,270)	3,202 (p=0,088)	1/24
HIPP	DA	0,004 (p=0,947)	1,254 (p=0,268)	0,071 (p=0,791)	1/27
	DOPAC+HVA/DA	0,055 (p=0,816)	0,000 (p=0,985)	0,688 (p=0,411)	1/27
	NA	1,317 (p=0,256)	0,037 (p=0,847)	0,862 (p=0,357)	1/28
	5HT	1,355 (p=0,250)	3,698 (p=0,060)	2,743 (p=0,104)	1/28
	5HIAA/5HT	0,395 (p=0,532)	0,875 (p=0,354)	0,067 (p=0,797)	1/28
SN	DA	1,756 (p=0,198)	0,056 (p=0,815)	0,335 (p=0,568)	1/27
	DOPAC+HVA/DA	0,134 (p=0,717)	2,881 (p=0,103)	3,383 (p=0,078)	1/27
	NA	0,058 (p=0,812)	2,591 (p=0,121)	0,015 (p=0,905)	1/26
	5HT	0,489 (p=0,492)	3,032 (p=0,095)	0,040 (p=0,843)	1/26
	5HIAA/5HT	3,293 (p=0,083)	0,668 (p=0,422)	2,277 (p=0,146)	1/25
VTA	DA	0,027 (p=0,871)	12,471 (p<0,01)	0,005 (p=0,942)	1/24
	DOPAC+HVA/DA	0,007 (p=0,933)	3,643 (p=0,068)	0,377 (p=0,545)	1/28
	NA	0,136 (p=0,716)	5,210 (p<0,05)	1,378 (p=0,251)	1/29
	5HT	0,857 (p=0,363)	7,130 (p<0,05)	2,811 (p=0,106)	1/29
	5HIAA/5HT	0,396 (p=0,535)	0,033 (p=0,858)	0,24 (p=0,629)	1/29
DR	DA	0,055 (p=0,817)	3,049 (p=0,094)	0,278 (p=0,603)	1/27
	DOPAC+HVA/DA	2,945 (p=0,101)	1,707 (p=0,205)	1,428 (p=0,245)	1/24
	NA	0,031 (p=0,862)	0,656 (p=0,427)	0,224 (p=0,640)	1/25
	5HT	6,361 (p<0,05)	2,978 (p=0,099)	0,310 (p=0,584)	1/23
	5HIAA/5HT	0,110 (p=0,744)	0,060 (p=0,809)	0,353 (p=0,559)	1/22
LC	DA	0,066 (p=0,799)	2,962 (p=0,098)	1,069 (p=0,311)	1/28
	DOPAC+HVA/DA	1,296 (p=0,266)	0,196 (p=0,622)	1,196 (p=0,285)	1/27
	NA	0,019 (p=0,892)	1,688 (p=0,206)	3,115 (p=0,090)	1/27
	5HT	0,575 (p=0,456)	3,573 (p=0,071)	0,756 (p=0,393)	1/27
	5HIAA/5HT	0,184 (p=0,672)	0,000 (p=0,996)	0,459 (p=0,506)	1/24

Abbreviations: Acb, nucleus accumbens; AMY, amygdala; CPu, caudate putamen; DR, dorsal raphe; HIPP, hippocampus; HYPO, hypothalamus; LC, locus coeruleus; mPFC, medial prefrontal cortex; SN, substantia nigra; VTA, ventral tegmental area.

### 4.3.6. OEA treatment affected CRF mRNA level in the AMY and oxy expression in the PVN of bingeing rats

Our previous studies demonstrated the crucial role of oxytocinergic neurotransmission in mediating the hypophagic effect of OEA [47] and the pivotal role of CRF system in sustaining binge eating behaviour in our rat model [68]. Since both oxy and CRF can be affected by frustration stress, we assessed the "pure" effects of OEA on stress response without the potential impact of food intake to evaluate whether the anti-bingeing effects of OEA in this model might be attributed to a reduced effect of stress exposure. To this aim, we measured both CRF and oxy mRNA by in situ hybridization of radiolabelled probes in the brains of NR+S and R+S rats treated with either OEA or veh and sacrificed at the end of the stress exposure (Fig. 4.1B, EXP. 4). As shown in the representative autoradiography reported in Fig. 4.6A and C, CRF mRNA signal was detected in the CeA and in the PVN.

The results of the densitometric analyses of CeA were statistically analysed by two-way ANOVA that revealed no effect of caloric restriction and no effect of treatment ($F_{restriction}$ = 3.026 df = 1/19, P = 0.098, $F_{treatment}$ = 0.169 df = 1/19, P =0.6856 respectively) but a significant interaction between the two factors ($F_{interaction}$ = 9.491 df = 1/19, P < 0.01). *Post hoc* analyses, demonstrated that OEA treatment was able to reduce CRF mRNA in the CeA of bingeing rats, without affecting the same parameter in the PVN (Fig. 4.6B and D, respectively), where the two-way ANOVA revealed no significant effect ($F_{restriction}$ = 0.455 df = 1/21, P =0.507, $F_{treatment}$ = 0.468 df = 1/21, P = 0.50, $F_{interaction}$ = 1.066 df = 1/21, P = 0.587).

This observation confirms our previous data supporting the crucial role of CRF system at extra-hypothalamic level in binge-like HPF consumption in our rat model [68].

We previously demonstrated that peripheral administration of OEA was able to activate a subpopulation of PVN neurons that included mostly, albeit not exclusively, oxytocinergic neurons [47]. Based on these observations, the last aim of our study was to evaluate whether the combination of caloric restriction, frustration stress exposure and OEA treatment could affect oxy mRNA expression in the PVN (Fig. 4.7A). The two-way ANOVA of data obtained from the semiquantitative densitometric analyses revealed a significant effect of food restriction ($F_{restriction}$ = 9.897 df = 1/232, P = 0.0051) no effect of treatment, ($F_{treatment}$ = 1.058 df = 1/232, P = 0.3159) and a significant interaction between the two factors ($F_{interaction}$ = 5.544 df = 1/232, P = 0.0288). The *post-*

hoc analyses demonstrated that oxy mRNA expression was significantly increased in bingeing rats treated with OEA (Fig. 4.7B).
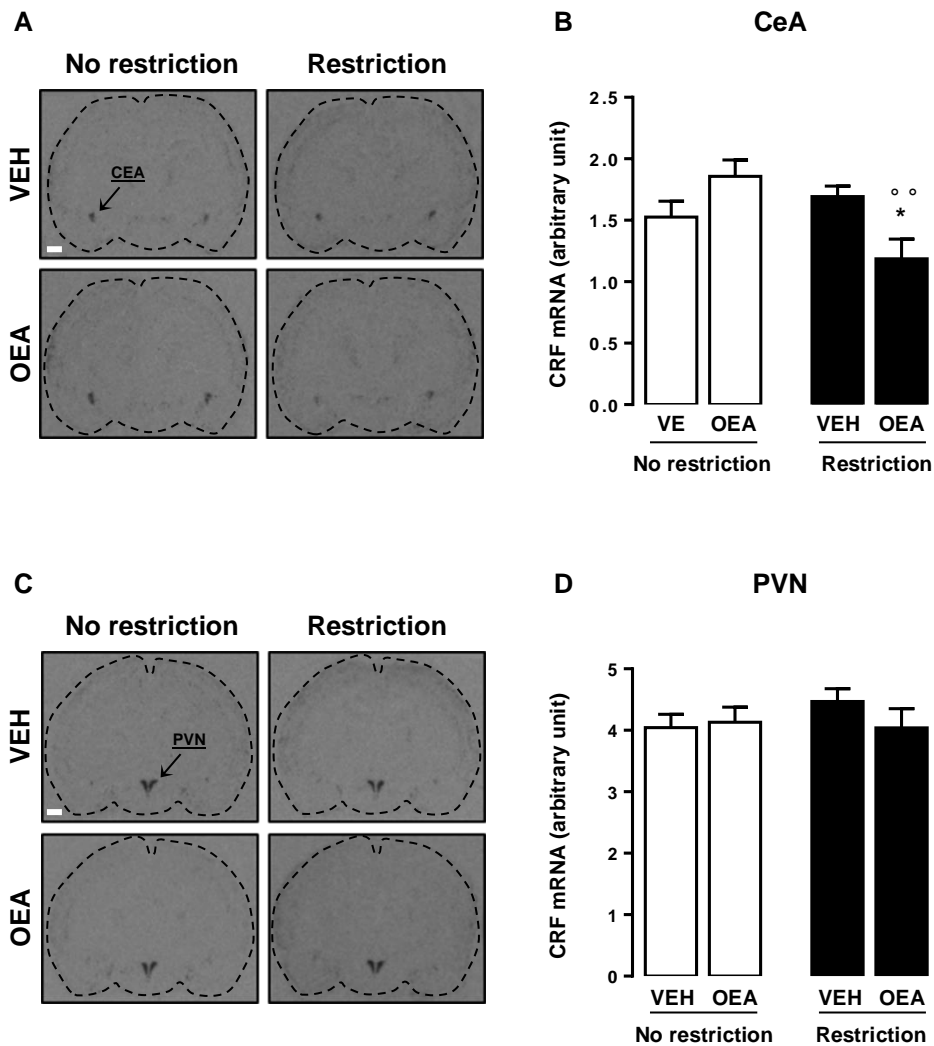
Fig. 4.6: Representative in situ hybridization images (scale bar = 1 mm) of corticotropin-releasing factor (CRF) mRNA expression within the central amygdala (CeA, **panel A**) and the paraventricular nucleus (PVN, **panel C**) of NR+S (non restricted – stressed) and R+S (restricted – stressed) rats treated with either vehicle (veh) or OEA (10 mg kg $^{-1}$, i.p.) and sacrificed 1 h after the treatment. Semiquantitative densitometric analyses of CRF mRNA in the CeA (**panel B**) and PVN (**panel D**) of NR+S and R+S rats treated with either veh or OEA (10 mg kg $^{-1}$, i.p.) and sacrificed 1 h after treatment. Data are expressed as mean ± SEM. *$P<0.05$ vs veh in the same diet regimen group; °°$P<0.01$ vs NR+S in the same treatment group (Tukey's *post hoc* test; CeA: N = 4-6, PVN: N = 5-6).

A

B   **PVN**
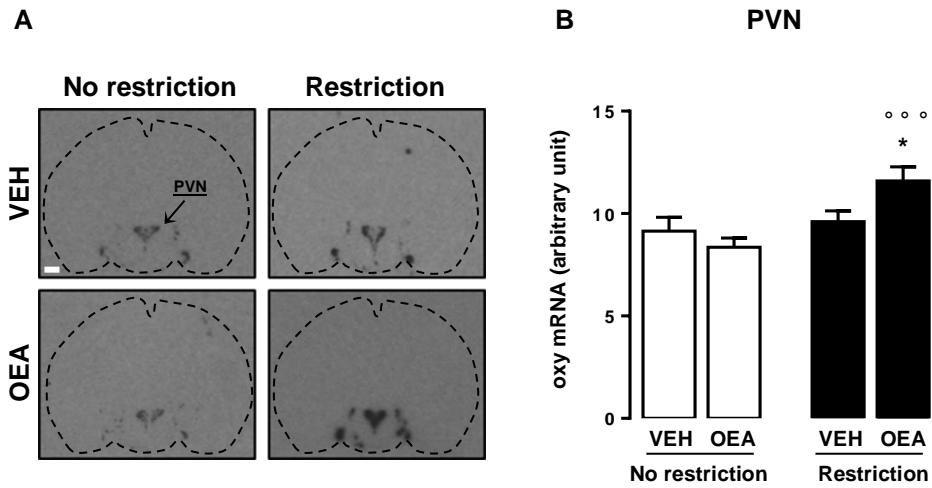
Fig. 4.7: Representative in situ hybridization images (scale bar = 1 mm) of oxytocin (oxy) mRNA expression in the paraventricular nucleus (PVN, **panel A**) of NR+S (non restricted – stressed) and R+S (restricted– stressed) rats treated with either vehicle (veh) or OEA (10 mg kg $^{-1}$, i.p.) and sacrificed 1 h after treatment. Semiquantitative densitometric analyses of oxy mRNA expression in the PVN (**panel B**) of NR+S and R+S rats treated with either veh or OEA (10 mg kg $^{-1}$, i.p.) and sacrificed 1 h after treatment. Data are expressed as mean ± SEM. *P<0.05 vs veh in the same diet regimen group; °°°P<0.001 vs NR+S in the same treatment group (Tukey's *post hoc* test; N = 5-6).

4.4. Discussion

This study demonstrates for the first time that OEA prevents binge-like palatable food consumption induced by stress in female rats with a history of food restriction, suggesting that this lipid signal might represent a potential target for the development of more efficacious and safer treatments for BED or for other eating disorders characterized by binge episodes.

The effect of OEA was dose- and time-dependent, being long-lasting at the dose of 10 mg kg^{-1} i.p.. According to previous reports from our laboratory and from other research groups [40,44,47,50,51], this dosage is able to induce satiety in both free-feeding and food-deprived rats, without causing motor impairment, malaise, pain or hormonal and body temperature alterations in adults rats. The same dosage is also able to reduce food intake in fasted/refed Magel2 knock out mice, an animal model of Prader-Willi syndrome, characterized by hyperphagia and overweight with respect to wild type littermates [82].

In particular, 10 mg kg^{-1} OEA selectively reduces HPF consumption in bingeing rats at all the time points considered and the HPF intake reduction ranges from a percentage of -62,7% at earliest time-point until -34,02% at 120 minutes, when, according with previous behavioural OEA studies [55], rats treated with OEA did not compensate the food they did not eat during the earlier time points.

The mechanism accounting for this selective anti-binge effect remains to be fully elucidated; it is known that the compulsive eating during the BED is also associated to a stimulation of the HPA axis and an elevation of CORT levels, indeed our rats display an increase of serum CORT levels at the end of the stress procedure which corresponds to the access to the HPF [66]. Interestingly, as reported in the introduction, OEA is able to influence the stress response by reducing the HPA axis activation, ACTH and CORT levels in an experimental paradigm inducing stress [61,83], thus suggesting that in our circumstances the behavioural effect of OEA might be attributed, at least in part, to its effect in dampening the HPA axis hyperactivation. Moreover, CORT is able to motivate drug-seeking behaviour in rats [84] and we hypothesize that in our rat model it might play a crucial role in compulsive behaviour towards food, which resembles the driving motivated behaviour typical of a state of addiction. On this regard, it has been shown that OEA and PPAR-α agonists decrease nicotine self-administration and nicotine seeking in rats and monkeys by preventing nicotine-induced excitation of dopaminergic neurons in the AcbSh [85]. Similarly, it has been shown that the inhibition of fatty acid

amide hydrolase enzyme, which increases the bioavailability of OEA, impedes nicotine-induced activation of neurons in the AcbSh [86]. Additionally OEA has been demonstrated to block cue-induced reinstatement of alcohol- self administration and - seeking behaviour [87] and to block the acquisition and expression of cocaine-induced conditioned place preference and cocaine-induced sensitization [88]. On the bases of these observations, we hypothesize that the inhibition of compulsive eating in our rat model exerted by OEA, resembles the capability of this compound to counteract the addiction-related behaviours described so far.

Moreover, in the present study we were unable to detect any significant effect of OEA on feeding behaviour of female rats belonging to the three other experimental groups, which did not show binge-like palatable food intake within the timeframe of the experiment. This observation suggested us that the anti-binge effect observed after OEA administration was not the expression of an anorexiant action due to satiety induction, as found in previous studies, but rather the consequence of the selective inhibition of "hedonic hunger", which refers to the compulsive consumption of food for pleasure and not to maintain energy homeostasis [89,90].

Based on this result, we focused our attention on the so called "dopamine motive system", according to the definition adopted by Dr. Wolkow and colleagues, that refers to the brain areas controlling the reinforcement and motivational aspects of feeding [72].

By analyzing neuronal activation of brain areas that partake directly or indirectly to this circuit, we found that OEA treatment is able to restore a "normal" brain activity, by reducing the stress-induced c-Fos increase in brain areas regulating the dopaminergic signalling namely the Acb, the CPu, and the SN. Interestingly opposite effects were observed within the VTA, where DA cell bodies projecting to the Acb through the mesolimbic pathway are located, and at the level of the PP (Fig. 4.8A). No effect has been observed within the VP, which sends gabaergic projections to the VTA, where it guarantees basal DA cell activity (tonic DA release) (Fig. 4.8A) [73]. The fact that OEA has no effect on the activation of the VP, is therefore consistent with its ability to increase VTA neuronal activity (see discussion in the text regarding tissue monoamine concentrations). Moreover, the OEA-induced c-Fos increase in the PP is also consistent with the increase of VTA neuronal activity in bingeing rats after OEA administration. In fact, the PP is implicated in promoting burst-firing of DA cells (phasic DA release, action potential) within the VTA, and an activation of the PP is strictly related to the activation of VTA dopaminergic neurons (Fig. 4.8A) [73]. We hypothesize that the results

obtained within the AMY, where OEA significantly decreases stress-induced c-Fos activation in bingeing rats, might refer to the ability of this lipid to counteract the neurological mechanism related to stress. At the level of the CeA, in fact, the CRF system, whose activation is crucially involved in frustration stress-induced binge eating in our rat model [67], coordinates the affective reactions to stress [91]. In keeping with this latter observation, and overlapping the trend obtained by the c-Fos analysis within the AMY, we found that OEA was able to decrease CRF mRNA at the level of the CeA in bingeing rats, while no significant differences were observed in CRF mRNA levels within the PVN (Fig. 4.8B), thus confirming recent findings demonstrating that hypothalamic CRF system is not sufficient to account for binge-like HPF consumption in our BED model [68]. Furthermore, the ability of OEA to increase c-Fos expression within the PVN of bingeing rats might be linked to the activation of oxytocinergic neurons (Fig. 4.8C), highly expressed in this brain area. The increased oxy mRNA levels observed in R+S rats treated with OEA is in accordance with this hypothesis and is in line with our previous studies indicating that the behavioural effect of OEA on food intake is linked to the activation of the central oxytocinergic system [47,58].

Brain DA signaling is essential for the reinforcing/rewarding properties of several stimuli, such as drug of abuse and food; additionally a large body of evidence suggests that brain DA regulates the entire body energy homeostasis [92–94]. Two are the brain dopaminergic pathways involved in hedonic feeding: 1) the nigrostriatal circuit, which maintains constant levels of fat ingestion by regulating the food consumption phase, and 2) the mesolimbic dopaminergic circuit, which plays a pivotal role in the drive towards high caloric palatable food (motivation to consume food, particularly energy-dense food) and is also associated to stress- and cue-induced feeding [95]. Previous studies from Tellez and collaborators [59] have linked the anorexigenic effect of OEA to its ability to restore a dorsal striatal DA release in diet-induced obese (DIO) mice displaying a deficit in nigrostriatal DA signalling in response to fat ingestion. Tellez's study suggested for the first time that OEA might regulate the reward value of fat by establishing a link between caloric-homeostatic and hedonic-homeostatic control of eating. In the present work we focused our attention at the AcbSh level, where the DA release is promoted by addictive drugs, palatable food and by acute stress challenge [96–99].

We observed that restricted and non restricted rats were characterized by similar DA baseline levels and similarly responded to stress, with a small pick increase of extracellular DA concentrations. Moreover, in both groups, HPF consumption was

unable to evoke DA release in accordance with previous observations showing that AcbSh DA responds only when the palatable food is consumed for the first time, differently from the core of the Acb (AcbC) where DA levels increase whenever the food is ingested and such increase is not related to the palatability or to the novelty [100]. We found that OEA treatment was able to significantly dampen DA release evoked by frustration stress within the AcbSh of both non-restricted and restricted rats.

This result is supported by previous observations suggesting that the activation of PPAR-α receptors (through OEA or the synthetic PPAR-α agonist WY14643 administration) dampens the activity of DA neurons within the VTA and decreases the nicotine-inducing DA release in the Acb [85,101]. At the basis of this response the authors hypothesize a rapid and non-genomic activity of PPAR-α receptors which consists in a phosphorylation of nicotinic receptors present on dopaminergic neurons of the VTA, by promoting their internalization and by abolishing the response of DA neurons to nicotine [102]. Additionally, we observed that peripheral OEA is able to revert also the effect of amphetamine in both restricted and non-restricted animals. In fact, we found that OEA significantly reduced DA release evoked by the subcutaneous challenge of amphetamine. Amphetamine promotes monoaminergic transmission by recruiting several mechanisms including the blocking of the DA, NA and 5-HT transporters (DAT, NET, and SERT respectively). Moreover, amphetamine acts as substrate of the monoamine transporters, competes with the neurotransmitter substrate and enter the presynaptic neurons. Inside the nerve terminals, amphetamine inhibits the vesicular monoamine transporter 2 (VMAT2). As result of these latter mechanism, amphetamine reverses the transport of monoamines by pumping them into the synapse [103]. This is not the first time that OEA has been associated to the effect induced by amphetamine-like compounds; for instance it has been demonstrated that OEA prevents MDMA-induced cognitive deficits in mice [104]. Moreover, the same authors demonstrated that OEA is also able to prevent MDMA-induced DAT loss; similarly, in the present work, we suggest that peripheral administration of OEA might prevent the reduction of DAT induced by amphetamine, and this effect might contribute to a restoration of a physiologic DA release. OEA might therefore have a protective role against the abnormalities evoked by psychostimulant drugs such as amphetamine, at least regarding tonic dopaminergic neuronal activity, which is possible to measure through brain microdialysis experiment [105]. In keeping with results obtained by brain microdialysis experiment, we found that OEA also reduces DA tissue levels within the Acb of

bingeing rats; interestingly opposite results were observed at the level of the VTA, where OEA induces a significant increase of DA tissue concentration in both restricted and non restricted animals without altering DA turnover. We think that these latter results might occur in virtue of OEA capability to activate the PP which, as previously mentioned in the text, sends excitatory glutamatergic inputs to the VTA where it mediates DA burst firing (phasic DA release) (Fig. 4.8A) [73].

The observation that the increase in DA VTA concentration induced by OEA does not produce a discernible increase in DA levels within the Acb was surprising for us, since burst firing is the main mechanism by which DA is released. However, we hypothesize that OEA-induced PP activation produces a massive DA release that will be highly located at the synaptic cleft level; this huge amount of DA stimulates reuptake mechanisms and the activation of presynaptic DA type 2 receptors (D2R), which would then result in a rapid downregulation of the dopaminergic tone (Fig. 4.8A). As far as the DA turnover, which is an index of neurotransmitter activity [106,107], we found that OEA dampens the DA turnover induced by frustration stress exposure in bingeing rats in both mPFC and AMY. On this regard it has been demonstrated that DA type 1 receptors (D1R), highly expressed within the mPFC, play a crucial role in food-related behaviour [108] by activating a population of glutamatergic neurons within the AMY [109], which, in turn, project to the Acb and modulate the appetitive drive motivation [110]. The increase of DA turnover that we observe in bingeing rats treated with veh is in line with previous study suggesting that the activation of D1 family receptors in the mPFC is involved in the stress-induced drug seeking, and in yohimbine-induced of food seeking [111]. We therefore hypothesize that OEA might dampen the hyperactivity of the dopaminergic system in the mPFC which, in turn, reflects a decrease in DA activity also within the AMY by restoring a physiological dopaminergic transmission. This is supported by the values of the turnover measured in bingeing rats treated with OEA, which are comparable to those of control animals (no restricted animals). As far as the serotonergic system, we found that OEA is able to enhance 5-HT transmission in most of the brain areas analysed selectively in bingeing rats. We think that the increase of 5-HT observed within the HYPO is consistent with its role in the control of appetite [112]; in fact drugs like d-fenfluramine, sibutramine, fluoxetine (selective serotoninergic reuptake inhibitor) (SSRIs)) and 5-HT$_{2C}$ receptor agonists significantly induce hypophagia in laboratory rodent and reduce caloric intake in both lean and obese humans [113]. We therefore hypothesize that the anti-binge effect induced by OEA in

bingeing rats might occur, at least in part, because of its effect in promoting a high serotonergic tone. Moreover, our previous study demonstrated that both Sibutramine and Fluoexetine are efficient in reducing HPF intake in this rat model of BED [66], further supporting that an increase of 5-HT tone might be a valid approach for the treatment of this pathology. Importantly, differently from sibutramine and fluoexetine, which are able to reduce HPF intake also in the other experimental groups (NR+NS, R+NS and NR+S), the effect induced by OEA is selective in reducing HPF intake in bingeing rats, without modifying HPF intake in control animals (NR+NS) or in animal that experienced food restriction or stress separately (R+NS and NR+S). Additionally, 5-HT$_{2c}$ receptors are located not only within the HYPO, where they modulate homeostatic feeding, but also within the VTA, Acb and mPFC where they regulate DA cell activity associated with several behaviours, including reward and impulse control [114–116]; such effect is supported by the finding that lorcaserin (a selective 5-HT2$_{2c}$ receptor agonist) reduces binge eating in mice by stimulating dopaminergic transmission [30]. Moreover, since the increase of serotonergic tone is also able to alleviate depressive-like symptoms [117], in keeping with very recent findings obtained by Costa and collaborators [60] we think that the high levels of 5-HT induced by OEA might also counteract the depressive-like state that often occurs in subject suffering from BED. Finally, we also found that OEA increases the noradrenergic tone in most of the brain region analysed. We hypothesize that the increase observed within the LC might reflect an increase in the NA synthesis exerted by OEA, since this is the brain area in which reside the cell bodies of noradrenergic neurons. Moreover, the OEA-induced NA increase within the HYPO is consistent with our previous study demonstrating that the behavioural effects of OEA are strictly dependent from the activation of a noradrenergic pathway connecting the brainstem to the HYPO [51]. Finally, our results are in keeping with previous study from others [53] demonstrating that i.p. OEA induces a dose-dependent increase in the levels of hypothalamic NA. NA, in fact, is released within the HYPO during consumption of a meal and correlates with the meal size thus suppressing food intake [118].
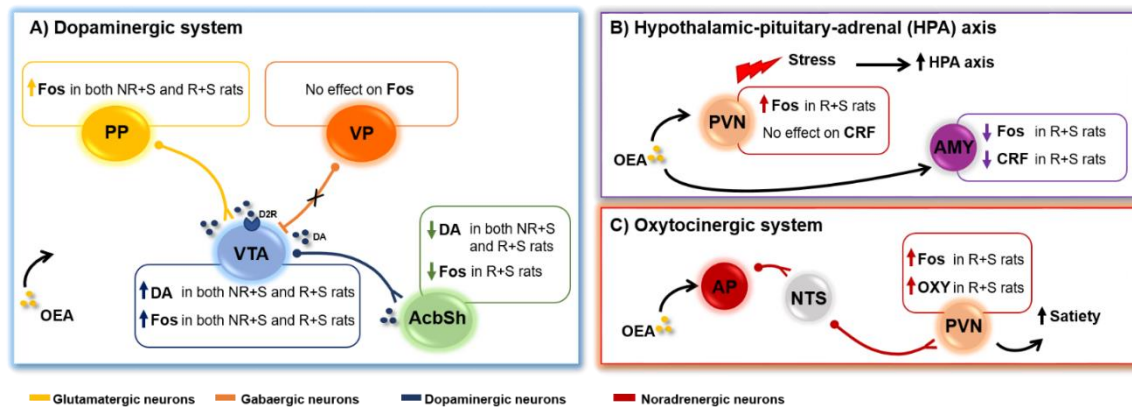
Fig. 4.8: Schematic drawing illustrating the putative interactions between OEA and the central dopaminergic system (panel A), the hypothalamic-pituitary-adrenal (HPA) axis (panel B) and the central oxytocinergic system (panel C).

Panel A: the OEA-induced Fos increase in the pedunculopontine nucleus (PP) produces a massive dopamine (DA) release at the level of the ventral tegmental area (VTA), this huge amount of DA activates presynaptic DA type 2 receptors (D2R) thus resulting in a rapid downregulation of the dopaminergic tone and leading to a lower release of DA in the shell of the nucleus accumbens (AcbSh) of both NR+S (non restricted – stressed) and R+S (restricted – stressed) rats as we observed by the microdialysis study. In accordance with this hypothesis, OEA does not induce an activation of the ventral pallidum (VP, in terms of Fos activation), which sends gabaergic projections to the VTA.
Panel B: The stress response is associated to a stimulation of the HPA axis. OEA is able to decrease Fos expression and CRF mRNA levels within the amygdala (AMY) in R+S rats, thus suggesting the ability of this lipid to counteract the neurological mechanism related to stress. No significant differences were observed in CRF mRNA levels within the PVN, thus suggesting that that hypothalamic CRF system is not sufficient to account for binge-like HPF consumption in our BED model.
Panel C: After being intraperitoneally administered, OEA activates noradrenergic neurons of the area postrema (AP), which in turn project to the nucleus of the solitary tract (NST). Noradrenergic neurons within the NST could then mediate OEA ability to increase Fos expression within the PVN of R+S rats. This effect is linked to the increased oxy mRNA levels observed in R+S rats after OEA treatment.

4.5. Conclusions

In conclusion, in the current study we provide evidence for the first time that the endogenous fatty-gut lipid OEA exerts a selective inhibitory effect on binge-like eating behaviour in female rats, by modulating key mechanism of the hedonic-homeostatic brain circuits. These conclusions broaden our current knowledge on the role played by OEA in the caloric-homeostatic control system and support the hypothesis that OEA might represent a novel potential pharmacological target for the treatment of aberrant eating patterns.

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Chapter V

Conclusions

5.1. Conclusions

Although further studies are necessary, the results collected in almost 20 years of both preclinical and clinical studies strongly support OEA as a promising candidate for the development of more effective and safe anti-obesity therapies.

The results of the present work represent a major advance in understanding the complex circuitry underlying OEA pro-satiety effect. In particular, through my thesis project we demonstrated, for the first time, that OEA is able to reach and permeate the brain parenchyma within few minutes after its peripheral administration, and we showed a crucial role of the AP as receptive brain region for circulating OEA. Moreover, we here demonstrated that OEA exerts a selective inhibitory effect on binge-like eating behaviour in female rats, by modulating key mechanism of the hedonic-homeostatic brain circuits and therefore it might represent a novel potential pharmacological target for the treatment of aberrant eating patterns.

Interestingly in the last few years, several clinical studies have been conducted to clarify the role of this lipid compound in the regulation not only of the satiety but also of the body weight and body fat. The majority of OEA studies in humans have been focusing on the effect of diet manipulation on endogenous OEA tone or on the correlation between OEA plasma levels and a variety of pathophysiological parameters such as body fat, exercise, BMI and satiety[1–6].

In virtue of its oral availability and its property in reducing body weight, OEA has been introduced into the weight loss supplement market in USA, under the name of RiduZone (OEA [200 mg]/capsule- the only FDA-acknowledged OEA supplement on the market). Indeed encouraging data collected from a retrospective analysis performed on 42 patients (14 male, 28 female) who took RiduZone 2–3 times/day for 4–12 weeks demonstrated that RiduZone induces an average of 7–8% decrease in BMI after 10 weeks of treatment [7].

Very recently, Payahoo and colleagues conducted a randomized, double-blind, placebo-controlled clinical trial to evaluate the effects of OEA supplementation on 60 healthy obese subjects.

The eligible patients assigned to the intervention group received two capsules of OEA ([125 mg]/capsule) a day for 60 days whereas the placebo group received the same amount of starch [8].

Beyond collecting anthropometric measurements, body fat and appetite sensations (hunger, fullness, the desire to consume food, and the desire to eat sweet/salty/fatty foods), PPAR-α gene expression was also measured during the present study.

The authors showed a significant reduction of body weight, BMI, waist circumference, and fat percent after 8 weeks of OEA treatment in the intervention group compared to the placebo group without showing any adverse events. These effects have been shown to be paralleled to the OEA-induced PPAR-α increase observed in the group of patients treated with OEA [8]. Interestingly, in this study OEA was also able to significantly decrease hunger and sweet food craving selectively in the intervention group. This latter results we think is in line with our study, further confirming the selective anti-binge property of OEA [8].

The same research group performed an additional clinical trial on obese individuals by using the same above-described experimental design showing OEA ability to improve obesity-related conditions such as inflammation and oxidative stress [9].

Finally it is noteworthy that both preclinical and clinical studies revealed that OEA does not induce any signs of anxiety nor does it change CORT levels; moreover, hypophagic effects of OEA are not accompanied by, malaise, visceral illness, changes of body temperature or nausea [10].

Taking together, the results of the above-cited human studies further support the relevance of our findings, thus opening novel perspectives for OEA potential clinical use for eating-related disorders.

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